Slutrapport

Complete information about the studies, materials and methods, results and the discussions can either be found in the doctoral thesis of Helena Back or the publications submitted with this final report.

Background

Equine herpesvirus (EHV) type 2 and 5 are gamma herpesviruses that belong to the genus *Percavirus*, with genome sizes of 184 kbp (Telford et al., 1993) and 182 kbp (Wilkie et al., 2015) respectively. EHV-2 and EHV-5 are closely related viruses and prior to 1987 EHV-5 was indistinguishable from EHV-2. However, studies identifying divergent profiles of restriction fragment length polymorphism facilitated differentiation of EHV-5 from EHV-2 (Browning & Studdert, 1987). Moreover further sequencing resulted in reclassification of EHV-2 and EHV-5 from beta herpesviruses to the family of slower growing gamma herpesviruses (Telford et al., 1993). Both EHV-2 and EHV-5 have been detected in the Icelandic horse population, where the horses have been isolated for more than 1000 years (Torfason et al., 2008), suggesting that the equine gamma herpesviruses are not a recently appearing microbial agents in horses. The clinical significance of infection with equine gamma herpesviruses remains unresolved, mainly because of its worldwide distribution and the high prevalence of infection in different horse populations but with only scattered reports of possible clinical problems related to these infections. EHV-5 has also increasingly been associated with the severe lung disease equine multinodular pulmonary fibrosis (EMPF) in adult horses.

Study population

The horses participating in the study were actively racing Standardbred trotters from four different professional training yards (TYs) in the area of Mälardalen, Sweden. The horses were monitored over 13 consecutively months and blood samples as well as nasal swabs were taken on monthly basis. All horses were training and racing according to their regular schedule during the study period and were at the time of recruitment healthy and well performing with a mean age of 3 years, (range 2-8 y, SD 1.33). The horses underwent clinical examination at each sampling occasion (performed by a single veterinarian throughout the study), where signs of respiratory disease, such as fever (>38.3^{o}C), nasal discharge and cough were recorded. Their racing performance was also evaluated at each sampling occasion with both objective and subjective methods (Back et al 2015). The health status was also monitored on a weekly basis throughout the study period.
In the genetic study of EHV-5, the selected NS were from eight horses with a high viral load of EHV-5 identified at two sampling occasions one year apart in the TYs. They were all healthy and well performing at both sampling occasions. In addition, the pre-mortem (lung biopsy) and post-mortem samples (lung) from the horse with EMPF were included.

Diagnostic methods

Quantitative PCR (qPCR)

Since no qPCR assay for EHV-2 and EHV-5 were available at the time when study III was initiated, we developed and validated two new qPCR assays targeting the conserved region of the DNA polymerase gene of each virus. Viral DNA was extracted from NS using a Magnatrix 8000+ robot and the extraction kit NorDiag Vet Viral NA (NorDiag AB, Hägersten, Sweden) according to the manufacturer´s instructions. Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Saint-Aubin, France) were used to amplify the selected regions of the nucleic acid, using the primers and the UPL-probes as are further described in Back et al 2015. The viral load within each sample, was calculated by linear regression using the ten-fold serial dilutions of known concentration of DNA copies from the validation of the qPCR assay and the given Cq values. DNA copies detected above the threshold at a quantification cycle (Cq) <40 were considered as a positive result.
The analysis of EHV-2 and EHV-5 were run in two separate assays in order to avoid the risk of failure to identify each of them if co-infection occurred. Moreover, the conserved DNA polymerase gene was used as the target gene to increase the possibility of the assay to detect the virus even if different strains were present. No cross reaction could be detected between the two PCR assays.

Next generation sequencing (NGS)
In study IV a region of the EHV-5 glycoprotein B (gB) in which high variability flanked with stable nucleotides could be identified was selected to design a new PCR assay, which were followed by NGS. By using this method of "deeper" sequencing, genetic variations in that particular segment of EHV-5 would be detectable and thereby enable unmasking of any multiple infections by several strains. The amplicon PCR and library preparation was performed according to the Illumina protocol "16S Metagenomic Sequencing Library Preparation" with smaller modifications and thereafter sequenced (NGS) using MiSeq Reagent Kit v.3 600 cycles on the MiSeq instrument (Illumina) and the "Generate FASTQ" workflow. The high quality reads obtained from the NGS were trimmed, merged, and phylogenetic analysis were performed in the software program Trimmomatic, COPE 1.1.2, MUSCLE and MEGA, which are described in more detail in the materials and methods of paper IV. The different clinical specimens included in study IV were coded with a unique letter for each horse followed by number "1" (first sampling occasion) or "2" (second sampling occasion) and the last number (1-3), illustrate the most (1) and less (3) common strain within each of the clinical specimens.

Endoscopy
In the study of presence and viral load of EHV-2 and EHV-5, 28 horses at the same TY underwent endoscopic examination (Endoscope pks 60914, Karl Storz, Tyttlingen, Germany), before and after each SFE from April to August 2011 to estimate the visual degree of upper respiratory inflammation. The purpose of the examination after the workload was to assess the lower airways for presence of fresh blood that could suggest exercise induced pulmonary haemorrhage. Images from each examination of the pharyngeal region and from the trachea at the level one-meter deep from the nostrils were taken and stored electronically for subsequent grading. The endoscopic findings were graded at the same occasion by an experienced equine clinician blinded to clinical and viral status of the horses. Endoscopic scoring of mucus accumulation was performed using the scale of 0-5 as previously described (Koblinger *et al.*, 2011; Gerber *et al.*, 2004). Accumulation of mucus grade 0 was considered normal whereas all grades >0 was deemed as excess accumulation of mucus. The score of pharyngeal inflammation was marked at a scale of 0-4 (Raker & Boles, 1978), where grades 0 and 1 were considered normal.

SAA
The acute phase proteins are synthesized in the liver during different inflammatory conditions or infectious diseases (Baumann & Gauldie, 1994). In equine medicine concentrations of the proteins fibrinogen and serum amyloid A (SAA) in serum are the inflammatory markers commonly used to detect nonspecific response to inflammatory or infection.
Concentrations of SAA (a major acute phase protein) have been shown to rapidly increase due to inflammatory stimulus, being elevated in less than 48 hours in horses infected with EIV (Hulten *et al.*, 1999). Because of its short half-life time in serum, reported in laboratory mice to vary between 30 min to 2 h (KluveBeckerman *et al.*, 1997; Tape & Kisilevsky, 1990; Hoffman & Benditt, 1983), the serum concentrations of SAA decrease rapidly after resolution of disease and have been suggested to well reflect recovery or the response to therapy (Hulten & Demmers, 2002). Moreover, compared to the traditional markers (WBC count or plasma fibrinogen) SAA has been found to be a more reliable marker in horses to monitor inflammation as well as being a prognostic indicator (Belgrave *et al.*, 2013).
Fibrinogen (a minor acute phase protein) is slower reacting and peaks within 1-2 weeks after stimulus and decrease slowly during recovery (Jacobsen *et al.*, 2005; Hulten *et al.*, 2002). This makes this biomarker less useful for detection and monitoring inflammation than SAA.
In this work the levels of SAA were analyzed in serum at the department of clinical chemistry at SLU using the immunoturbidometric assay (Hillstrom *et al.*, 2010), where a level of SAA>20 mg/L was considered positive for systemic inflammation (Jacobsen & Andersen, 2007).

Results and discussion
Of the monthly NS´s from the 63 racing Standardbred trotters followed over a year 74% (492/663) were positive to EHV-5 and 30% (196/663) were identified with EHV-2. Furthermore, as illustrated in table 1 of the samples contained both EHV-2 and EHV-5.

None of the horses were positive at all sampling occasions to EHV-2 whereas for EHV-5 none were completely negative. This high prevalence of both EHV-2 and EHV-5 (table 1), is in agreement with previous studies conducted elsewhere (Hue *et al.*, 2014; Wang *et al.*, 2007). Moreover the observation that EHV-5 could be detected in 100% of the horses at least once during the year illustrates the highly endemic situation of this virus in actively racing Standardbred trotters. Some of the horses were however negative at all sampling occasions to EHV-2 (14%).

The role of EHV-2 and EHV-5 in clinically detectable disease and the performance of the athletic horse is debated and information about the pattern of viral load regarding gamma herpesviruses in horse population is scanty. In this comprehensive work the presence of EHV-2 or EHV-5 could not be associated with either clinical respiratory disease or poor performance, which suggests that simply shedding of equine gamma herpesviruses has little, if any, relationship to impaired performance. Furthermore could not levels of SAA be correlated to presence or viral load of EHV-2 and/or EHV-5.

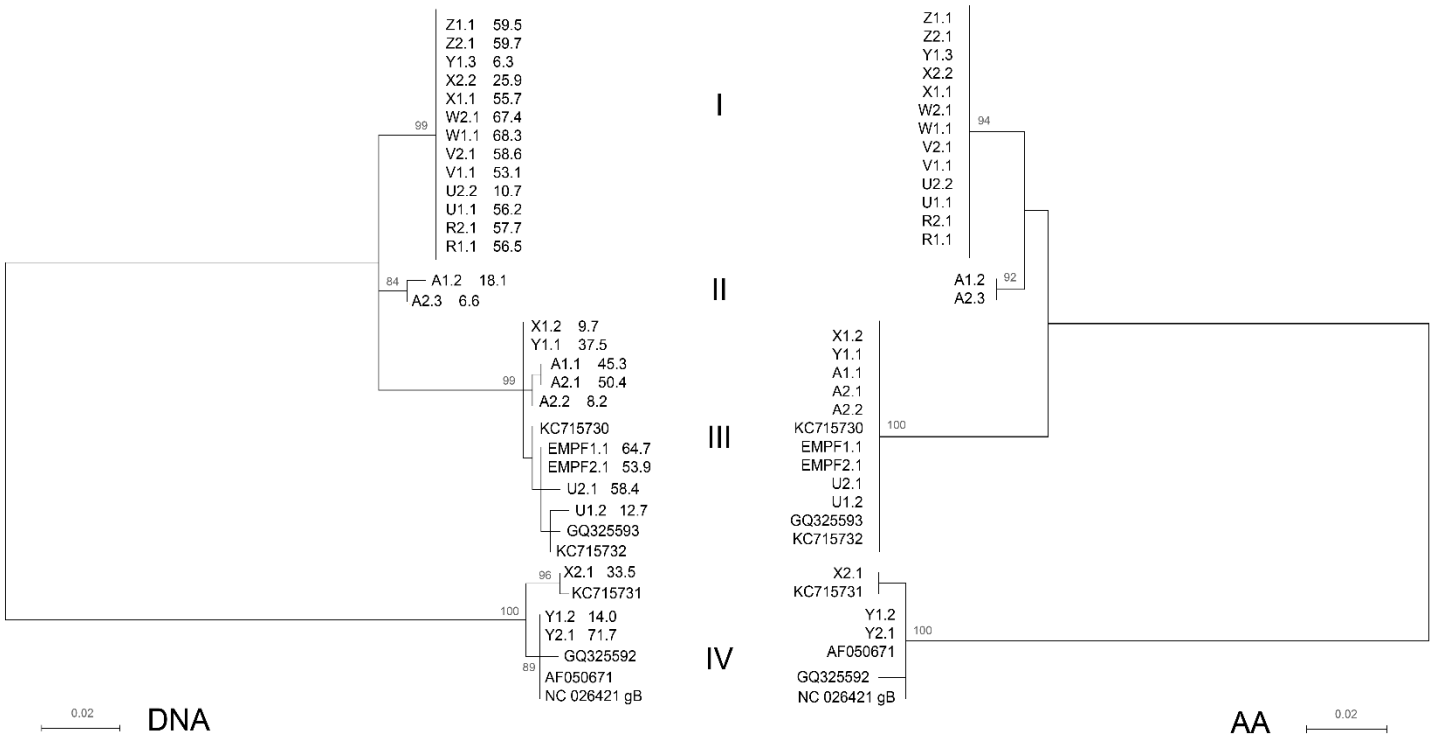
	Training yard 1 (N=348)	**Training yard 2 (N=124)**	**Training yard 3 (N=151)**	**Training yard 4 (N=40)**	**Total (N= 663)**
Neg samples EHV-2	238 (68%)	83 (67%)	110 (73%)	36 (90%)	467 (70%)
Pos samples EHV- 2	110 (32%)	41 (33%)	41 (27%)	4 (10%)	196 (30%)
Neg samples EHV-5	63 (18%)	49 (40%)	49 (32%)	10 (25%)	171 (26%)
Pos samples EHV-5	285 (82%)	75 (60%)	102 (68%)	30 (75%)	492 (74%)
Pos samples EHV-2 & 5 (co-detection)	100 (28%)	35 (28%)	37 (25%)	4 (10%)	176 (27%)

The numbers and proportions of samples (N) collected on multiple occasions from 63 high performing trotters in Sweden that tested positive or negative for equine herpesvirus type 2 (EHV-2) and/or EHV-5 by the qPCR assays. Data were obtained from horses at four different training yards and from the entire study population.

Recent case reports have suggested an association between EHV-5 and EMPF. The pathogenesis of EMPF is however not fully understand and since a high prevalence of EHV-5 also has been described in healthy horses, genetic analysis could provide further understanding of the virus and its interaction with the host. EHV-5 has a large genome which over the years has been little investigated and it was only recently that the whole genome has been sequenced (Wilkie *et al.*, 2015). Against this background, NGS was used in our work to further analyse a segment of the EHV-5 gB gene over time in nine individual horses (eight healthy, one with EMPF) to explore any genetic differences in the EHV-5 detected in horses included in our studies.

The genetic characteristics of gB gene from EHV-5 strains present in samples collected from one EMPF case and in nasal secretions from eight healthy horses sampled twice at one year of interval were investigated. In the 18 samples were 27 different nucleotide sequences (i.e. strains), representing 11 unique sequence types of the partial gB gene of EHV-5, identified. Some individual horses were infected by up to three different strains at the same time. Phylogenetic analysis of the strains resulted in detection of four separate clusters which are here suggested as genotypes (I-IV) of the EHV-5 gB gene. Two of the identified genotypes (I and II) did not match with any previously described strains. The diversity between strains in the phylogenetic tree within the four divergent genotypes was less

than 2% (0 nucleotides (I), 2 nucleotides (II), 0-3 nucleotides (III), 0-8 nucleotides (IV)). Between the genotypes, apart from genotype I and II that diverged by only 9 nucleotides, the divergence ranged from 19-81 nucleotides, where genotype IV diverged most (by 81 nucleotides between genotype III and IV).



Phylogenetic trees based on DNA sequences (left) and translated amino acid (aa) sequences (right) showing four suggested genotypes (I-IV) of EHV-5 gB gene segment identified in nine Swedish horses. The individual strains and sequences are shown within genotypes. Only strains that included >5% of the reads in each clinical specimen were incorporated in the tree. The different clinical specimens are coded with a unique letter for each horse followed by number "1" (first sampling occasion) or "2" (second sampling occasion) and the last number (1-3), illustrate the most (1) and least (3) common strain within each of the clinical specimens. On the left side proportions of the trimmed and paired reads for the genotypes are specified in percentage. 1000 bootstrap replicates were performed for each tree and obtained values are shown where to bootstrap support is >80%. Scale bar corresponds to 2% of the total variation.

The four genotypes were distinctly separated on nucleotide level, as well as on amino acid (aa) level. In total five different aa sequences were identified, where genotype I-III resulted in three different aa sequences for each genotype, and the strains from genotype IV resulted in two unique aa sequences. The results show that aa sequences within each genotype seem to be strongly conserved, but between genotypes they differ considerably. This might affect the structural or conformational constraints on this gB segment, possibly causing genotypes to interact differently with the host.

Three different patterns of EHV-5 infection (based on the partial gB gene) over time were detected in this study. The strains referred to in this section are illustrated in the phylogenetic tree. Infection of a single identical strain (genotype I) on both sampling occasions occurred in four horses (R, V, W and Z), which illustrates a high stability of the viral strains over time. he second pattern was infection of two strains from different genotypes with varying proportions, which was illustrated in two horses (A and U). Horse U shed two strains, one from genotype I and the other from genotype III at both sampling occasions, but the proportions shifted over time. In horse A, also the proportions of the

strains (genotype II and III) varied over time as illustrated in table 2, but not as striking as for horse U. This highlights the influence of the host-virus-environment interaction and may also be explained by the viability of different strains.
In the third pattern appearance and loss of different strains over time was observed in two of the horses (X and Y). Horse X was first identified with strains of genotype I and III, one year later an identical sequence from genotype I was detected together with a new strain classified as genotype IV, and the strain of genotype III was absent. Horse Y first harbored three strains, which were classified in three different genotypes (I, III and IV). One year later genotype IV could be detected. This third pattern feature that the number of strains and genotypes that infect adult animals can be variable, but also the high stability of strains over time. Moreover, the described changes in horse X suggests that infection with a new strain of EHV-5 occurs. Further studies are needed to investigate whether this stability is mainly related to host, virus and/or environmental factors.
The observations in our work shows a range of interactions between EHV-5 and the host over time, where the viruses persist in some horses and others have a more dynamic infection pattern including strains from different genotypes. The eight healthy horses were at the same TY and presumably under the very similar environmental infection pressure. Therefore, the different patterns of viral interaction with the host suggests that individual characteristics such as strain and the genetic background or the immune responses to EHV-5 in the host, may play important roles in the viral dynamic over time. The dynamic infection of EHV-5 was described for the first time in this work, however it remains to further investigate their possible effect on the equine health in a larger study population.

As illustrated in the phylogenetic tree, the sample from the EMPF horse was classified as genotype III, and that it was highly similar (one nucleotide divergence) to the nucleotide sequence (KC715730) obtained from another EMPF case diagnosed in USA (Williams *et al*., 2013). However, other healthy horses in our study IV were also infected with strains of the same genotype. Furthermore, the EMPF case was infected with only one strain of genotype III, whereas healthy horses in which genotype III was detected were co-infected with multiple strains simultaneously, and it cannot, be excluded that genotypes or combinations of strains might be of clinical importance.

Oral presentations (Helena Back)
Hästsektionens vinterkurs. Februari 2014. Sundsvall, Sweden
”Betydelsen av lågvirulenta virus och subkliniska luftvägsinfektioner hos svenska travhästar”

Hippocampus lunchseminarium, SVA. 2014
“Viral load in nasal secretion of equine herpesviruses 2 and 5 in actively racing Standardbred trotters”

The annual Swedish veterinary conference. November 2014, Uppsala, Sweden.
”Luftvägsviroser: Diagnostik, behandling och aktuell forskning”

The Dorothy Russel Havemeyer Foundation IAD Workshop (Equine Inflammatory Airway Disease) October 2014, Normandy, France.
“Viral load in nasal secretion of equine herpesviruses 2 and 5 in actively racing Standardbred trotters”

Nordic Clinical Research Workshop, poor performance in horses. June 2015, SLU, Uppsala, Sweden.
“Viral load in nasal secretion of equine herpesviruses 2 and 5 in actively racing Standardbred trotters”

Hästforskarträff, SLU. September 2015, Uppsala, Sweden
“Low Virulent Respiratory Viruses in Standardbred Trotters, relationship to health and athletic performance”

The annual Swedish veterinary conference. November 2015, Uppsala, Sweden
“Viral load in nasal secretion of equine herpesviruses 2 and 5 in actively racing Standardbred trotters”

Peer reviewed publications

Back H, Ullman K, Treiberg Berndtsson L, Penell J, Ståhl K, Valarcher J-F & Pringle J (2015). Viral load in nasal secretion of equine herpesviruses 2 and 5 in actively racing Standardbred trotters: temporal relationship of shedding to clinical findings and poor performance. *Veterinary Microbiology* 179(3-4), 142-148.

Back H*, Ullman K*, Leijon M, Söderlund R, Penell J, Ståhl K, Pringle J & Valarcher J-F (2015). Genetic variation and dynamics of infections of equine herpesvirus type 5 (EHV-5) in individual horses (accepted in Journal of General Virology).
*shared first authorship

Doctoral thesis

Low Virulent Respiratory Viruses in Standardbred Trotters, relationship to health and athletic performance
http://pub.epsilon.slu.se/12569/
The doctoral thesis was defended at SLU 25th of September 2015 by Helena Back, DVM.