Frequent presence of hepaci and pegiviruses in commercial equine serum pools

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Abstract

Novel viruses belonging to the genera *Hepacivirus* and *Pegivirus* have recently been discovered in horses and other animal species. Viral genomes of non-primate hepaciviruses (NPHV), equine pegivirus 1 (EPgV 1) and Theiler's disease associated virus (TDAV) were detected in a horse serum routinely used for cell culture propagation in our laboratory. Therefore, a study was carried out to further investigate the presence of these human Hepatitis C virus (HCV) related viruses in equine serum based products used in veterinary medicine and for research and to characterize the viral genomes. Without exception all commercially available equine sera purchased for cell culture propagation (n=6) were tested positive for NPHV, EPgV 1 or TDAV genomes by qRT-PCR. Molecular analyses of one single commercial horse serum from Europe confirmed multiple viral genomes, including two TDAV genomes significantly different from the only published TDAV sequence. Furthermore, multiple batches of horse serum pools (n=35) collected for manufacturing of biological products turned out to be positive for NPHV and EPgV 1 genomes. Nevertheless, the final commercial products (n=9) were exclusively tested qRT-PCR negative. Field samples (n=119) obtained from two premises located in the same region as the donor horses were analyzed, demonstrating the frequent presence of NPHV and EPgV 1, but the absence of TDAV genomes.

The presence of NPHV, EPgV 1 and TDAV in commercial equine sera and serum based products could have considerable consequences for biosecurity and may also bias the outcome of research studies conducted with related viruses.

Introduction

Recently, novel members of the family *Flaviviridae* have been discovered in horses, belonging to the genera *Hepacivirus* and *Pegivirus* (Burbelo et al., 2012; Chandriani et al., 2013; Kapoor et al., 2013a; Pfaender et al., 2014a). Non-primate hepacivirus (NPHV) infections in horses have some features in common with hepatitis C virus (HCV) infections in humans and thus might be interesting as a surrogate model (Pfaender et al., 2014b; Ramsay et al., 2015; Scheel et al., 2015a; Scheel et al., 2015b). In addition to NPHV, two different species of pegiviruses in horses have been recently identified, the equine pegivirus 1 (EPgV 1) and the "Theiler´s Disease Associated Virus" (TDAV) (Chandriani et al., 2013; Kapoor et al., 2013a). In contrast to NPHV and EPgV 1 where no disease connection is known, TDAV is associated with a fulminant serum hepatitis ("Theiler´s disease") occurring after treatment with equine serum products (Chandriani et al., 2013; Kapoor et al., 2013a). About 0.9%-2.5 % of equine sera from different European countries were found to contain NPHV RNA (Lyons et al., 2014; Pfaender et al., 2014b). An overall NPHV RNA prevalence of approx. 2.5% was observed in Northern Germany, but a significantly higher prevalence of 10.4% was found in Thoroughbred horses (Pfaender et al., 2014b). Reasons for this observation remain elusive, but it can be speculated that genetic determinants or alternatively an increased exposure to the pathogen might be responsible. Interestingly, a comparatively high NPHV genome prevalence of 22.6% (7/31 sera) was recognized in Japanese-born domestic horses (Tanaka et al., 2014). EPgV 1 genomes were detected with 9.5% prevalence (7/74 horses) in the US and approximately 3.8% prevalence (12/328 horses) in Scotland, England and France (Kapoor et al., 2013a; Lyons et al., 2014). Despite the first identification of TDAV in horses with Theiler's Disease in the USA, hitherto no further TDAV has been reported (Chandriani et al., 2013).

The presence of hepaci- and pegiviruses in equine sera could have significant consequences. Horse sera are commonly used for the production of anti-sera. Such anti-sera are licensed for

various treatments of different animal species including tetanus prophylaxis as well as for treatment of humans with snake antivenom immunoglobulins and botulism antitoxin (Anonymous, 2015). In addition, other biologicals like modified live vaccines might contain infectious NPHV, EPgV and TDAV, in particular when the cell lines used for virus propagation are grown under supplementation of horse serum. Transmission via blood products and contact with infectious blood is well documented for human hepacivirus (Hepatitis C Virus, HCV) and human pegivirus (hPgV, formerly GBV-C or HGV) (Feucht et al., 1997; Fiordalisi et al., 1997; Roth et al., 1997). A recent study demonstrated that transmission of NPHV by plasma transfusion is possible and that a hepatitis can be induced in horses (Ramsay et al., 2015). It was recognized for a long time that administration of equine blood products can induce “Theiler’s disease” in horses and such a case was also the source for the discovery of TDAV in 2013 (Chandriani et al., 2013).

Starting point of the study presented here was the detection of NPHV, EPgV 1 and TDAV genomes in a single commercial horse serum that we routinely use for the propagation of cell cultures used for infection studies. This observation prompted us to investigate the presence of NPHV and pegivirus genomes in equine sera in order to shed light on the biosecurity aspect of commercially available horse sera and in products containing horse sera. Independently of the geographic origin, high amounts of NPHV and pegivirus RNA could be detected in serum batches used for commercial purposes. Furthermore, the presence of TDAV in commercially available equine sera of European and South American origin is demonstrated.

Materials and methods

Serum samples

Undefined serum pools of different geographic origin were tested (n=41), including commercially available equine sera for cell culture propagation (n=6) and serum pools (n=35)

from serum donor horses. Geographical origin of equine sera for cell culture propagation is given as certified by the manufacturer (Table 2). The donor horses originate from one premise in Northern Germany. For both kinds of serum samples information about the number of individual sera present in these pooled sera is not available. Finally, five different batches of the commercially available tetanus anti-serum (company Wirtschaftsgenossenschaft Deutscher Tierärzte; WDT) and four other veterinary products (Prevaccinol, MSD; Feliserin Plus, IDT; ProteqFlu-Te, Merial; Equilis Tetanus Vaccine, Merial) were screened for the presence of viral genomes. Individual horse sera (n=119) were obtained from the Clinic of Horses, University of Veterinary Medicine Hannover, Germany, and were already investigated for presence of NPHV genomes in a previous study (Pfaender et al., 2014b). The sera were taken as part of routine health management in two premises of the same region where the premise of donor horses is located (Northern Germany) and analyzed for the presence of viral genomes to obtain information about the regional epidemiological situation in the field.

Genome detection by multiplex real-time reverse transcription (RT)-PCR

RNA from serum samples was prepared with the QIAamp Viral RNA kit according to the manufacturers' recommendations. During RNA purification, 10 µl internal control RNA (*intype* IC-RNA, Qiagen, diluted 1:1 in RNA-safe buffer) was added. Five microliters (µl) of the final RNA preparation were mixed with 20 µl of Superscript III One-Step RT mastermix (Invitrogen) containing 2 µl of the primer/probe mix (Table 1).

For detection and simultaneous differentiation between EPgV 1 and TDAV-, a primer pair was designed matching with conserved regions of the reference sequences [KC145265, KC410872]. Specific probes enabled a differentiation without the need for subsequent sequencing. The primer/probe mix included 10 pmol/µl of the generic primers and 1.25 pmol/µl of the probes for specific identification of EPgV 1 (WST-189 probe) and TDAV

(TDAV-199 probe) viral genomes. Furthermore, an internal control based on an EGFP transcript was included (2.5 pmol/µl primers, 1.25 pmol/µl probe) as described previously (Hoffmann et al., 2006). The synthetic internal control RNA (EGFP) allows to determine the individual PCR performance and to indicate possible inhibitions. In addition, a previously published NPHV-specific real-time RT-PCR was set-up with the same internal control RNA as a duplex real-time PCR (Burbelo et al., 2012).

Quantification of genome loads

To obtain defined RNAs that serve as copy standards in qRT-PCR, partial 5'NTR sequences of NPHV and EPgV 1 were amplified and subsequently cloned into the plasmid pCR2.1 (Invitrogen). As template for PCR reaction, RNA obtained from the serum of a horse (designated "WST") with an EPgV 1/NPHV co-infection was used (Pfaender et al., 2014b). The cloned TDAV sequence was synthesized according to the only available TDAV sequence [KC145265] representing the amplicon generated with the previously published primers EVT-146/ EVT-146 (Chandriani et al., 2013). *BamH*I linearized plasmids were used to transcribe *run-off* RNAs of defined size with a T7 transcription kit (MEGAscript T7, LifeTechnologies). After removal of plasmid DNA by Turbo DNase (MEGAscript T7, LifeTechnologies) RNA was subsequently purified with the MegaClear kit (Ambion) and the amount of RNA was determined photometrically (NanoDrop). Mean values of three measurements were used to prepare RNA dilutions as copy standards for real-time RT-PCRs.

Molecular characterization of viral genomes

A generic conventional RT-PCR detecting a conserved part in the NS3 coding region (amplicon: 355 nucleotides) of EPgV 1 [KC410872] and TDAV [KC145265] was developed to confirm the results of the real-time PCR (targeting the 5´-NTR) and to unravel the diverse EPgV 1 and TDAV pegivirus sequences. This conventional RT-PCR was performed with the

NS3-specific primers TDAV-3744_fw and TDAV-4098_rev (Table 1) in a Thermostart mastermix (Thermo Fischer Scientific). For amplification of NPHV sequences, the previously established primers EQNS3OS, EQNS3OAS, EQNS3IS, EQNS3IAS were employed and PCR was performed utilizing the LongAmp Taq DNA polymerase (NEB) (Lyons et al., 2012). The resulting PCR products encompassing NS3 specific sequences (amplicon NPHV: 173 nucleotides, amplicon EPgV 1 and TDAV: 355 nt) were cloned (TOPO TA kit, Invitrogen) and used for subsequent Sanger sequencing (LGC genomics, Berlin). As described previously, genetic distances of the partial NS3 encoding sequences were calculated with the Kimura 2-parameter substitution model and phylogenetic trees were calculated by the Neighbor-Joining method (Postel et al., 2012). In addition, accuprime polymerase was used according to the manufacturers' recommendations (Life Technologies, Darmstadt) for amplification of ~4 kB fragments derived from EPgV 1 and TDAV genomes (5'NTR-NS3 region) by semi-nested PCR. In the first round, the generic primers EVT-146/TDAV-4098r were used, in the second PCR round the forward primer EVT-146 was combined with a reverse primer specific to the different TDAV variants, designated B2 and B10 (primers TDAV-B2_4002r, TDAV-B10_4002r). Double stranded Sanger-Sequencing was performed by primer walking using TDAV-B2 and TDAV-B10 specific primers as listed in Table 1.

Results

Development of a highly sensitive pegivirus specific triplex real-time RT-PCR

The designed primers proved to bind specifically to synthetic run-off transcripts of TDAV as well as EPgV 1 RNA. Analytical sensitivity of the triplex real-time RT-PCR for EPgV 1 and TDAV genome detection was approx. 10 genome equivalents per reaction. High amounts of EPgV 1 or TDAV RNA (10^5 genome equivalents/PCR reaction) did not produce cross-reactivity of the probes. Moreover, results of the 5'NTR-specific real-time multiplex RT-PCR were confirmed by the conventional PCR targeting to the NS3 region. Comparison of both

assays with the EPgV 1 positive individual hose sera, revealed the sensitivity to be 100-1000 times higher compared with the conventional PCR, which was primarily designed to produce larger PCR products for genetic typing. Due to the lack of individual sera positive for TDAV it was not possible to test the accuracy of the multiplex real-time RT-PCR with respect to TDAV genome detection. An internal control allowed validating the RNA purification and amplification process of each individual reaction.

Molecular characterization of viral genomes from one cell culture serum

The developed triplex real-time PCR revealed high viral genome titers in a commercial horse serum routinely used for cell culture propagation in our laboratory (Table 2, serum #6). Five additional cell culture sera were purchased and all of them turned out to be positive for at least one viral genome species (Table 2, sera #1-5). All serum batches were positive for EPgV 1 RNA, including a fetal horse serum, which was the only one to be negative for NPHV genome (Table 2). NPHV genome titers were very low in commercial sera originating from Germany and Chile. TDAV-like pegivirus genomes were found in three serum pools originating from the same company, but certified to be of different geographical origin, namely Germany, Italy and Chile. The TDAV RNA loads from the serum pool derived from Germany were near the detection limit of the real-time PCR, whereas the Italian horse serum #6 contained comparably high amounts of EPgV 1 and TDAV genomes (1.0×10^5 and 1.3×10^5 copies/ml), respectively. Molecular characterization was performed to unravel the different viral genomes in this serum of Italian origin (Fig. 1). NPHV-specific nested PCR, subsequent cloning and analysis of partial NS3 encoding sequences (127 nucleotides) obtained from 20 independent clones (M42, M43, M45, M46, M49, M50, M54, M58, M60, M61, M76, M78, M79, M83, M88, M97, M100, M102, M108, M110) revealed the presence of at least six different NPHV genome types in this commercial serum (Fig. 1A). Accordingly, six GenBank entries were created (corresponding to clones with underlined

numbers; GenBank KT795396-KT795401) representing in some cases several clones with identical sequences (Fig. 1A). All of the identified NPHV sequences were different to the sequence obtained from the "WST" NPHV RT-PCR control [KT795395]. Genetic differences of the sequences obtained from the commercial horse serum were within the range of previously reported genetic diversity and displayed genetic differences of up to 10% to previously reported sequences (M79 and KP640275) and up to 21% difference among each other (M79 and M88). For characterization of the pegivirus genomes, a newly established conventional RT-PCR with subsequent cloning and sequencing was performed. From 20 sequenced plasmids with partial NS3 encoding sequences (313 nucleotides), nine were found to contain EPgV 1 sequences (clones M1, M4, M5, M6, M7, M8, M9, B1, B7) and eleven were most similar to the TDAV reference sequence (clones M2, M3, M10, B2, B3, B4, B5, B6, B9, B10, B11). Plasmids M6 and M9 contained identical EPgV 1 sequences and plasmids B2, B3, B4, B5, B6 and B9 contained identical sequences. For clones with identical sequences only one GenBank entry was created (unterlined clone numbers, GenBank KT795381-KT795394). The generated pegivirus sequences segregated into four distinct phylogenetic groups (Fig. 1B). All of the nine EPgV 1 sequences were distinct to the EPgV 1 reference sequence "C0035" (6-10% genetic distance) and the PCR control "WST" [KT795380]. EPgV 1 sequences displayed up to 12% genetic distance among each other, but only for two sequences this led to one single altered amino acid. The eleven TDAV sequences were all clearly distinct to the reference sequence which originated from an American horse serum (15-16% genetic distance). Interestingly, the obtained sequences clustered in two phylogenetic groups (12-13% genetic distance to each other) represented by four (group 3; represented by clone B2) and seven (group 4; represented by clone B10) independent clones (Fig. 1B). The 42-44 nucleotide differences compared to the TDAV reference sequence were almost exclusively synonymous mutations as only in three of eleven sequences one or two amino acid residues (out of 104) were changed.

By design of TDAV specific primers it was possible to sequence 3,887 nucleotides of the 5`NTR-NS3 region from one TDAV genome (group 3) directly from the amplicon obtained in the semi-nested PCR with the TDAV-B2 specific reverse primer TDAV-B2_4002r. Direct sequencing of the amplicon obtained in the semi-nested PCR with the TDAV-B10 specific reverse primer TDAV-B10_4002r gave no clean signals and therefore separation of the B10 variant from accompanying TDAV sequences was not possible. The obtained TDAV sequence of variant B2 (GenBank KT795391) showed an overall genetic distance of 9% on nucleotide level to the published TDAV sequence. The deduced amino acid sequence of 1153 amino acids revealed a high degree of conservation with only 22 changes in amino acid composition resulting in 98% identity to the TDAV reference sequence from the US and about 38% identity to the EPgV 1 reference sequence. The same range of identity in the amino acid compositions (37-40%) was observed when compared to pegiviruses of bat, simian and human origin.

Presence of viral genomes in serum pools of donor horses and in equine serum-derived veterinary products

To further estimate the possible biosecurity risk of biologicals produced with equine sera, 35 undefined horse serum pools used as raw sera for manufacturing of veterinary products were analyzed for the presence of viral RNA species. Out of the 35 serum pools (number and volumes of individual sera not known), 24 pools (68.6%) were tested positive for NPHV genomes and 25 pools (71.4%) contained EPgV 1 genomes, respectively. Combining the calculated genome titers with the results obtained with the six sera for cell culture propagation (n=41 sera, Fig. 2), the calculated mean genome loads were higher for NPHV (9.2×10^5 copies/ml) than for EPgV 1 (4.8×10^4 copies/ml). Even though the majority of serum pools contained high loads of viral genomes, the five different final batches of tetanus antiserum

(WDT) as well as the four other veterinary products (Prevaccinol, MSD; Feliserin Plus, IDT; ProteqFlu-Te, Merial; Equilis Tetanus Vaccine, Merial) were tested negative.

Genome prevalence of individual equine serum samples

Individual horse sera from the field (n=119) were included in the study to monitor the regional epidemiological situation with respect to virus prevalence and to validate the finding of genome titer differences between EPgV 1 and NPHV observed in the pooled sera from donor horses. Sera from 10 out of 119 horses (8.4 %) originating from two premises in Northern Germany revealed to be NPHV RNA positive as reported previously (Pfaender et al., 2014b). All RNA positive sera originated from a premise with Thoroughbred horses resulting in an extraordinary high prevalence of 12.8 % (10/78). In contrast, EPgV 1 genomes were detected in 16 sera (13.5%) from both premises (suppl. Fig.). No apparent differences in EPgV 1 RNA prevalence were observed between the premise with Warmblood horses (12.2 %) and the premise with Thoroughbreds (14.1%). In all cases, multiplex PCR identified the pegiviruses as EPgV 1 and no TDAV was detected. Interestingly, four out of ten (40%) NPHV positive samples revealed a simultaneous infection with EPgV 1 (suppl. Fig.). The mean value of genome loads was conspicuously higher for NPHV (1.5×10^7 genome equivalents/ml) than for EPgV 1 (1.7×10^5 genomes/ml) (Fig. 2).

Discussion

Novel pegi- and hepaciviruses have been recently discovered in various animal species including horses, dogs, cattle, rodents, and bats (Baechlein et al., 2015; Burbelo et al., 2012; Chandriani et al., 2013; Corman et al., 2015; Drexler et al., 2013; Gagnieur et al., 2014; Kapoor et al., 2013a; Kapoor et al., 2013b; Quan et al., 2013; Sibley et al., 2014). The aim of our study was to determine a potential risk associated with the presence of NPHV and pegiviruses in medical and research products for life sciences that contain equine serum.

Here, it was demonstrated that one single cell culture serum purchased for cell culture propagation contained fairly high amounts of multiple NPHV, EPgV 1 and TDAV genomes. Detailed genetic characterization revealed a close relationship of NPHV and EPgV 1 sequences to recently published sequences. The presence of TDAV genomes was of particular interest, as TDAV genomes were detected for the first time in equine sera from outside the USA, namely in commercial serum pools from Chile, Germany and Italy. As all sera were purchased from the same company, a cross-contamination of two sera with low viral genome content (from Chile and Germany) during manufacturing process cannot be excluded. A further verification was not possible as no TDAV sequences could be obtained from these two sera. In contrast, the higher TDAV genome loads in the commercial serum from Italy enabled a more detailed molecular characterization. Due to the high complexity of this pooled serum (containing at least six different NPHV, nine EPgV 1 and two TDAV genomes) and possibly also due to sample quality (long term storage of serum), it was not possible to establish complete TDAV genomes by deep-sequencing of prepared RNA (data not shown). Nevertheless, genotyping of partial NS3 encoding sequences revealed two TDAV variants (named B2 and B10) that are clearly distinct at nucleotide level. The nucleotide sequences of the European TDAV variants B2 and B10 are also significantly different from the TDAV sequence from the USA, whereas the respective deduced amino acid sequences are almost identical, suggesting highly conserved functions of the viral proteins. This result was confirmed by determination and analysis of a 3,887 nt long sequence fragment of the TDAV-B2 genome. Several attempts to generate further long-range PCR amplicons of the two TDAV genomes were unsuccessful. Nevertheless, the genetically distinct TDAV genomes from Europe and Chile were detected by the newly developed triplex real-time PCR and the conventional NS3-specific PCR, thus proven to be useful tools to screen for pegivirus RNA and to discriminate between EPgV 1 and TDAV genomes. Of note, analysis of the generated sequences revealed that previously used TDAV specific primers (EVT-162/EVT-163) are not

306 targeting well conserved regions in the NS3 coding region of the TDAV genome (Chandriani
307 et al., 2013; Lyons et al., 2014). The primer EVT-162 showed 1 and 4 mismatches, the primer
308 EVT-163 displayed 3 and 4 mismatches compared to the B2 and B10 TDAV sequences,
309 respectively. The generic primers used in the presented study targeting to the 5'NTR and to
310 the NS3 coding region were able to detect EPgV 1 as well as TDAV, including the different
311 TDAV variants so far known. Nevertheless, determination of further TDAV sequences will be
312 necessary to prove the suitability and broad reactivity of the designed primers/probes.
313 With respect to the biosecurity aspect, the presence of viral genomes in cell culture sera
314 illustrates that these commercial sera can contain a mixture of different viruses comprising
315 NPHV, EPgV 1 and also the more rare TDAV and therefore can be regarded as potentially
316 infectious for susceptible hosts. Different viral genomes were found independently of the
317 geographical origin of the serum pools. This observation is in line with reports on NPHV
318 genomes found in commercial equine serum pools originating from New Zealand and the US
319 (Burbelo et al., 2012; Scheel et al., 2015a). Contaminations of commercial equine sera with
320 EPgV 1 and TDAV have not been reported so far, but data from field sera are available. A
321 recently published study reported a 3.7% (12/328 horses) prevalence of EPgV 1 viraemic
322 horses in different European countries, but no infections with TDAV (Lyons et al., 2014). In
323 the presented study, analysis of two large horse breeding premises in Northern Germany
324 showed a uniformly EPgV 1 prevalence of 13.5% independently on the observed differences
325 in NPHV prevalence, but here also no TDAV was detected. Although the available data
326 suggest that TDAV infections occur less frequently than infections with EPgV 1 or NPHV,
327 our results demonstrate that the risk of TDAV presence in commercial equine sera pools
328 nevertheless is not negligible. So far, infectivity of the sera could not be determined as
329 permissive cell lines for these novel viruses are not available. However, the high number of
330 genome equivalents found in the different serum pools suggests a considerable risk of
331 infection for susceptible hosts. On the other side, all selected batches of veterinary products

and commercial anti-sera tested negative for viral RNA. This indicates that the viral RNAs were removed during the manufacturing processes of the tested products which make a residual infectivity very unlikely. In consequence, absence of viral RNA in equine raw sera or proper treatment of biological products including robust virus inactivation should be ensured to prevent NPHV, EPgV 1 and TDAV transmission via medical products. Elucidation of host ranges, transmission routes and their efficacy will require further investigations and finally provide a solid basis to assess the risk of infection via the use of equine derived biologicals.

Many cell culture systems are dependent on the supplementation of serum. Serum, tissues and fluids of bovine origin are often used in biotechnology and for the production of medical products. Contaminations with ruminant pestiviruses, members of a related genus in the family *Flaviviridae*, are a frequently recognized problem worldwide (Castro et al., 1997; Levings and Wessman, 1991; Uryvaev et al., 2012). In some countries, due to legal regulations, the absence of pestiviruses in bovine sera has to be demonstrated (Anonymous, 2001; Blumel and Stuhler, 2010; Robertson, 2006). In the EU, a "Guideline on the use of bovine serum in the manufacture of human biological medicinal products" as well as regulations for testing veterinary vaccines were implemented to improve the safety of biological products for use in humans and animals (Anonymous, 2010, 2012; Bruckner, 2010). To avoid the risk of pestivirus-positive sera, cell lines can be adapted to equine sera or serum-free medium. The alternative use of equine sera in cell culture is of particular interest for research groups studying pestiviruses, HCV or other related viruses (Yanagi et al., 1996). Besides the biosecurity aspect, the presence of viral RNA, infectious virus or cross-reacting antibodies in sera used for cell-culture propagation might also interfere with research studies, in particular those dealing with HCV and related viruses. Moreover, contamination of sera with viral RNAs must also be considered when applying highly sensitive, unbiased methods like deep-sequencing from cell culture derived virus isolates.

In conclusion, the recent discovery of equine NPHV, EPgV 1 and TDAV requires a thorough evaluation of potential risks regarding the application of equine sera. Procedures for testing of equine raw sera and blood derived products should include specific assays for detection of these viruses. A careful risk assessment requires determination of transmission routes and detailed investigations of the host range of these novel pegi- and hepaciviruses.

Conflict of interest statement

The authors declare that they have no competing interests.

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480

481 **Table 1: Primers and probes used in this study.**

Primer/probe	Sequence (5´-3´)	Target	Purpose	Ref.
EVT-146	AGGGTTCTTCGGGTAAATCC	5´NTR	triplex Pegivirus real-	1
EPgV-314r	TCGKCGAGCYACAGACCGT		time RT-PCR	4
TDAV-199 probe [TEX-BHQ2]	TGTTTTGGGTTCAGGGCAGTAG GGGCA			
WST-189 probe [6FAM-BHQ1]	TGTTGTGATTGTGTTAGGGCAG GTGGCA			
Qanti-5UF1	GAGGGAGCTGGAATTCGTGAA	5´NTR	duplex NPHV	2
Qanti-5R1	GCAAGCATCCTATCAGACCGT		real-time RT-PCR	
NPHV probe [6FAM-TAMRA]	CCACGAAGGAAGGCGGGGGC			
EGFP1-F	GACCACTACCAGCAGAACAC	EGFP	internal control for	3
EGFP2-R	GAACTCCAGCAGGACCATG		real-time RT-PCR	
EGFP probe [HEX-BHQ1]	AGCACCCAGTCCGCCCTGAGCA			
TDAV-3744f	GGAGCCCGGAGCGCATGGGTA	NS3	sequencing amplicon,	4
TDAV-4098r	TGGCAGGGACAAGGGTGGACT		sequencing	
TDAV-B10_4002r	CATCCGTGACGGTCTGGGCA	NS3	semi-nested PCR, sequencing	4
TDAV-B2_4002r	CATCCGTGACAGTTTGAGCG		semi-nested PCR, sequencing	4
TDAV-B2_1070f	GGAAATTGTCTGGGGTTTGC		sequencing	4
TDAV-B2_1204r	ACGAGCATGATCGCAAGGTG		sequencing	4
TDAV-B2_1932f	ACCAAGCAGAGGTGGAATGC		sequencing	4
TDAV-B2_2055r	TAGTGCGTCCAGCCAAAAGC		sequencing	4
TDAV-B2_2818f	TATTTGCTTGGGCGGTGTTG		sequencing	4
TDAV-B2_3021r	CAACCTCGTTAGGCCAAACC		sequencing	4

482 1, Chandriani *et al*., 2013;

483 2, Burbelo *et al.*, 2012;

484 3, Hoffmann *et al.*, 2006

485 4, this study.

Table 2: Pegivirus- and NPHV-RNA in commercially available pooled hose sera used for cell culture propagation.

Sample characteristics			Result of qRT-PCR [genome equivalents/ml serum]		
Serum ID	Specification	Origin	NPHV	EPgV 1	TDAV-
1	Horse Serum, sterile	Canada	1,4E+04	7,3E+05	-
2	Horse Serum	Chile	1,4E+03	5,9E+04	-
3	Horse Serum, inactive	Chile	7,2E+01	5,1E+04	5,1E+03
4	Fetal Horse Serum	Costa Rica, Brazil, Mexico	-	1,4E+03	-
5	Horse Serum	Germany	5,0E+01	1,8E+05	9,7E+02
6	Horse Serum	Italy	1,5E+04	1,0E+05	1,3E+05

Figure 1: Genetic characterization of NPHV (A) and pegivirus (B) genomes found in a single commercial horse serum of Italian origin.

Neighbor-joining tree of partial NS3 encoding sequences of NPHV (A) and pegiviruses (B) obtained from one commercial pooled horse serum (serum ID 6, Table 2). For phylogenetic analysis of NPHV (A), sequences (127 nucleotides) from 20 recombinant plasmids with viral RNA derived PCR amplicons (bold) were compared with related NPHV reference sequences obtained from GenBank [Acc. No.]. For pegivirus phylogeny (B) pegivirus specific sequences (313 nucleotides) obtained from 20 plasmids are compared with the reference sequences of EPgV 1 "C0035" and TDAV and selected pegivirus sequences from other host species [Acc. No.]. "WST" pegivirus [KT795380] and hepacivirus [KT795395] sequences were obtained from a German horse with a co-infection and served as positive control in the RT-PCRs. To increase clarity, pegivirus sequences are summarized in sequence groupsrepresenting EPgV 1 (group 1 = seven sequences, group 2 = two sequences) and TDAV (group 3 = seven sequences; group 4 = two sequences). Only significant bootstrap values (>700) of major nodes are shown. The phylogenetic trees were generated with the software Dendroscope (Huson and Scornavacca, 2012).

Figure 2: Genome loads of NPHV and pegivirus RNA positive sera.

Box-and-whisker plots depicting the quartiles of genome copies/ml serum calculated for undefined serum pools comprising raw sera from donor horses and commercial sera for cell culture propagation (n=41) as well as for individual horse sera (n=119). Results of quantitative real-time RT-PCR assays specific for NPHV, EPgV 1 and TDAV are shown (GE = genome equivalents). Mean values are indicated by diamonds.

Supplementary Figure: Individual genome titers of NPHV and pegivirus RNA positive horse sera.

Mean of duplicates obtained from NPHV and pegivirus-specific quantitative real-time-RT-PCRs of 119 individual horses sera from two premises (A: Thoroughbred horses n=78, B:

515 Warmblood horses n=41). Given is the logarithm of genome equivalents (GE) per milliliter

516 serum.