Vertical transmission of hepatitis C virus-like non-primate hepacivirus in horses

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Non-primate hepacivirus (NPHV), a recently discovered hepatotropic virus infecting horses, is phylogenetically the closest known homologue of hepatitis C virus (HCV). The main route for acquiring HCV infection in childhood is vertical transmission. However, nothing is known about the natural mode of transmission for NPHV. To investigate the possibility of vertically transmitted NPHV infection in horses, 20 Thoroughbred broodmares and their foals were monitored during foaling season 2015 until 6 months post-partum. Prepartal serum was taken from the mares, and during foaling umbilical cord blood and colostrum samples were collected. Postnatal serum samples were taken from the foals after delivery. In addition, serum was taken at 3 and 6 months after foaling from all mares and foals. Samples were analysed for the presence of NPHV RNA by quantitative real-time PCR and for the presence of anti-NPHV NS3 antibodies by luciferase immunoprecipitation system. Identified NPHV isolates were sequenced and phylogenetic analysis of the viral glycoproteins was used to track the course of naturally occurring infections and the circulation of distinct isolates within the herd. At parturition, 16 mares were seropositive, including four viraemic mares. Vertical transmission occurred in one of these four mare–foal pairs. Interestingly, NPHV isolates of newly infected foals and mares after 3 and 6 months cluster in their respective pasture herds suggesting another horizontal route of transmission.

INTRODUCTION

Non-primate hepacivirus (NPHV) is a hepatotropic equine virus, which was described for the first time in 2011 (Burbelo et al., 2012; Kapoor et al., 2011). NPHV was initially detected in respiratory samples of domestic dogs and was tentatively designated as canine hepacivirus (CHV) (Kapoor et al., 2011). However, in subsequent investigations it became apparent that a majority of horses carried the virus (Burbelo et al., 2012; Drexler et al., 2013; Lyons et al., 2012, 2014; Pfaender et al., 2015). Consequently, the virus has been assigned to horses as the presumed natural host and was subsequently renamed as NPHV (Burbelo et al., 2012; Kapoor et al., 2011; Lyons et al., 2012). Recently, several new members of the hepaciviruses have been identified in other mammalian species, including bats, rodents, non-human primates and cattle (Baechlein et al., 2015; Corman et al., 2015; Drexler et al., 2013; Firth et al., 2014; Kapoor et al., 2013; Lauck et al., 2013; Lyons et al., 2014; Quan et al., 2013; Scheel et al., 2015). NPHV has been assigned to the family Flaviviridae and to the genus Hepacivirus. To
date, it represents the closest known homologue of hepatitis C virus (HCV). NPHV has a high prevalence in horses with 2–7% carrying NPHV RNA and 30–40% seropositivity (Pfaender et al., 2014; Burbelo et al., 2012). Most studies report a subclinical course of infection with only mild elevation of liver enzymes at seroconversion in some affected horses (Lyons et al., 2012; Pfaender et al., 2015). Nevertheless, there have been reports about severe hepatitis in horses with concurrent detection of NPHV RNA, indicating NPHV may represent a potential causative agent for equine serum hepatitis (Ramsay et al., 2015; Reuter et al., 2014). In addition to NPHV, the equine pegivirus 1 (EPgV 1) and Theiler’s disease-associated virus (TDAV) were identified in horses representing new related species of the family Flaviviridae within the genus Pegivirus (Chandriani et al., 2013; Kapoor et al., 2013; Postel et al., 2016). The clinical relevance of both viruses remains unclear, although TDAV has been discovered in the context of an outbreak of equine serum hepatitis (Chandriani et al., 2013). So far, for all three equine viruses, the natural route of transmission still remains elusive.

HCV is a blood-borne virus and transmission occurs mainly via the parenteral route (Alter, 2007). Nevertheless, alternative modes of transmission have been described to contribute to the spread of HCV. Among these, vertical transmission represents the leading route of acquiring a childhood HCV infection. Mother-to-child transmission (MTCT) occurs in 5% of infected mothers and affects prepartally viraemic mothers with only few exceptions (Tово et al., 2016). Exact timing and mechanisms of MTCT are unclear, although intruterine and intrapartum transfer of HCV has mainly been described (Mast et al., 2005; Resti et al., 1998; Tovo et al., 2016).

This study aimed to investigate the incidence of vertical transmission of NPHV, TDAV and EPgV 1 in horses. Serum samples from 20 mare–foal pairs were taken at parturition as well as at 3 and 6 months post-partum and analysed for the presence of viral RNA of NPHV, TDAV and EPgV 1 as well as anti-CHV/NPHV NS3 antibodies. Identified NPHV isolates were sequenced to identify the circulation of certain isolates within a herd.

**RESULTS**

**Vertical transmission of NPHV from mare to foal**

In this study, we investigated the vertical transmission of NPHV from mare to foal. Therefore, 20 Thoroughbred broodmares and their foals were monitored before parturition until 6 months post-partum. Prepartal serum of the mares, umbilical cord blood, colostrum and postnatal serum of the foals was taken at the time of foaling. All collected samples were analysed for the presence of NPHV RNA by a quantitative SYBR Green based real-time PCR (qRT-PCR). As shown in Fig. 1, four out of 20 mares (3.0, 10.0, 18.0 and 19.0) carried NPHV RNA at parturition (Fig. 1, left panel). Of note, out of the four respective foals, foal 3.1 was NPHV RNA positive after birth (Fig. 1, right panel). In three other respective foals, no virus could be detected. In addition, anti-CHV/NPHV NS3 antibodies were determined by luciferase immunoprecipitation system (LIPS) revealing that 16 out of 20 mares were seropositive before parturition (Table 1). Analysis of the colostrum and umbilical cord blood of the seropositive mares revealed that all colostrum samples were seropositive for NPHV-specific antibodies (Table 1). However, umbilical cord blood samples were seronegative (Table 1), indicating passive transfer of antibodies from the mares to their foals via colostrum. Representative for the passive transfer in the 16 mare–foal pairs, anti-CHV/NPHV NS3 antibodies are illustrated for mare–foal pair 3, 10 and 19 in Fig. 2. For mare–foal pair 18, no colostrum and umbilical cord blood samples could be collected. Interestingly, for mare–foal pair 3, where NPHV RNA was postnatally detected in the foal 3.1, NPHV RNA was also detected in the umbilical cord blood (Fig. 2, upper left). In contrast, umbilical cord blood of pairs 10 and 19 were negative for NPHV RNA, as were all other umbilical cord blood samples (Table 1). A summary of the virological analysis of all 20 mare–foal pairs is displayed in Table 1. In conclusion, a case of vertical transmission from mare to foal could be identified for NPHV.

**Maternal NPHV infection was incoherent from placentitis at parturition**

Next to serum, colostrum and umbilical cord blood, placenta tissue samples were collected after physiological separation of the placenta. From mare 18.0, no placenta samples could be taken. The placenta represents a transient connective organ between the mare and its foetus during gestation. First, placenta samples were analysed by qRT-PCR revealing the absence of NPHV RNA in all tissue samples (data not shown). Next, histological analyses were conducted with most of the placenta samples being macroscopically and histologically unremarkable. In Fig. 3, histological pictures of the pregnant and non-pregnant horn of the placenta from the prepartally NPHV RNA-positive mares 3.0, 10.0 and 19.0 are illustrated. Placenta samples from mare 3.0 and 10.0 were histologically unremarkable (Fig. 3). However, in the pregnant horn of mare 19.0, a purulent and necrotizing placentitis (Fig. 3, lower right panel, marked with arrows) was diagnosed. In addition, placenta tissues of the remaining 16 mares were histologically investigated. Next to mare 19.0, another mare (15.0) had histological signs of placentitis in the pregnant horn (data not shown). Yet, at parturition mare 15.0 was NPHV RNA negative, whereas mare 19.0 was NPHV RNA positive (Table 1), thus prepartal viraemia in mares and the occurrence of placentitis at parturition were likely incoherent.

**Course of NPHV infection in mares and foals**

To monitor the course of infection, additional serum samples of all mares and foals were taken approximately 3 and 6 months after foaling. All samples were investigated for the presence of NPHV RNA, anti-CHV/NPHV antibodies...
Table 1) and liver-specific enzymes [γ-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH) and aspartate amino transferase (AST)]. The course of NPHV infection as well as the related liver-specific enzyme activities for the four prepartally NPHV RNA-positive mares and their respective foals are shown in Fig. 4. Three of these initially viraemic mares (3.0, 10.0 and 19.0) also carried viral RNA in their follow-up serum samples 3 and 6 months post-partum (Fig. 4a, b, d). These three mares had higher viral loads compared with mare 18.0 that cleared the infection after foaling (Fig. 4c). All of the four mares were seropositive at all investigated time points. Foal 3.1 was NPHV RNA positive directly after birth and stayed viraemic for the whole monitored time period. In addition, anti-CHV/NPHV NS3 antibodies were detected at all three time points (Fig. 4a).

NPHV isolates cluster in their respective pasture herds

In Table 1, details on the status of NPHV RNA and anti-CHV/NPHV NS3 antibody for all mare–foal pairs at all tested time points are given (Table 1). Of note, seven foals were newly infected within the first 6 months of life (8.1, 9.1, 10.1, 16.1, 17.1, 19.1 and 21.1). Of these, foals 8.1, 16.1 and 21.1 were viraemic after 3 months, whereas the other four foals were NPHV RNA-positive after 6 months. Foal 21.1 got a plasma transfusion 1 day post-partum. Since the donor plasma was tested NPHV RNA positive (data not shown), this is the likely route of NPHV infection for this individual foal. Interestingly, for most of the foals being seropositive after birth, a marked decrease of maternal anti-CHV/NPHV NS3 antibodies was observed at the age of 6 months. In addition to new infections in foals, viral RNA was detected in two initially NPHV RNA-negative mares (8.0 and 16.0) within the following 6 months after foaling.

To track viral NPHV clusters in the horse cohort, phylogenetic analysis of sequences from the viral glycoprotein region (E1E2) were performed (no sequence was obtained for foal 21.1). As shown in Fig. 5, the identified isolates form three distinct clades. The isolates of mare–foal pair 3 cluster within one clade. Identical sequences were detected in the umbilical cord blood and the postnatal serum of foal 3.1, with only a few nucleotide exchanges compared to the isolate in maternal serum (Fig. 5). Isolates in the foal’s serum differed between parturition and subsequent samples; however, in the follow-up samples 3 and 6 months post-partum, sequences were identical to those in the maternal serum (Fig. 5). Isolates in the foal’s serum differed between parturition and subsequent samples; however, in the follow-up samples 3 and 6 months post-partum, sequences were identical to those in the maternal serum (Fig. 5). Another cluster consists of the identified isolates from mare–foal pair 10 and foal 9.1, 6 months post-partum. The third cluster consists of NPHV isolates identified in mare–foal pair 16, 8 and 19 and foal 17.1. Sequences from identified NPHV isolates in the six newly RNA-positive foals cluster in the respective pasture groups of mare–foal pairs with similar isolates were identified in RNA-positive horses from the same herd.
Table 1. Results for the detection of NPHV RNA and anti-CHV/NPHV NS3 antibodies in serum, umbilical cord blood and colostrum samples

All collected samples were analysed for the presence of NPHV RNA and anti-CHV/NPHV NS3 antibody by qRT-PCR and LIPS, respectively. NPHV RNA titres are displayed as RNA copies ml$^{-1}$ and NPHV RNA-negative samples as dash. Anti-CHV/NPHV NS3 antibody values are given as relative light units (RLU), whereas values above the cutoff are highlighted in grey.

<table>
<thead>
<tr>
<th>Mare (X.0)</th>
<th>NPHV RNA (copies ml$^{-1}$)</th>
<th>Anti-CHV/NPHV NS3 antibodies (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parturition 3 months 6 months</td>
<td>Serum† Umbilical cord blood Colostrum Serum 3 months 6 months</td>
</tr>
<tr>
<td>1.0</td>
<td>–</td>
<td>– – –</td>
</tr>
<tr>
<td>1.1</td>
<td>–</td>
<td>– – –</td>
</tr>
<tr>
<td>2.0</td>
<td>No samples → mare-foal pair excluded</td>
<td>– – – – – – – – – – – – – –</td>
</tr>
<tr>
<td>2.1</td>
<td>–</td>
<td>– – –</td>
</tr>
<tr>
<td>3.0</td>
<td>1.06E+07</td>
<td>2.10E+06</td>
</tr>
<tr>
<td>3.1</td>
<td>8.23E+06</td>
<td>2.66E+06</td>
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<tr>
<td>4.0</td>
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<tr>
<td>7.0</td>
<td>–</td>
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<tr>
<td>7.1</td>
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<td>– – –</td>
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<tr>
<td>8.0</td>
<td>–</td>
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<tr>
<td>8.1</td>
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<tr>
<td>10.0</td>
<td>3.86E+06</td>
<td>8.23E+06</td>
</tr>
<tr>
<td>10.1</td>
<td>–</td>
<td>– – –</td>
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<td>16.0</td>
<td>–</td>
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<tr>
<td>16.1</td>
<td>–</td>
<td>1.73E+06</td>
</tr>
<tr>
<td>17.0</td>
<td>–</td>
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DISCUSSION

To the authors' knowledge, natural routes of NPHV transmission among horses have not been reported to date. In this study, we investigated a possible vertical transmission of NPHV infection by monitoring 20 Thoroughbred broodmares during foaling season 2015. We identified vertical transmission in one foal by detection of NPHV RNA in prepartal serum of the respective mare and importantly in umbilical cord blood as well as post-suckling serum of the respective foal taken 14 h post-partum. NPHV RNA could also be detected in three additional mares before foaling; however, no transmission of the viral RNA to their respective foals occurred. Future studies are required to confirm the occurrence of vertical transmission of NPHV infection in horses. Moreover, no vertical transmission of NPHV infection could be observed.

NPHV is genetically closely related to HCV. Vertical transmission of HCV represents the primary route of acquiring HCV infection in childhood. MTCT has almost exclusively been described in prepartally viraemic and seropositive mothers, occurring in 5% of deliveries (Tovo et al., 2016). However, the mechanism and exact time point of vertical transmission of HCV remain elusive. An intrauterine infection has been suggested by one study, which reported one third of the HCV cases in children. However, further studies are required to confirm this finding.

NPHV appears to be genetically similar to HCV, and vertical transmission of NPHV in horses may occur in a similar manner. Future studies are required to investigate the exact mechanism and time point of vertical transmission of NPHV in horses.

Table 1. cont.

<table>
<thead>
<tr>
<th>Mare (X.0)</th>
<th>Foal (X.1)</th>
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<tbody>
<tr>
<td></td>
<td>NPHV RNA (copies ml⁻¹)</td>
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<tr>
<td></td>
<td>Parturition</td>
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<tr>
<td>Serum</td>
<td>Umbilical cord blood</td>
</tr>
<tr>
<td>17.1</td>
<td>–</td>
</tr>
<tr>
<td>18.0</td>
<td>6.78E+04</td>
</tr>
<tr>
<td>18.1</td>
<td>–</td>
</tr>
<tr>
<td>19.0</td>
<td>6.36E+06</td>
</tr>
<tr>
<td>19.1</td>
<td>–</td>
</tr>
<tr>
<td>20.0</td>
<td>–</td>
</tr>
<tr>
<td>20.1</td>
<td>–</td>
</tr>
<tr>
<td>21.0</td>
<td>–</td>
</tr>
<tr>
<td>21.1</td>
<td>–</td>
</tr>
</tbody>
</table>

*Serum at parturition was taken from mares (X.0) in the last week before foaling.
†Serum at parturition was taken from foals (X.1) approximately 14 h postnatally.

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HCV-infected children carrying HCV RNA within the first 3 days of life (Mok et al., 2005). Additionally, a high maternal viraemia before delivery has been discussed as one potential risk factor for MTCT of HCV (Steininger et al., 2003; Yeung et al., 2001) and interestingly, the mare 3.0 in which vertical transmission of NPHV occurred also had the highest viral load of the four prepartally viraemic mares.

Of note, in contrast to the invasive haemochorial placentaion in humans, the epitheliochorial equine placenta provides a highly developed placental barrier. Thus, equine maternal and foetal blood circulations are completely separated during gestation (Chucri et al., 2010; Enders & Blankenship, 1999). Therefore in horses, umbilical cord blood can be understood as equivalent for pre-suckling serum of the foal. In the placenta of mare–foal pair 3, no NPHV RNA was detected and no signs of placentitis were seen at parturition indicating that vertical transmission of NPHV occurred without an infection and inflammation of the placental tissue itself.

In addition to viral RNA, all mare–foal pairs were tested for the presence of anti-CHV/NPHV NS3 antibodies. Transfer of NPHV-specific antibodies to the foal via colostrum could be shown in this study for all seropositive mares. This was expected, since the colostrum represents a known route of antibody transfer in horses, whereas the placental barrier hampers intrauterine transfer of macromolecules as immunoglobulins from the mare to its foetus (Chucri et al., 2010; Jeffcott, 1972). Interestingly, the detection of
maternal anti-CHV/NPHV NS3 antibodies in the foals decreased from the age of 3 months onwards, which might explain a higher susceptibility of the foals to an NPHV infection. Next to foal 3.1, another seven foals became NPHV RNA positive within their first 6 months of life. Of those, six foals received maternal anti-CHV/NPHV NS3 antibodies by passive transfer. However, the neutralizing properties of anti-NPHV antibodies still need to be investigated in future studies. Two of the foals (10.1 and 19.1) that were tested NPHV RNA positive after 6 months were born to the other two chronically infected mares (10.0 and 19.1).

To obtain a better understanding of the circulating NPHV isolates in the study cohort, all NPHV RNA-positive samples were sequenced for E1E2 and phylogenetically analysed. Interestingly, the NPHV isolates detected in foals 10.1 and 19.1 were similar to the respective isolate of their mother. Therefore, next to a horizontal mode of transmission, a vertically acquired infection with delayed viraemia cannot be excluded. Although, first infection studies reported the detection of viral RNA 1 week after experimental inoculation in both, adult and immunocompetent foals (Ramsay et al., 2015), the incubation period for NPHV field infection remains unknown. Therefore more studies are required to investigate the course of natural infection, especially in the young horse.

Notably, identified sequences in postnatal serum and umbilical cord blood from the vertically infected foal 3.1 were identical. These sequences differed slightly from the isolates in the prepartal maternal serum that were again identical with follow-up serum samples from the mare and foal (Fig. 5). A potential explanation is a foetal bottleneck infection in utero or at parturition and a postnatal superinfection of the foal with the whole load of maternal quasispecies. Quasispecies are described for HCV as a group of similar but non-identical genetic viral variants in infected individuals, but still need to be verified for NPHV (Hoofnagle, 2002; Simmonds, 2004). Interestingly, a pattern similar to the transmission in our mare–foal pair 3 has been described for MTCT of HCV, with transfer of only a few maternal quasispecies variants to the infant (Indolfi et al., 2013; Kudo et al., 1997). The postnatal superinfection of foal 3.1 points to an alternative horizontal mode of NPHV transmission. This hypothesis is also supported by the distribution pattern of NPHV isolates in newly infected mares.
Fig. 4. Course of infection in four mare–foal pairs 3, 10, 18 and 19 over the monitored period of 6 months. At parturition as well as at 3 and 6 months, post-partum serum from each mare and foal was taken and analysed for the presence of anti-CHV/NPHV NS3 antibodies (grey triangle) and NPHV RNA (black bullet) by LIPS and qRT-PCR, respectively. The cutoff limit for the LIPS was determined as described before and is illustrated as a grey dashed line. The detection limit of the qRT-PCR is depicted as a black dashed line. For each mare and foal, liver-specific parameters (GLDH, GGT and AST) are shown at the three time points as unfilled symbols, with the following reference ranges set by the laboratory: GLDH <145 IU l\(^{-1}\), GGT <140 IU l\(^{-1}\) and AST <800 IU l\(^{-1}\). (a) Mare–foal pair 3, (b) mare–foal pair 10, (c) mare–foal pair 18 and (d) mare–foal pair 19.
and foals. These cluster in the corresponding herds that pastured together during the monitored time period, indicating a horizontal route of field infection (Fig. 5). Each herd contained a persistently infected horse, representing a possible origin of infection. Further investigations are necessary to determine the routes of NPHV transmission. Arthropod-borne infection and medical treatment with contaminated blood products or instruments have been discussed (Burbelo et al., 2012; Pfänder et al., 2015; Postel et al., 2016; Pybus & Thévé, 2015), but airborne or smear infection need to be taken into account as well.

In conclusion, this study provides for the first time evidence for a vertically acquired NPHV infection in a foal. Due to the high number of newly infected foals within their first months of life, another horizontal route of transmission seems likely.

**METHODS**

**Animals.** During foaling season 2015, 21 Thoroughbred broodmares between the ages of 6 and 18 years were monitored at a stud farm in Germany. In the following study, the mares are numbered from 1.0 to 21.0 and their respective foals from 1.1 to 21.1. The mares foaled between March and May 2015. For foaling and a subsequent period of 2–10 days, mares were kept in a separate stable. Until foaling they had daily access to pasture in groups of 2 to 7 horses, but after foaling mare–foal pairs had discrete grazing hours. A few days post-partum, mare–foal pairs were brought to different stables with fluctuating herds for daily grazing within the first 2 months. Afterwards, the pasture groups remained mainly in the same composition.

**Samples.** Mare–foal pairs were monitored around parturition until 1 day post-partum. At each sampling time point, a general examination of the mares and foals was performed. Prepartal serum was taken from mares in the last week before foaling as part of a routine blood check. During parturition, umbilical cord blood was

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**Fig. 5.** Phylogenetic analysis of the NPHV E1E2 region. Nearly full-length viral glycoprotein E1E2 sequences (1451 bp) for all 23 identified NPHV isolates were aligned, and phylogenetic analysis was conducted using the number of differences method (Nei & Kumar, 2000) implemented in the neighbour-joining algorithm (Saitou & Nei, 1987). One thousand pseudo-replicate datasets were generated to estimate robustness of branching orders within the phylogeny (bootstrapping) (Felsenstein, 1985) with significant values (>70 %) shown on branches. All sequences are available in GenBank (accession numbers KX064762–KX064784). Branch lengths are relative to the scale bar and are in units of the number of base differences per sequence. Multiple sequence alignment and phylogenetic analysis were conducted using MEGA6 software (Tamura et al., 2013). The herds in which the mares and foals were pastured together are marked with brackets in the tree.
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Table 2. Detection of EPgV 1 RNA by qRT-PCR in four mare–foal pairs

<table>
<thead>
<tr>
<th>Mare (X.0)</th>
<th>EPGV 1 RNA (copies ml⁻¹ serum)</th>
<th>Time point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At parturition</td>
<td>3 months post-partum</td>
</tr>
<tr>
<td>4.0</td>
<td>1.64E+04</td>
<td>4.60E+04</td>
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<tr>
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<td>7.0</td>
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</tr>
<tr>
<td>7.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15.0</td>
<td>8.30E+01</td>
<td>No sample</td>
</tr>
<tr>
<td>15.1</td>
<td>–</td>
<td>Euthanized</td>
</tr>
<tr>
<td>16.0</td>
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<tr>
<td>16.1</td>
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collected. In addition, pre-suckling colostrum samples were milked. Placenta samples (1×1 cm) originating from four locations (uterine body, uterine body in proximity to the cervix, pregnant horn and non-pregnant horn) were taken and stored at −20°C and in 4% buffered formalin, respectively. The formalin-fixed samples were transferred to 70% ethanol after 48 h and embedded in blocks of paraffin within the following weeks. No prepartal blood could be drawn from mare 2.0, and therefore mare–foal pair 2 was excluded from the study. Post-suckling serum samples were taken from all foals approximately 14 h postpartum as part of a routine blood check. At 3 and 6 months after foaling, serum samples were taken from the mare–foal pairs with ethical approval by Lower Saxony’s official authorities (LAVES file no. 15A 535). Serum and colostrum samples were immediately frozen at −20°C for approximately 1 week before they were transferred to −80°C until further analyses.

RNA extraction and cDNA synthesis. Viral RNA extraction from serum, umbilical cord blood and colostrum was performed using the High-Pure Viral RNA kit (Roche) according to the manufacturer’s recommendations. For RNA extraction from placenta tissues, 40–200 mg of the frozen placenta was homogenized in 1 ml TRIzol (Invitrogen) with an Ultra-Turrax® T 25 rotor-stator tissue homogenizer (Ika, Laboratory Supply Network) and homogenized according to the manufacturer’s instructions. For the SYBR Green based qRT-PCR, extracted RNA was transcribed into cDNA by applying the PrimeScript RTMaster Mix Kit (Takara) with random primers provided by the kit. All cDNA samples were stored at −20°C until further analysis.

qRT-PCR of NPHV RNA. All cDNA samples were analysed in duplicates for the presence of NPHV RNA via quantitative real-time PCR (qRT-PCR). Therefore, SYBR Premix Ex Taq II (Takara) was utilized with NPHV-specific primers targeting the 5’UTR, which have been described before (Burbo et al., 2012). A standard curve for the quantification of RNA copies was assessed by serial dilution of a plasmid containing the NPHV 5’UTR as described earlier (Pfaender et al., 2015). Read-out was performed with a LightCycler 480 (Roche).

Multiplex qRT-PCR of TDAV RNA and EPgV 1 RNA. Extracted RNA was analysed for the presence of TDAV RNA and EPgV 1 RNA by a probe-based multiplex real-time PCR (RT-PCR) with specific primers (EVT-146, EPgV-314r) and probes for EPgV 1 [WST-189 probe (6FAM-BHQ1)] and TDAV [TDAV-199 probe (TEX-BHQ2)] as described before. Dilution series of run-off transcribed RNAs were used as copy standards to determine the titres of pegivirus genomes (Postel et al., 2016).

Histopathological examination of placenta samples. For histology, placenta tissue from four localizations (pregnant horn, non-pregnant horn, placental body and placental body close to cervix) was routinely processed in paraffin wax, cut at 3 µm thickness, and stained with haematoxylin and eosin.

Sequencing of NPHV-RNA-positive serum samples. Extracted RNA was transcribed into cDNA using the High Fidelity cDNA Synthesis kit (Roche) according to manufacturer’s recommendations with 4 µl (100 µM) of the E1E2-specific primer A-O-hepaci-NS2 (5’-CAATA TTCAAGGACCATTAAC-3’). All cDNA samples were stored at −20°C until further analysis. A nested PCR was performed with a T100 Thermal Cycler (Bio-Rad). For the outer PCR, primers A-O-hepaci-NS2 and S-O-EQSUTRIAS (5’-CTGATAGGATGCTTGCGAGGG-3’) have been utilized. Afterwards, a touchdown PCR was conducted as inner PCR with two sets of the following primers: F-3.1_S6M_NPHVseqVT (5’-ATC TTGTGCCGTTTGTGGCA-3’) with R-3.1_S6M_NPHVseqVT (5’-AGCACAGACCCCATGAGGAC-3’) and F-19.0_NPHVseqVT (5’-CTCTA TTTCTTACTGCTGCTG-3’) with R-19.0_NPHVseqVT (5’-AGCA GCCAATGAAAGGAAAGA-3’). The Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) was used for all PCR reactions. Information about the thermal cycling parameters is available upon request. After amplification, the samples were loaded on an agarose gel and amplification products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Sanger sequencing was performed in both directions (GATC Biotech AG) and a consensus sequence was generated for each amplicon. Multiple sequence alignments were generated for nearly full length of the E1E2 region (1451 bp) with MEGA software by MUSCLE alignment (Tamura et al., 2013). Identified sequences are available in GenBank (GenBank accession numbers KKX064762–KKX064784).

Lips. Serum and colostrum samples were analysed for the presence of anti-CHV/NPHV NS3 antibodies by the LIPS as described before (Burbo et al., 2012; Pfaender et al., 2015). Relative light units (RLU) were measured with a plate luminometer (LB 960 Centro XS3; Berthold). A cutoff was determined by the mean value plus threefold SD of wells containing only buffer A, the Renilla-luciferase (RUC)-CHV helicase fusion protein and A/G beads.

Liver-specific enzymes in serum samples. Serum samples were analysed by the central laboratory of the small animal clinic at the Justus Liebig University of Veterinary Medicine in Gießen (Germany) for GLDH, GGT and AST. The following reference ranges were indicated by the laboratory: GLDH <145 IU l⁻¹, GGT <140 IU l⁻¹ and AST <800 IU l⁻¹.

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