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Frequent occurrence of nonprimate hepacivirus infections in Thoroughbred breeding horses – A cross-sectional study for the occurrence of infections and potential risk factors

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ABSTRACT

Recently, several new hepaciviruses have been identified of which the nonprimate hepacivirus (NPHV) – the closest relative to hepatitis C virus (HCV) discovered to date – is highly prevalent in horses. However, potential risk factors for the transmission of NPHV among horses remain still unknown. Therefore, the objective of this study was to investigate the occurrence of NPHV infections in Thoroughbreds in northern and western Germany and to identify potential risk factors associated with NPHV infections. Using a cross-sectional study design, a total of 733 serum samples from Thoroughbred broodmares and stallions from northern and western Germany were analyzed for the presence of anti-NPHV nonstructural protein 3 (NS3) antibodies and NPHV RNA using the luciferase immunoprecipitation system (LIPS) and a quantitative real-time PCR, respectively. Information regarding signalment, stud farm, breeding history and international transportation history of each horse were collected and evaluated. A frequent occurrence of NPHV was found in the study population with 453 seropositive horses (61.8%) and 134 horses (18.3%) carrying NPHV RNA. Furthermore, statistical analysis revealed that the probability of being infected decreased for horses with a transportation history with increasing age by 20% each year. For horses that stayed in Germany no association between age and infection could be observed. In conclusion, the high occurrence of NPHV infections in breeding Thoroughbreds suggests circulating NPHV infections, endemic herds or persistent shedding in these animals and revealed the association of age and international transportation as risk factor for NPHV infections.

1. Introduction

In recent years, new hepaciviruses belonging to the family of *Flaviviridae* within the genus *Hepacivirus* have been identified in domestic dogs (El-Attar et al., 2015; Kapoor et al., 2011), horses (Burbelo et al., 2012; Pfaender et al., 2014, 2015; Scheel et al., 2015b), cattle (Baechlein et al., 2015; Corman et al., 2015), rodents (Drexler et al., 2013; Firth et al., 2014; Kapoor et al., 2013), bats (Drexler et al., 2013; Quan et al., 2013) and non-human primates (Lauck et al., 2013). Among these, nonprimate hepacivirus (NPHV) was described to infect horses and represents the closest phylogenetic relative of hepatitis C virus (HCV) to date (Pfaender et al., 2014; Scheel et al., 2015b). HCV is a major human pathogen with 3–4 million people worldwide getting newly infected every year (Mohd Hanafiah et al., 2013) and about 350,000 people dying from severe consequences of HCV infection such as liver cirrhosis or hepatocellular carcinoma (Davila et al., 2004; Perz et al., 2006). However, the development of a prophylactic or therapeutic vaccine is hampered by the lack of an immunocompetent animal model (Billerbeck et al., 2013).

NPHV prevalence in horses diverged from 20% to 40% seropositiv-

Abbreviations: NPHV, nonprimate hepacivirus; LIPS, luciferase immunoprecipitation system; HCV, hepatitis C virus; CHV, canine hepacivirus; Abs, antibodies; SD, standard deviations; RLU, relative light units; OR, Odds ratios; CI, confidence interval; EHV, equine herpesvirus; cDNA, complementary DNA; qRT-PCR, quantitative real-time PCR

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ity and 2–36% viraemia between different studies (Burbelo et al., 2012; Figueiredo et al., 2015; Lu et al., 2016; Lyons et al., 2014; Matsuu et al., 2015; Pfaender et al., 2015; Pronost et al., 2016; Tanaka et al., 2014). Similar to HCV a hepatotropism has been described for NPHV (Pfaender et al., 2015; Scheel et al., 2015a). In the majority of cases a subclinical hepatitis has been reported with elevated liver enzymes at seroconversion (Pfaender et al., 2015; Ramsay et al., 2015; Scheel et al., 2015c). However, Reuter et al. (2014) described the occurrence of severe hepatitis in horses with concurrent detection of NPHV RNA. In contrast to humans infected with HCV, infected horses were often able to eliminate the virus (Pfaender et al., 2015; Ramsay et al., 2015; Scheel et al., 2015c) although chronically infected horses have been reported in some studies (Lyons et al., 2014; Matsuu et al., 2015; Pfaender et al., 2015; Ramsay et al., 2015; Reuter et al., 2014; Scheel et al., 2015c). Potential risk factors predisposing individual horses to NPHV infection are unknown. Direct transmission during breeding is possible in virus infections of horses and has been described e.g. in equine arteritis virus infection (Timoney et al., 1987). In humans chronic stress can predispose to viral infection (Salleh, 2008), similarly, this can be hypothesized for horses. Therefore, gender, age, state of reproduction, breeding history and international transportation history were chosen as potential risk factors associated with infection.

Since a higher prevalence of NPHV infections in Thoroughbred horses compared to other breeds has been reported (Pfaender et al., 2015), this study aimed to investigate the occurrence of NPHV infections among Thoroughbreds in northern and western Germany to subsequently identify potential risk factors for NPHV infections. To this end, a total of 733 serum samples from Thoroughbred broodmares and stallions were analyzed for the presence of anti-NPHV NS3 antibodies and NPHV RNA, respectively. Furthermore, statistical analysis of risk factors potentially affecting the prevalence of NPHV infections was performed to investigate natural routes of virus transmission.

2. Materials and methods

2.1. Study design

In Germany, a total of 1450 broodmares and 80 stallions are registered as breeding horses at The German Thoroughbred Studbook Authority (Cologne). In a cross-sectional study, a total of 710 Thoroughbred broodmares and 23 stallions stabled on stud farms in northern and western Germany (Lower Saxony, North Rhine-Westphalia, Hamburg, Schleswig-Holstein) were examined in autumn 2014. In this region, all horses presented for the annual fertility monitoring were investigated, if the owners confirmed participation in the study and the horses were obedient. Although only horses from northern and western Germany were assessed, the study population represented 49% of all registered brood mares and 29% of all registered stallions.

2.2. Serum samples

A total of 733 blood samples from Thoroughbred broodmares and stallions stabled on stud farms in Northern and Western Germany (Lower Saxony, North Rhine-Westphalia, Hamburg, Schleswig-Holstein) were collected after verbal agreement of the horse owners. The samples were transferred to the laboratory within 12 h after collection. Serum was prepared by centrifugation (Universal 320®, Hettich, Tuttlingen, Germany) for 6 min at 3000 rounds per minute. All samples were stored at −80 °C until further analysis.

2.3. Detection of anti-NPHV NS3 antibodies

Samples were analyzed for the presence of anti-NPHV NS3 antibodies (Abs) using the luciferase immunoprecipitation system (LIPS) as described before (Burbelo et al., 2012; Pfaender et al., 2015). Relative light units (RLU) were measured in a plate luminometer (LB 960 XS3; Berthold, Freiburg, Germany). For calculation of sensitivity, a cut-off limit, analogous to Burbelo et al. (2012) and Pfaender et al. (2015) was determined, which was derived from the mean value plus 3 standard deviations (SD) of the replica samples containing only buffer A, Renilla luciferase (Ruc) extract and protein A/G beads.

2.4. Detection of NPHV RNA

Viral RNA was extracted from all serum samples using the High Pure Viral RNA Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Next, viral RNA was transcribed into complementary DNA (cDNA) by applying the PrimeScriptRTMaster Mix Kit (TaKaRa, Kusatsu, Japan). All cDNA samples were stored at −20 °C until further analysis. For the SYBR Green based quantitative real-time polymerase chain reaction (qRT-PCR) the SYBR®Premix Ex Taq™ II kit (TaKaRa, Kusatsu, Japan) was used in combination with the previously published primers Quanti-5UF1 and Quanti-5UR1 targeting the 5′ untranslated region (5′UTR) (Burbelo et al., 2012; Gather et al., 2016). A serial dilution of a plasmid containing the NPHV 5′UTR was generated as standard row for the quantification of NPHV. Measurement of fluorescence was conducted with a LightCycler 480 (Roche, Mannheim, Germany).

2.5. Data collection

Based on laboratory results, four different groups regarding the state of NPHV infection of the 733 horses were distinguished: seronegative and NPHV RNA negative [Abs−/RNA−]; seropositive and NPHV RNA negative [Abs+/RNA−]; seropositive and NPHV RNA positive [Abs+/RNA+] and seronegative and NPHV RNA positive [Abs−/RNA+]. Information regarding gender, age, state of reproduction, breeding history and international transportation history of the study population was received from the database of the Association for breeding and racing of Thoroughbreds (Cologne, Germany). Age was recorded as a continuous variable for descriptive analysis and additionally horses were assigned to four age groups of similar size, 3–6 year old horses (n = 171), 7–10 year old horses (n = 206), 11 to 15 year old horses (n = 233) and 16 to 29 year old horses (n = 123), respectively. The state of reproduction was categorized into brood mares (i.e. mares that had been covered for at least one previous season), maiden mares and stallions. Breeding history in the year of examination was either documented as covered or not covered. The transportation history included whether a horse was transported internationally in the year of investigation (yes or no) as well as the respective transport destination. In addition, the number of horses on each stud farm was inquired. The stock size was categorized in 3 groups of similar sizes including 36 farms with 1 to 9 horses, 34 farms with 10 to 39 horses and 19 stud farms with over 40 horses.

2.6. Statistical analysis

All statistical analyses were performed using SAS, version 9.3 (SAS Institute, Cary, North Carolina). A description of variables was performed with respect to the four groups of state of infection. Logistic regression models were performed to assess potential risk factors (age, stock size of the stud farm, state of reproduction, breeding history and transportation history) on the occurrence of infection. For this, the outcome was defined as dichotomous variable with animals tested negative for NPHV [Abs−/RNA−] and animals tested positive [Abs ± /RNA+]. Therefore, [Abs+/RNA−]-horses were not considered in the regression analysis, because no information about infections in the past were available. Potential associations between the explanatory variables were investigated by determination of the Chi-Square test or coefficient of determination (R^2, association between a quantitative and a qualitative variable). In a first step, univariate logistic regression