Viraemic frequencies and seroprevalence of non-primate hepacivirus and equine pegiviruses in horses and other mammalian species

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Non-primate hepacivirus (NPHV), equine pegivirus (EPgV) and Theiler’s disease associated virus (TDAV) are newly discovered members of two genera in the Flaviviridae family, Hepacivirus and Pegivirus respectively, that include human hepatitis C virus (HCV) and human pegivirus (HPgV). To investigate their epidemiology, persistence and clinical features of infection, large cohorts of horses and other mammalian species were screened for NPHV, EPgV and TDAV viraemia and for past exposure through serological assays for NPHV and EPgV-specific antibodies. NPHV antibodies were detected in 43% of 328 horses screened for antibodies to NS3 and core antibodies, of which three were viraemic by PCR. All five horses that were stablemates of a viraemic horse were seropositive, as was a dog on the same farm. With this single exception, all other species were negative for NPHV antibodies and viraemia: donkeys (n=100), dogs (n=112), cats (n=131), non-human primates (n=164) and humans (n=362). EPgV antibodies to NS3 were detected in 66.5% of horses, including 10 of the 12 horses that had EPgV viraemia. All donkey samples were negative for EPgV antibody and RNA. All horse and donkey samples were negative for TDAV RNA. By comparing viraemia frequencies in horses with and without liver disease, no evidence was obtained that supported an association between active NPHV and EPgV infections with hepatopathy. The study demonstrates that NPHV and EPgV infections are widespread and enzootic in the study horse population and confirms that NPHV and potentially EPgV have higher frequencies of viral clearance than HCV and HPgV infections in humans.

INTRODUCTION

Hepatitis C virus (HCV) was discovered over 20 years ago in the serum of a patient with non-A, non-B hepatitis. HCV is a major cause of chronic liver disease, hepatocellular carcinoma and liver cirrhosis, infecting approximately 3% of the world’s population (Alter et al., 1999; Perz et al., 2004; Shepard et al., 2005). Human pegivirus (HPgV) is a lymphotropic virus that causes little or no apparent disease in humans despite persistently infecting an estimated 5% of the human population (Berg et al., 1999).

HCV and HPgV are positive-stranded RNA viruses with genomes approximately 9000 bases in length which are classified into two separate genera of the family Flaviviridae, Hepacivirus and Pegivirus respectively (King et al., 2011; Stapleton et al., 2011). Until recently, the only other virus classified as a member of the genus Hepacivirus was GBV-B, detected in a laboratory tamarin (Muerhoff et al., 1995; Simons et al., 1995a) in which it caused hepatitis and could be experimentally transmitted to certain other New World primate species (Bukh et al., 2001). GBV-B has, however, never been detected in any other captive or wild primate populations, in South America or elsewhere (Simmonds, 1999).
In horses (equine pegivirus, EPgV; Kapoor et al., 2013). By contrast, pegiviruses infect a diverse range of mammals including humans, New World monkeys, chimpanzees (SPgV) and bats (BPgV) (Adams et al., 1998; Birkenmeyer et al., 1998; Epstein et al., 2010; Kapoor et al., 2013a; Quan et al., 2013; Simons et al., 1995b).

Very recently, several studies of dogs, horses, wild rodents, bats and non-human primates (NHPs) have revealed much greater viral diversity of members of the genera Hepacivirus and Pegivirus (Burbelo et al., 2012; Drexler et al., 2013; Kapoor et al., 2011, 2013a; Lauck et al., 2013; Lyons et al., 2012; Quan et al., 2013). Homologues of HCV were first detected in domestic dogs and horses, described as non-primate hepativiruses (Burbelo et al., 2012; Lyons et al., 2012), subsequently in several species of bats (bat hepativirus, BHV; Quan et al., 2013) and rodents (rodent hepativirus, RHV; Drexler et al., 2013; Kapoor et al., 2013b), and most recently in the black-and-white colobus monkey (Lauck et al., 2013). While clearly similar in genome organization to HCV and containing homologues of each of the structural and non-structural genes characterized in HCV, these new animal viruses are remarkably divergent in sequence from both HCV and GBV-B, and are likely to qualify as putative additional species within the genus Hepacivirus. At least two lineages of rodent hepativiruses additionally contain an internal ribosomal entry site (IRES) that more closely resembles those of pegiviruses than the type IV IRESs found in all other hepativiruses (Drexler et al., 2013).

Similarly, several new pegiviruses have been described in horses (equine pegivirus, EPgV; Kapoor et al., 2013a) several species of rodents (RPgV; Drexler et al., 2013; Kapoor et al., 2013b) and further species of bats (Quan et al., 2013). In contrast to the close host associations of primate and horse pegiviruses, rodent viruses were highly genetically heterogeneous and lineages were interspersed with viruses infecting other mammalian species, providing evidence against the previous hypothesis for virus–host co-evolution (Sharp & Simmonds, 2011).

Despite the differences in pathogenicity between HCV and HPgV infections, both are capable of establishing persistent infections in humans, as can non-primate hepativirus (NPHV) in horses, with persistent viraemia detected in a horse serially sampled over 6 months (Lyons et al., 2012). Research in the USA recently detected and characterized EPgV viraemia in horses presenting with elevated liver enzymes and healthy controls (Kapoor et al., 2013a). The study further established the ability of EPgV to establish persistent infections in two horses in a healthy Alabama herd, while four others cleared viraemia and other animals presented with new infections. Preliminary findings suggest that EPgV is not strictly hepatotropic and that it is detectable in lymph tissue and peripheral blood mononuclear cells (Kapoor et al., 2013a).

Finally, a recent study has demonstrated infection of horses with another pegivirus, genetically distinct from EPgV (Chandriani et al., 2013). The virus, termed Theiler’s disease associated virus (TDAV), was proposed as the aetiological agent of acute hepatitis observed in horses following administration of equine plasma (Chandriani et al., 2013). TDAV was detected in three separate outbreaks of acute serum hepatitis in horses in the USA, and subsequent inoculation of horses with TDAV-positive plasma resulted in several weeks of viraemia preceding liver disease.

In the current study we investigated frequencies of active and past infection of horses and other mammalian species by NPHV, and extended screening for EPgV and TDAV to horses and donkeys. We also made a preliminary investigation of the potential association of NPHV and EPgV infections and hepatopathy in horses.

RESULTS

NPHV serology screening

To investigate frequencies of current infection and past exposure to NPHV, we developed an ELISA using recombinant proteins expressed from the NS3-helicase domain and from the core gene (Table S3 available in the online Supplementary Material). This two-stage assay was used to screen a large study group of horses and other mammalian species (328 horses, 100 donkeys, 116 dogs, 132 cats, 362 human, 164 NHPs; Table 1). All samples were screened in blocking concentrations of soluble non-recombinant pGex-2t Escherichia coli lysate, and reactivity to NS3/core-expressing and non-expressing controls was compared with minimize assay non-specificity. The cut-off for the NPHV assay was set conservatively as the mean serological reactivity (measured at 405nm) plus three SDs of unreactive samples (NS3: 0.128 + 3(0.053) = 0.287; core: 0.136 + 3(0.048) = 0.280), and was used to categorize samples as anti-NPHV positive or negative.

All samples were screened for both anti-NS3 and anti-core IgG antibodies. Samples were considered positive if above the cut-off in both ELISAs; those that were solely NS3

| Table 1. NPHV and pegivirus RNA and antibodies detected by PCR and ELISA, respectively |
|---------------------------------|------------|------------|------------|------------|
| Species                        | NPHV      | EPgV      | TDAV      |
| Horses                         | PCR+ Ab+  | PCR+ Ab+  | PCR+      |
| Hepatopathy                    | 0/111     | 53/111    | 7/111     | 62/111 0   |
| Control                        | 3/217 89/217 | 5/217 130/217 | 0         |
| Donkeys                        | 0/100 0/100 | 0/100 0/100 | NA        |
| Humans                         | 0/362 0/362 | 0/164 0/164 | NA        |
| Non-human primates             | 0/113 1/113 | 0/131 0/131 |          |
| Dogs                           | 0/131 0/131 |           |          |

+, Positive; NA, not applicable; Ab, antibody.
antigen-positive or core antibody-positive were classified as indeterminate (Table 2). A plot of the serology results for the NPHV NS3 absorbance at A405 nm (A) against NPHV core absorbance at A405 nm (Fig. 1) demonstrated good concordance between reactivity to NS3 and core antigens ($R^2$ = 0.5285). Overall, 142 from 327 horse samples tested in both assays were confirmed positive (43.3%) and 19 (5.5%) were reactive in only one assay. In view of their indeterminate status, the latter 19 samples were excluded from subsequent analyses of disease status. All samples from horses previously identified as viraemic were reactive with both antigens by ELISA (Lyons et al., 2012; Table 3).

All samples from all other mammalian species were unreactive in both NS3 and core ELISAs with the exception of a single sample taken from a dog that had come into regular contact with the previously reported NPHV viraemic horse (Table 3; A: NS3, 1.003 and core, 0.6255) (Lyons et al., 2012). Serial samples collected from a persistently viraemic horse showed declining antibody levels over a 1-year period (horse 3; Figs 1, 2 and Table 3). Over the initial 6-month period, viral loads were elevated but declining as previously reported (Lyons et al., 2012). At the time of final sampling, RNA was undetectable indicating viral clearance, but the horse remained seropositive. Similarly, all five contact horses co-housed with the viraemic horse were non-viraemic but seropositive (Table 3).

**Infection frequencies of EPgV in horses and donkeys.**

An assay to detect antibodies to EPgV was developed by expression of the region of NS3 in *E. coli* (pET-28b) homologous to that used for NPHV antibody screening. Recombinant protein was used in an indirect ELISA format using the same blocking and control antigens used for NPHV screening to minimize assay non-specificity. Due to the lack of a comparable core region for EPgV, Western blots (WB) using recombinant NS3 protein were used to confirm the presence of antibody in samples reactive to the EPgV-NS3 ELISA. Samples from 328 horses and 100 donkeys were initially tested (Table 1). Using an $A$ threshold of the mean serological reactivity (0.130 + 3 SD) of unreactive samples (0.262), 63 % of samples from horses but none of the 100 donkey samples were identified as reactive by ELISA (Fig. 3). Screen-positive samples were tested for anti-NS3 antibodies by WB, of which 88 % were confirmed and considered positive (58.5 %). NS3 ELISA-reactive samples that were negative in the supplementary WB assay were considered as indeterminate and not included in analyses of disease associations.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Sample date</th>
<th>Viral load*</th>
<th>NS3 NPHV</th>
<th>Core NPHV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A405nm</td>
<td>A405nm</td>
</tr>
<tr>
<td>1</td>
<td>1998</td>
<td>1.3 x 10^5</td>
<td>0.65</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>1997</td>
<td>4.4 x 10^5</td>
<td>0.55</td>
<td>0.66</td>
</tr>
<tr>
<td>3a†</td>
<td>Dec 2011</td>
<td>4.8 x 10^7</td>
<td>0.90</td>
<td>0.69</td>
</tr>
<tr>
<td>3b</td>
<td>Mar 2012</td>
<td>2.1 x 10^5</td>
<td>0.67</td>
<td>0.68</td>
</tr>
<tr>
<td>3c</td>
<td>Mar 2012</td>
<td>7.1 x 10^4</td>
<td>0.51</td>
<td>0.62</td>
</tr>
<tr>
<td>3d</td>
<td>Dec 2012</td>
<td>Negative</td>
<td>0.50</td>
<td>0.41</td>
</tr>
<tr>
<td>3e</td>
<td>Apr 2013</td>
<td>Negative</td>
<td>0.37</td>
<td>0.38</td>
</tr>
<tr>
<td>4‡</td>
<td>July 2013</td>
<td>Negative</td>
<td>1.00</td>
<td>0.63</td>
</tr>
<tr>
<td>5§</td>
<td>Mar 2012</td>
<td>Negative</td>
<td>0.61</td>
<td>0.50</td>
</tr>
<tr>
<td>6§</td>
<td>Mar 2012</td>
<td>Negative</td>
<td>0.76</td>
<td>0.70</td>
</tr>
<tr>
<td>7§</td>
<td>Mar 2012</td>
<td>Negative</td>
<td>0.79</td>
<td>0.66</td>
</tr>
<tr>
<td>8§</td>
<td>Mar 2012</td>
<td>Negative</td>
<td>0.77</td>
<td>0.59</td>
</tr>
<tr>
<td>9§</td>
<td>Mar 2012</td>
<td>Negative</td>
<td>0.70</td>
<td>0.69</td>
</tr>
</tbody>
</table>

*RNA copies ml^-1 determined by qRT-PCR.
†All samples labelled '3' refer to serial samples from a previously identified NPHV viraemic horse taken between December 2011 and April 2013 (Lyons et al., 2012).
‡Dog and §horses in regular contact with Horse 3.

**Table 3. Serological reactivity in horses with NPHV viraemia detected by PCR**

**Table 2.** (a) Confirmation of serology-reactive samples by confirmatory ELISAs and Western blot. (b) NPHV and EPgV RNA detection in serology-positive horses

<table>
<thead>
<tr>
<th>NPHV</th>
<th>EPgV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>NS3 Ab +</td>
<td>NS3 Ab−</td>
</tr>
<tr>
<td>Core Ab +</td>
<td>142</td>
</tr>
<tr>
<td>Core Ab−</td>
<td>3</td>
</tr>
<tr>
<td>PCR +</td>
<td>3</td>
</tr>
<tr>
<td>PCR−</td>
<td>0</td>
</tr>
</tbody>
</table>

Ab, antibody.
seven horses (four mares, two geldings, one unknown, aged 5–29 years with one of unknown age) from separate individual premises (Table 4). Seven of 12 EPgV viraemic horses were from the hepatopathy group; four of these had only serum biochemical but no clinical indication of hepatopathy, while clinical information for the other three was unavailable. Five of 12 EPgV viraemic horses were from the control horse group, being clinically healthy and having no prior indication of hepatopathy. However, subsequent testing of the viraemic control horses revealed that two of seven had serum biochemical evidence of mild hepatopathy. Thus, overall nine of 12 EPgV viraemic horses had serum biochemical evidence of hepatopathy, namely elevation in gamma-glutamyl transferase (GGT) (8/12), glutamate dehydrogenase (GLDH) (3/12) and/or bile acids (n=2; Table 4), but all six for which there was available clinical information had no clinical signs of hepatopathy or other systemic disease. Viral load was determined by qPCR against an NS3 transcript standard and ranged from $4.09 \times 10^5$ to $1.98 \times 10^9$ RNA copies ml$^{-1}$ (Table 4). Repeat sampling of five of the initially viraemic horses demonstrated persistent infections which were maintained over a 4-month period, although with declining viral loads (Table 4). There was little evidence for hepatopathy maintained over that period with liver enzyme levels largely within the normal range, with the exception of mildly elevated GGT/GLDH/bile acids in two cases (Table 4). Proportions of EPgV seropositive horses in the hepatopathy (62/111) and control (130/217) groups were not significantly different (Fischer’s exact test, $P=0.55$; Table 1).

### Frequency of TDAV viraemia

Previously published primers targeting the conserved NS3 helicase and NS5B RdRp of TDAV (Chandriani et al., 2013; EVT-162/163 and EVT-180/181/83) were used to screen all 328 horses and 100 donkeys. All samples were PCR negative using the previously published primer sets. TDAV-NS3 specific primers designed as part of this study and tested against a TDAV-NS3 transcript control also failed to detect the presence of viral RNA by nested PCR in all horse and donkey samples (Table 1). It remains to be determined whether TDAV is present in equine blood products or in horses or other species outside the USA.

## DISCUSSION

Infection with HCV and its genetically related GBV-B virus was, until recently, thought to be confined to humans and experimentally infected New World primates, respectively. The detection of canine hepacivirus (CHV) and NPHV in dogs and horses, respectively, was the first indication of a wider host range for hepacivirus infection (Burbelo et al., 2012; Kapoor et al., 2011; Lyons et al., 2012). More recently, detection and characterization of a much wider and diverse range of hepaciviruses were found in bats, rodents and an Old World primate (Drexler et al., 2013; Kapoor et al., 2013a, b; Lauck et al., 2013; Quan et al., 2013). These new discoveries have fundamentally revised our knowledge of viral diversity and host ranges of viruses in this genus, their epidemiology and pathogenesis.

Detection of NPHV infection was initially determined by PCR screening and deep sequencing (Kapoor et al., 2011; Lyons et al., 2012) and serological detection of CHV-NS3 antibodies to detect past infections (Burbelo et al., 2012). Recombinant protein expressed from the helicase domain of CHV NS3 was used as an antigen in a luciferase immunoprecipitation system (LIPS) to screen a range of species (Burbelo et al., 2012). In the current study, recombinant protein was expressed from the evolutionarily conserved NS3 helicase and core regions of NPHV and used in

![Fig. 1.](image)

**Fig. 1.** Association between serological reactivities to NS3 and core antigens used for ELISA screening in horse samples. Samples in red indicate previously reported viraemic horses. Pink indicates seroreactivity over a period of 1.5 years of serial sampling of a single viraemic case. The black line indicates the overall declining trend in the combined NS3 and core antibody detection in horse 3 (NPHV, RNA positive; Table 3). The green symbol indicates the seropositive sample from a dog. Serological reactivity was measured at A405nm.
ELISAs to determine seroprevalence of NPHV in horses, donkeys, cats, dogs, NHPs and humans. Using two separate ELISAs to detect NPHV antibodies, and considering only samples reactive in both assays to be confirmed as sero-positive, substantially improved the likely specificity of the assays compared with those based on single antigens. We additionally set assay thresholds conservatively to further prevent misclassification of samples.

The weak seropositivity reported in a single cow by the LIPS method (Burbelo et al., 2012) could not be confirmed by a PCR assay. A second serological assay was developed to estimate seroprevalence to EPgV by expression of the NS3 helicase; in the absence of a homologous core protein, all screen-positive samples were confirmed by WB to minimize non-specificity. Seroprevalence data provide evidence for past virus exposure, although as discussed below, the potential occurrence of re-reversion (loss of detectable antibodies over time) means that they represent minimum estimates for frequencies of past infection in study populations.

NPHV antibodies were detected in 43 % of horses, which is comparable to the 35 % previously reported in the USA (Burbelo et al., 2012). These findings indicate NPHV is enzootic and widely circulates in horse populations in the UK, France and the USA. These exposure frequencies are substantially higher than for HCV in human populations, even in regions of past epidemic spread such as in Pakistan and Egypt, where maximum seroprevalences of approximately 4 % and 15 %, respectively, have been described (Averhoff et al., 2012; Miller & Abu-Raddad, 2010; Qureshi et al., 2010). These suggest likely differences in transmission efficiency between these two hepacivirus species and are indicative of non-parenteral routes of NPHV transmission between horses. The low frequency of viraemia in seropositive horses (3/142; 2.1 %) also contrasts with much higher rates of HCV persistence in humans, where over 50 % of anti-HCV seropositive individuals are actively infected (Crofts & Aitken, 1997; Nalpas et al., 1992; Sugitani et al., 1992). Through serial sampling of one study horse (horse 3; Table 3), we were able to document declining viral loads and clearance of RNA over a 1-year period from an initially viraemic horse, while remaining seropositive. Furthermore, the observation that NPHV antibodies declined over time in horse 3 (Table 3) and may ultimately become undetectable indicates that the NPHV seroprevalence observed in this study may underestimate the level of prior NPHV infections in the equine population. This limitation of serology assays to estimate past exposure frequency and seroprevalence of NPHV and EPgV

Table 4. Viral loads, serological reactivity and indices of liver damage (GGT, GLDH) and liver function (bile acids) in EPgV viraemic horses.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Viral load*</th>
<th>EPgV Ab A405nm</th>
<th>Western blot</th>
<th>GGT† (&lt;42 U l⁻¹)</th>
<th>GLDH† (&lt;12 U l⁻¹)</th>
<th>Bile acid† (0–12 µmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.46 × 10⁶</td>
<td>0.44</td>
<td>Positive</td>
<td>0</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2.79 × 10⁷</td>
<td>1.39</td>
<td>Positive</td>
<td>2</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>2.34 × 10⁶</td>
<td>0.88</td>
<td>Positive</td>
<td>9</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>1.98 × 10⁶</td>
<td>0.97</td>
<td>Positive</td>
<td>44</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>3.19 × 10⁶</td>
<td>0.83</td>
<td>Positive</td>
<td>42</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>2.79 × 10⁷</td>
<td>0.81</td>
<td>Positive</td>
<td>40</td>
<td>21</td>
<td>10.6</td>
</tr>
<tr>
<td>7</td>
<td>3.06 × 10⁵</td>
<td>0.85</td>
<td>Positive</td>
<td>21</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>8</td>
<td>1.26 × 10⁶</td>
<td>0.81</td>
<td>Positive</td>
<td>23</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>1.15 × 10⁹</td>
<td>0.36</td>
<td>Negative‡</td>
<td>156</td>
<td>50</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>1.26 × 10⁹</td>
<td>0.12</td>
<td>Negative‡</td>
<td>136</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>NA</td>
<td>0.49</td>
<td>Positive</td>
<td>126</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>NA</td>
<td>0.55</td>
<td>Positive</td>
<td>749</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>NA</td>
<td>0.45</td>
<td>Positive</td>
<td>243</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>NA</td>
<td>0.42</td>
<td>Positive</td>
<td>120</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Viral loads were determined by qRT-PCR.
†Elevated values are shown in bold.
‡Viraemic horses which were concluded as being antibody negative (Horse 7: ELISA and WB negative) or indeterminate (Horse 8: WB negative).
NA, Not applicable.

Horses 1–5 are in the control group and horses 6–12 are in the hepatopathy group. Horses 1–5 were sampled on three occasions.

http://vir.sgmjournals.org
extends to HCV. Antibodies to HCV also decline over time, sometimes to undetectable levels, several years after virus clearance (Takaki et al., 2000). NPHV antibodies were also detectable in all stablemates indicating previous infections among all horses on the farm.

All other species tested (Table 1) were seronegative with the exception of a single dog (Table 1, 3) which had direct contact with a previously reported NPHV viraemic horse (Lyons et al., 2012; Fig. 2 and Table 3). Identification of a seropositive dog in the UK provides the first evidence that NPHV may transmit to other species, and is concordant with the initial report of CHV/NPHV infections in dogs in the USA, identified in association with an outbreak of respiratory infections in separate geographically isolated dog kennels (Kapoor et al., 2011).

It remains to be determined conclusively how NPHV infections were transmitted between the farm horses. Virus transmission between these animals may have occurred through close contact from co-housing in the same stable, or be the result of exposure to contaminated blood in wounds or on farm surfaces, or the result of exposing the horses to contaminated needles during extensive travel abroad. Further studies on NPHV are warranted to confirm whether or not it utilizes transmission routes comparable to those of HCV; the high seroprevalences observed among horses in this and previous studies (Burvelo et al., 2012) suggests not.

A serological assay for EPgV-specific antibodies assay was developed to determine exposure frequencies in the same cohort of horses. Anti-EPgV NS3 antibodies were confirmed by WB in 58.5% of horses (192/328). This exposure frequency is substantially higher that the seroprevalence of HPgV in humans, globally estimated at 2–13% in healthy blood donors (Blair et al., 1998; Gutierrez et al., 1997; Pilot-Matias et al., 1996a; Tacke et al., 1997). However, higher rates have been described in groups with frequent sexual contact, such as up to 46% of HIV infected homosexual men (Pavlova et al., 1999; Williams et al., 2004) and 18% among prostitutes (Scallan et al., 1998). It is necessary to note that in contrast to the anti-EPgV-NS3 assay, seroprevalence rates in humans have been measured based on anti-E2 antibodies and antibodies to the NS3 protein of HPgV have not been measured during or after
viraemia. To date no studies have investigated pegivirus seroprevalence in bats or rodents.

Active pegivirus infection was detected in 12/328 (3.8 %) of horses, while all other species were EPgV RNA negative (Tables 1, 4). This detection frequency was not significantly different from the original survey of horses in the USA, where seven PCR-positive horses were found among 74 tested (9.5 %; \( P = 0.061 \)) (Kapoor et al., 2013a). These data are also similar to published estimates of active infection frequencies in the human population, which are approximately 5 % in the developed world with upwards of 20 % viraemia recorded in some developing countries (Mohr & Stapleton, 2009; Pavlova et al., 1999; Polgreen et al., 2003).

Ten of the 12 RNA positive samples were seropositive for EPgV (Table 4); antibodies were not detectable by ELISA or WB for the remaining two RNA positive samples (Table 4, Fig. 3). Indeed, over the 4 month period within which horse samples were collected (Table 4), antibody levels were generally maintained despite a general declining trend.

<table>
<thead>
<tr>
<th>Host</th>
<th>Horse</th>
<th>Rodent</th>
<th>Bat</th>
<th>Human</th>
<th>Simian</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPgV</td>
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<td>HPgV</td>
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<td>NPHV</td>
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Fig. 4. Phylogenetic analysis of (a) NS3 and (b) NS5B of EPgV sequences amplified from screen-positive study animals. Maximum-likelihood trees of nucleotide sequences were constructed using Tamura–Nei models of estimated distances calculated by using the program MEGA version 5.2. Evolutionary distances are shown on the scale bar above each tree. Datasets were bootstrap resampled 500 times to indicate robustness of branching (values \( > 70 \% \) shown on branches). Abbreviations detailed above correspond to; BPgV-bat pegivirus, EPgV-equine pegivirus, TDAV-Theilers disease associated virus, RPgV-rodent pegivirus, SPgV- simian pegivirus, NPHV-nonprimate hepacivirus.
in viral load. These findings contrast with reports of absent anti-E2 antibody in humans actively infected with HPGV and seroconversion on clearance of viraemia (Gomara et al., 2010; Pilot-Matias et al., 1996b; Schwarze-Zander et al., 2006; Tan et al., 1999; Van der Bij et al., 2005).

Persistent EPgV infection was evident from the repeated detection of viraemia in serial horse samples screened over a 4 month period in five horses (Table 4). Persistence of infection is characteristic of other pegiviruses, including SPgV (Simons et al., 1995b). Similarly, while immune competent individuals typically clear HPgV infections, this occurs over relatively prolonged time frames, years after primary infection (Berg et al., 1999; Tanaka et al., 1998).

The novel pegivirus TDAV was recently identified as the potential agent responsible for acute equine serum hepatitis (Chandriani et al., 2013). The USA study investigated an outbreak of acute serum hepatitis associated with the administration of equine antiserum to the botulinum toxin and found that 8/17 horses that received the antitoxin 2 preparation developed symptoms of acute hepatitis. While the screening of the study population of horses and donkeys for TDAV RNA failed to detect active infections, including in those horses with hepatopathy (Table 1), no animals screened were known to have received equine blood derived products. These findings do not therefore preclude the possibility that TDAV may indeed circulate in the UK equine population, but further investigation of this may be more effectively focused on those that have received equine blood derived products.

The classification and renaming of members of the Pegivirus genus was partly in response to the accumulating failure to demonstrate any link between human infection with HPgV and hepatitis, making a description such as ‘hepatitis G virus’ inappropriate (Stapleton et al., 2011; Takikawa et al., 2010). Data from this study facilitated some preliminary analysis of potential associations of active NPHV and EPgV infection with hepatopathy in horses. Serum biochemical evidence of hepatopathy was identified in 1/3 horses with NPHV viraemia and 9/12 horses with EPgV viraemia, but further study is required to determine whether the hepatopathy was attributable to NPHV and EPgV infections or to another of the many causes of equine hepatopathy. The prevalence of NPHV and EPgV viraemia and seropositivity were not significantly different in horses with hepatopathy versus the control group, suggesting that these viruses are unlikely to represent a common cause of hepatopathy in the study population. A previous study detected an increased frequency of EPgV viraemia (25%, 3/12) in horses with serum biochemical evidence of hepatopathy compared with healthy controls (6.4%, 4/62) but the authors also concluded that their study was insufficient to determine the health relevance of EPgV infection (Kapoor et al., 2013a). However, combining data from the current study and this previous study provides statistically significant evidence for this association with combined infection frequencies of 8.1% (10/123) in horses with hepatopathy compared with 3.2% (9/279) in control horses (Fisher’s exact test; P=0.042). This marginally significant association and the prior detection of viral RNA in the liver and lymph of infected horses (Kapoor et al., 2013a) provides preliminary data suggesting that EPgV may be a cause or contributory factor in equine hepatopathy. However, the observation that all three NPHV and all six EPgV viraemic horses for which clinical data were available were clinically healthy contrasts with HCV infections in humans, which can cause significant liver damage even in early stages of infection (Alter et al., 1995; Engel et al., 2007; Fierer et al., 2008). However, HCV infection can go undetected for decades in many individuals and in some cases elevated liver enzyme levels are not observed (Alberti et al., 1992; Mathurin et al., 1998; Pradat et al., 2002; Puoti, 2003; Puoti et al., 1997); therefore it is possible that NPHV infection may play an as yet undetermined role in equine hepatic disease. Further study is required to clarify potential disease associations with NPHV and EPgV infections in horses.

This study provides tools for the development of diagnostic assays for viraemia and antibody screening that will assist future research into their transmission, disease associations and tissue tropisms. The presence of hepacivirus and pegivirus species in dogs, horses, rodents and bats sheds light on the possible evolutionary history of HCV and HPgV, in which potential cross-species transmission and zoonotic origins suddenly become more plausible. Continued PCR and serology based screening of other mammalian taxa across a range of geographical locations, including areas of endemic HCV infection, is required to address these gaps in our understanding of the evolution of hepacviruses and pegiviruses.

**METHODS**

**Samples.** Serum or plasma samples (n=1197) were collected from 362 humans (Cameroon), 163 NHPs (Cameroon), 328 horses (Scotland, England and France), 100 donkeys (England), 113 dogs (Scotland) and 131 cats (Scotland). Samples from humans and NHPs were collected in Cameroon by Metabiota (formerly Global Viral Forecasting Initiative) staff in Cameroon and included 11 gorillas, 62 chimpanzees and 91 Old World monkeys as previously outlined (Lavoie et al., 2012; Lyons et al., 2012; Sharp et al., 2010). Samples from horses, donkeys, dogs and cats were archived excess diagnostic samples collected at the Royal (Dick) School of Veterinary Studies, Edinburgh, Liphook Equine Hospital Laboratory, Hampshire, and The Donkey Sanctuary, Sidmouth. Animals were of mixed breed, age and sex. Donkeys, dogs and cats had a wide range of diseases that prompted diagnostic sampling. Horses were categorized into a hepatopathy group (n=111) and a control group (n=217). The hepatopathy group comprised horses that had been investigated for suspected liver disease and which had biochemical evidence of hepatopathy, as indicated by serum/plasma concentrations of gamma-glutamyltransferase (GGT), glutamate dehydrogenase (GLDH) and/or bile acids exceeding the laboratory reference upper level. Hepatopathy was attributed to a wide range of causes. The control group comprised horses for which there was no clinical suspicion of hepatopathy, including clinically healthy horses and horses that were sampled for...
investigation of a wide range of diseases excluding hepatopathy. Samples were collected between 1995 and 2013, and were anonymized prior to testing.

**NPHV, EPgV and TDAV viraemia screening.** All samples were screened for NPHV by PCR with previously published primers and transcript controls (Lyons et al., 2012). Horse and donkey samples were screened for EPgV and TDAV RNA. For detection of EPgV RNA, nested PCR primers were designed targeting the viral NS3 helicase based on published sequences and used in addition to published primer sets (Burbelo et al., 2012). To validate the EPgV and TDAV PCR, RNA transcripts were generated from a plasmid containing PCR amplified partial NS3 cDNA by using an Ambion T7 transcription kit (Promega). Transcripts were purified with an RNaseasy kit (Qiagen), and concentrations were determined by using a NanoDrop 2000 (NanoDrop Products). RNA extractions were performed on 140 μl of plasma using a QIAamp viral extraction kit (Qiagen) according to the manufacturer’s instructions and eluted in a final volume of 60 μl.

RNA was converted to cDNA using random hexamers with a Superscript III Reverse Transcription System (Life Technologies) and then used in nested PCR with previously published NPHV NS3 primers (Lyons et al., 2012) in addition to newly designed EPgV NS3 and 5’UTR, and TDAV-NS3 primer sets (Table S1) and amplified using two rounds of 35 cycles at 94 °C for 18 s, 50 °C for 21 s, and 72 °C for 1.5 min; and one cycle of 72 °C for 5 min, with 2 μl of first-round amplicon added to the second round. TDAV qPCR screening was carried out as previously published (Chandriani et al., 2013). The EPgV and TDAV NS3 transcript was tested by using both EPgV and TDAV NS3 primer sets and used as a control in screening, with sensitivity of 0.5–5 RNA copies in a reaction (Tables S1, S4).

Positive second-round PCR amplicons were sequenced as previously published (Lyons et al., 2012) and analysed using SGE version 1.1 software (Simmonds, 2012).

**Viral RNA quantification.** Quantitative real-time PCR (qRT-PCR) was used to determine viral loads of positive samples using a standard calibration curve from a dilution series of the N35 transcript. Dilutions of EPgV and TDAV NS3 transcript were prepared from concentrations of 10⁶ to 1 copy μl⁻¹; 5 μl transcript RNA was used to generate cDNA using random hexamers and reverse transcription. Five microlitre aliquots of cDNA were assayed in triplicate for the positive samples in the same way. To quantify viral loads of positive samples, EPgV NS3IS and EPgV NS3HAS primers were used with 4 μl cDNA in a SensiFAST SYBR Hi-ROX kit (BioLine) as per the manufacturer’s instructions with the exception that the annealing temperature was reduced to 50 °C and the extension time extended to 15 s. Samples were analysed in triplicate and fluorescence measured using a Rotor-Gene Q system (Qiagen). Viral loads were read from the standard curve generated and converted to RNA copies μl⁻¹ based on the sample volume used in extraction and elution of the RNA.

**Synthesis of NPHV and EPgV NS3 and core recombinant proteins.** Nested PCR primers for NPHV and EPgV NS3 helicase were designed based on published sequences, with appropriate restriction sites for subcloning added to the inner primers (Table S2). PCRs were performed using GoTaq (Promega) in accordance with the manufacturer’s instructions, by using the conditions outlined above for both rounds.

Amplicons were cloned into a pGex-2T or pET28B vector for production of glutathione-S-transferase and polyhistidine fusion proteins respectively (Amersham Pharmacis Biotech, Merck Novagen). Briefly, the plasmid and the inserts were digested with BamHI and EcoRI according to the manufacturer’s instructions (Promega) and gel purified using a PureLink Gel Extraction kit (Invitrogen). The ligation reaction was carried out using T4 Ligase (Bioline) at an insert:vector ratio of 4 and transformed into JM109 competent cells (Agilent) for propagation. Recombinant clones were selected by antibiotic resistance and were confirmed by PCR and sequencing. High concentrations of plasmid were purified from overnight cultures of ampicillin resistant an OD600 of 0.6 colonies using a Wizard Plus SV Miniprep DNA Purification System according to the manufacturer’s instructions (Promega).

One Shot BL21(DE3) pLytS chemically competent E. coli (Invitrogen) were transformed with plasmid as per the manufacturer’s instructions. From overnight cultures of antibiotic resistant colonies a fresh 1:10 dilution was prepared and grown to an OD of 0.6 before induction at 20 °C for 3 h with 1 mM IPTG. LB medium was removed by centrifugation at 2000 g for 15 min and cell pellets were weighed and lysed with BugBuster Master Mix (Merck Millipore) containing Roche complete ULTRA protease inhibitors, as per the manufacturer’s instructions. Cell lysate was recovered by centrifugation at 12000 g for 15 min at 4 °C. Soluble and insoluble fractions were analysed by SDS-PAGE for the presence of recombinant protein.

**ELISA.** All samples were tested for antibodies to NPHV. Horse and donkey samples were also tested for antibodies to EPgV. High-bind 96-well ELISA plates (Greiner Bio-One) were coated overnight with BugBuster cell lysates (0.5 μg of recombinant protein in 100 μl of carbonate buffer) or an equivalent volume of protein isolated from cells infected with non-recombinant pGex-2T collected and processed in parallel with the NS3 and core proteins. Plates were washed with 250 μl of 1 % Tween 20/PBS (v/v), and coated wells were blocked with 150 μl of 3 % BSA/PBS (w/v) at room temperature for 2 h. After one round of washing, test samples were diluted 1:1000 in 100 μl of 3 % BSA/PBS (w/v), pre-incubated with 10 μl of non-recombinant pGex2T cell lysate, and added to the wells and incubated for 1 h. The wells were washed six times with 250 μl of 1 % Tween 20/PBS (v/v) and incubated each time for 15 min, and then were incubated for 30 min with 100 μl of HRP-conjugated goat anti-horse immunoglobulin G antibody (Serotec) or the appropriate species specific anti-IgG diluted 1:4000 in 2 % BSA/PBS (w/v). After four rounds of washing, plates were developed by adding 70 μl of the HRP substrate (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid; Liquid Substrate System) to each well in accordance with the manufacturer’s protocol (Sigma). Plates were allowed to develop for 20–25 min and were read at 405 nm. The immunoreactivity of samples to control wells was compared and the mean control plus three times the SD was established as the cut-off. Samples confirmed as NPHV NS3 antibody positive were confirmed by second ELISA targeting NPHV core antigen (Tables S2, S3). EPgV antibody positive samples were confirmed by Western blot analyses.

**Western blot analysis.** Western blots were carried out to confirm all EPgV seropositive cases in the absence of a second confirmatory ELISA for EPgV. Whole–cell lysate and enriched protein samples were analysed on 10 %/16 % SDS–PAGE gels. The gels were either stained with Coomassie blue (Sigma) or blotted onto Protran BA 85 nitrocellulose membranes (Whatman) by use of a semidy 1 électroblotter. Membranes and were blocked overnight in 4 % milk powder (MP)/0.05 % Tween 20/TBS (v/v). The membranes were then incubated at room temperature for 1 h with serum obtained from horses identified as EPgV seropositive and diluted 1:2000 in 4 % MP/0.05 % Tween 20/TBS (v/v). After six washes in 0.05 % Tween 20/TBS (v/v) for a total of 2 h, the membrane was incubated with HRP-conjugated goat anti-horse immunoglobulin G antibody (Serotec) diluted 1:35 000 in 4 % MP/0.05 % Tween 20/TBS (v/v). After four washes in 0.05 % Tween 20/TBS (v/v) for a total of 1 h, bound antibody was visualized using electrochemiluminescent prime Western blotting detection reagent in accordance with the manufacturer’s protocol (Amersham).
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REFERENCES


