Tropism of human pegivirus (formerly known as GB virus C/hepatitis G virus) and host immunomodulation: insights into a highly successful viral infection

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Abstract

Human pegivirus (HPgV; originally called GB virus C/hepatitis G virus) is an RNA virus within the genus *Pegivirus* of the family *Flaviviridae* that commonly causes persistent infection. Worldwide, ~750 million people are actively infected (viraemic) and an estimated 0.75–1.5 billion people have evidence of prior HPgV infection. No causal association between HPgV and disease has been identified; however, several studies described a beneficial relationship between persistent HPgV infection and survival in individuals infected with human immunodeficiency virus. The beneficial effect appeared to be related to a reduction in host immune activation. HPgV replicates well *in vivo* (mean plasma viral loads typically $>1\times10^7$ genome copies ml^{-1}); however, the virus grows poorly *in vitro* and systems to study this virus are limited. Consequently, mechanisms of viral persistence and host immune modulation remain poorly characterized, and the primary permissive cell type(s) has not yet been identified. HPgV RNA is found in liver, spleen, bone marrow and PBMCs, including T-and B-lymphocytes, NK-cells, and monocytes, although the mechanism of cell-to-cell transmission is unclear. HPgV RNA is also present in serum microvesicles with properties of exosomes. These microvesicles are able to transmit viral RNA to PBMCs *in vitro*, resulting in productive infection. This review summarizes existing data on HPgV cellular tropism and the effect of HPgV on immune activation in various PBMCs, and discusses how this may influence viral persistence. We conclude that an increased understanding of HPgV replication and immune modulation may provide insights into persistent RNA viral infection of humans.

Introduction

Human pegivirus (HPgV) is an RNA virus within the Pegivirus A species and family *Flaviviridae* (Adams *et al.*, 2013; Stapleton *et al.*, 2012a). The virus was originally called hepatitis G virus (HGV)/GB virus type C (GBV-C). The letters 'GB' represented the initials of a surgeon with acute hepatitis whose sera caused hepatitis in marmosets (Deinhardt *et al.*, 1967). Subsequent studies found that this virus did not cause hepatitis and there was no

direct evidence that the surgeon ('G. B.') was infected with HPgV. Thus, the virus (HGV/ GBV-C), along with several related primate and bat viruses (GBV-A, GBV-C$_{czp}$, GBV-D), was assigned to a new genus (*Pegivirus*) and renamed as HPgV (Adams *et al.*, 2013; Stapleton *et al.*, 2012a). HPgV has a 9.4 kb positive-sense ssRNA genome that is organized similarly to hepatitis C virus (HCV) (Fig. 1). HPgV and HCV are unusual amongst RNA viruses in that they commonly cause persistent infection in immune-competent humans.

Although HPgV is not as efficient at establishing persistent infection as HCV, an estimated 25% of infections persist and the other 75% clear viraemia within 2 years of infection (Gutierrez *et al.*, 1997; Tanaka *et al.*, 1998a). The prevalence of HPgV viraemia in cross-sectional serum surveys of healthy blood donors in developed countries is between 1 and 5 %, whilst up to 20% of blood donors in developing countries have active infection (Mohr & Stapleton, 2009). The rate of HPgV viraemia is also higher amongst individuals with blood-borne or sexually transmitted infections. Based on cross-sectional prevalence data, there are ~750 million people with HPgV viraemia worldwide (Stapleton *et al.*, 2014). Anti-HPgV antibodies are not usually detected during active infection, but antibody against the envelope glycoprotein E2 appears following viral clearance (Gutierrez *et al.*, 1997; Tacke *et al.*, 1997; Tanaka *et al.*, 1998a; Thomas *et al.*, 1997). These E2 antibodies appear to provide partial protection against reinfection (Elkayam *et al.*, 1999; Tillmann *et al.*, 1998). Thus, antibody to E2 represents a marker of prior infection, although antibody levels may decrease and become undetectable over time (Gutierrez *et al.*, 1997; Tacke *et al.*, 1997; Tanaka *et al.*, 1998a). As 12–20% of blood donors from developed countries have HPgV E2 antibodies (Gutierrez *et al.*, 1997; Tacke *et al.*, 1997) and a higher percentage of healthy blood donors in developing countries have E2 antibodies (reviewed by Mohr & Stapleton, 2009), the data suggest that ~1.5–2.5 billion people are currently infected or have evidence of prior HPgV infection, making this virus a major contributor to the human virome. Thus, HPgV shares infection rates with many viruses, including papillomaviruses, herpesviruses and torque teno viruses (TTVs), frequently found in healthy individuals (Okamoto, 2009).

HPgV is transmitted by exposure to infected blood, sexual exposure or by maternal–foetal transmission (Bhanich Supapol *et al.*, 2009; Hino *et al.*, 1998; Kleinman, 2001; Lin *et al.*, 1998; Ohto *et al.*, 2000; Stapleton, 2003). Although HPgV has not been conclusively shown to cause any human disease (Bhattarai & Stapleton, 2012; Mohr & Stapleton, 2009), several studies observed an association between HPgV viraemia and an increased risk of non-Hodgkin's lymphoma (Chang *et al.*, 2014; Civardi *et al.*, 1998; De Renzo *et al.*, 2002; Ellenrieder *et al.*, 1998; Giannoulis *et al.*, 2004; Keresztes *et al.*, 2003; Krajden *et al.*, 2010; Michaelis *et al.*, 2003; Nakamura *et al.*, 1997). In contrast, HPgV is associated with a beneficial effect in human immunodeficiency virus (HIV) infection, and most studies and a meta-analysis found prolonged survival in HIV-infected individuals co-infected with HPgV compared with those without HPgV viraemia (Heringlake *et al.*, 1998; Nunnari *et al.*, 2003; Tillmann *et al.*, 2001; Toyoda *et al.*, 1998; Williams *et al.*, 2004; Xiang *et al.*, 2001; Zhang *et al.*, 2006). A recent study of incident, transfusion-related HPgV infection in HIV-infected individuals confirmed a survival benefit in those subjects who acquire HPgV through blood transfusion (Vahidnia *et al.*, 2012). As a result of this beneficial interaction in HIV co-infected subjects, numerous studies designed to understand how HPgV confers the ascribed survival benefit have been reported.

As with HCV, HPgV does not grow well *in vitro*, yet replicates extremely well *in vivo* with mean plasma viral loads typically $> 1 \times 10^7$ genome copies ml^{-1}. Nevertheless, due to the poor growth of the virus *in vitro*, study of this virus is difficult, resulting in several unanswered questions about HPgV persistence and how the virus interacts with the host.

Many acute viral infections induce sterilizing T- and B-cell immune responses that clear the infection and result in immunological memory leaving the host resistant to reinfection. Following acute infection by many DNA viruses, such as the herpesviruses, actively replicating virus is mostly cleared by the host immune response; however, residual foci of quiescent (latent) infection remain, providing a source for occasional reactivation (Welsh & Waggoner, 2013). Whilst HIV and other retroviruses have a DNA intermediate that integrates into host cell chromosomal DNA enabling persistence, it remains unclear how strictly cytoplasmic RNA viruses such as HCV and HPgV evade immune clearance. Several viral mechanisms have been proposed as contributors to immune escape for HCV, including the presence of two hypervariable regions on the envelope (E2) protein (one in a location shown to be involved in viral entry), mutation of immunodominant T-cell epitopes (Keck *et al.*, 2009, 2014), interference of several HCV proteins with intracellular signalling pathways resulting in reduced innate and adaptive immune responses, and chronic stimulation of T-cells leading to T-cell exhaustion (reviewed in Burke & Cox, 2010; Lemon, 2010). In contrast, HPgV envelope proteins do not have any hypervariable regions; the virus displays limited sequence variability within persistently infected hosts in protein regions predicted to be T-cell epitopes, and is associated with reduced T-cell activation and exhaustion (Mohr & Stapleton, 2009; Stapleton *et al.*, 2012b). Complicating studies of HPgV persistence, the receptor(s) for HPgV have not been identified and the primary permissive cell(s) are not fully characterized. Nevertheless, HPgV RNA is detected in several types of PBMCs (Chivero *et al.*, 2014), and the relationship between HPgV infection and immune function, and how this relates to viral persistence in chronic infection *in vivo*, remain to be clarified. Understanding the life cycle and tropism of this relatively non-pathogenic virus may provide insights into virus-mediated interference in co-infected individuals and potentially identify a component of the microbiome that influences human immune responses. This review summarizes our current understanding of HPgV tropism and the contribution of HPgV infection of human immune cells (T-and B-lymphocytes, NK-cells, and monocytes) to host immune modulation.

HPgV tropism

HPgV was initially thought to be hepatotropic based on its identification in individuals with non-A, non-B, non-C hepatitis; however, subsequent epidemiological studies failed to find a conclusive association with either acute or chronic hepatitis (Fan *et al.*, 1999; Pessoa *et al.*, 1998; Tucker *et al.*, 2000). Although several early studies found HPgV RNA in liver biopsies, virus was subsequently detected in spleen, bone marrow, cerebrospinal fluid and PBMCs, as noted above (Chivero *et al.*, 2014; George *et al.*, 2006; Kisiel *et al.*, 2013; Mellor *et al.*, 1998; Pessoa *et al.*, 1998; Radkowski *et al.*, 2000; Tucker *et al.*, 2000). Studies attempting to find evidence of HPgV replication in liver tissue were either negative or inconclusive (Berg *et al.*, 1999; Fan *et al.*, 1999; Laskus *et al.*, 1997). Specifically, RNA levels of the hepatotropic HCV were consistently higher in the liver of HCV/HPgV co-

infected individuals relative to plasma, whilst the inverse was true for HPgV RNA. Furthermore, the median liver/serum ratio of HPgV RNA was <1.0, consistent with serum contamination of liver tissue (Pessoa *et al.*, 1998), and several additional studies did not find evidence of HPgV replication in the liver (Berg *et al.*, 1999; Fan *et al.*, 1999; Laras *et al.*, 1999; Pessoa *et al.*, 1998). HPgV negative-strand RNA, indicative of viral RNA replication, was not detected in liver samples, but was detected in bone marrow and spleen in autopsy or biopsy samples (Laskus *et al.*, 1997; Radkowski *et al.*, 2000; Tucker *et al.*, 2000). Furthermore, HPgV serum RNA levels did not decrease significantly following liver transplantation in an HCV/HPgV co-infected individual, whilst HCV RNA levels transiently decreased (Berg *et al.*, 1999). Table 1 summarizes selected studies showing HPgV replication as measured by the negative-strand RNA replication intermediate.

Life cycle

Based on similarities in genome organization and amino acid homology with HCV, it is likely that multiple attachment and entry receptors are involved in HPgV entry. HCV utilizes many receptors *in vitro*, including the low-density lipoprotein receptor (LDLr), scavenger receptor class B type I, CD81, claudin-1, occludin, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), signal transducer Harvey rat sarcoma viral oncogene homologue, Niemann–Pick C1-like 1 and transferrin receptor 1 (Evans *et al.*, 2007; Lozach *et al.* 2003; Martin & Uprichard, 2013; Moradpour *et al.*, 2007; Ploss *et al.*, 2009; Sainz *et al.*, 2012; Zeisel *et al.*, 2013; Zona *et al.*, 2014). Although little is known about HPgV entry, early studies suggested that the LDLr is involved in HPgV and HCV entry (Agnello *et al.*, 1999; Wünschmann *et al.*, 2000). Consistent with this, HPgV RNA in plasma is associated with lipids (Xiang *et al.*, 1998), including lipid-associated microvesicles that exhibit properties of exosomes (Bhattarai *et al.*, 2013). Given the universal distribution of LDLr on human cells, lipid-associated virus and/or serum microvesicle uptake may involve the LDLr (Wünschmann *et al.*, 2006). As there are no efficient *in vitro* replication systems, detailed studies on the HPgV life cycle have not been performed. Nevertheless, based on the related HCV, it is predicted that there is post-entry acidification of the endosomal vesicle leading to a conformational change in the virion with subsequent fusion of the viral and cellular membranes.

A potential alternative mechanism of HPgV entry may involve serum HPgV RNA-containing vesicles that have properties of exosomes (Chivero *et al.*, 2014). Exosomes are microvesicles of endocytic origin that are emerging as novel particles employed by cells and viruses to transmit viral and cellular RNAs and proteins to cells (Meckes & Raab-Traub, 2011). As HPgV and other members of the genus *Pegivirus* do not appear to encode a nucleocapsid protein at the N terminus of the polyprotein (Xiang *et al.*, 1998; Stapleton *et al.*, 2011), cellular-derived microvesicles (which carry ~50% of the HPgV RNA found in plasma; Bhattarai *et al.*, 2013) may be involved in virus release and/ or cellular infection. Supporting this hypothesis, HPgV RNA-containing microvesicles positive for an exosomal marker (cellular CD63) were able to deliver viral RNA to uninfected PBMCs and viral RNA replicated within these cells *ex vivo* (Chivero *et al.*, 2014). Other viruses such as hepatitis A virus and HCV have also been shown to utilize exosomes for viral transmission and

intercellular communication (Bukong *et al.*, 2014; Dreux *et al.*, 2012; Feng *et al.*, 2013; Ramakrishnaiah *et al.*, 2013).

HPgV infects diverse haematopoietic cell types *in vitro*

The primary site(s) of replication and cellular receptors for HPgV have not been identified, despite the passage of nearly 20 years since discovery. As noted above, early studies proposed that the virus was hepatotropic; however, several lines of evidence suggest that the virus is lymphotropic (Fogeda *et al.*, 1999; George *et al.*, 2006; Xiang *et al.*, 2000, 2001). Specifically, PBMCs from HPgV-infected individuals maintained in culture *ex vivo* release virus into culture media and virus production can persist for at least 35 days in culture (Fogeda *et al.*, 1999; George *et al.*, 2003; Rydze *et al.*, 2012). Furthermore, serum-derived virus from infected individuals is capable of infecting PBMCs *in vitro* (Chivero *et al.*, 2014; Xiang *et al.*, 2000).

In cell culture, HPgV replication is at best intermittent and produces low titres of intracellular RNA or virus released into cell culture media. The best-characterized *in vitro* methods described to date for HPgV replication utilize primary PBMCs (Fogeda *et al.*, 1999; George *et al.*, 2003, 2006; Rydze *et al.*, 2012; Xiang *et al.*, 2000). Unfortunately, production of HPgV by donor lymphocytes demonstrates considerable donor and isolate variability (George *et al.*, 2003), and quantification of HPgV in various peripheral blood cell types detects only 1–10 copies per 100 cells on average. Thus, a very small proportion of PBMCs appear to support replication (Chivero *et al.*, 2014). Long-term passage of HPgV in PBMCs for more than four or five passages has not been described even though the virus is non-cytopathic.

In a recent study, HPgV RNA was detected in T- and B-lymphocytes, NK-cells, and monocytes (Chivero *et al.*, 2014). HPgV RNA was present in all 14 study subjects' PBMCs, and in highly purified populations of T- and B-lymphocytes (>98% purity), consistent with previous studies (George *et al.*, 2006; Ruiz *et al.*, 2010). Amongst CD4$^+$ and CD8$^+$ T-cells, viral RNA was present in naive, central memory and effector memory subpopulations, with the highest concentrations reported in the naive (CD45RA$^+$) T-cells compared with central memory or effector memory T-cells (Chivero *et al.*, 2014). This finding is consistent with selective HPgV infection of naive T-cells that subsequently differentiate into effector or memory cells, or alternatively the infection of T-cells regardless of differentiation state. If infection is selective for naive T-cells, it would suggest that there are differences in expression of HPgV receptor(s) or that restriction factor(s) are expressed following differentiation. Alternatively, the data could be interpreted to suggest that HPgV infects haematopoietic precursor cells that maintain infection during differentiation into T- and B-cells. If the latter hypothesis is correct, HPgV infection may also reduce the frequency of naive cell differentiation into effector cells by decreasing activation and proliferation.

Although HPgV replicates efficiently in humans, with mean serum viral loads typically $>1\times10^7$ genome equivalents ml^{-1}, replication is inefficient *in vitro* and the production of virus by lymphocytes maintained in culture *ex vivo* is reduced following T-cell activation (George *et al.*, 2003; Rydze *et al.*, 2012). Consistent with an interaction between HPgV and cell activation, several clinical studies observed a reduction in T-cell activation and

proliferation markers in HIV-infected subjects with HPgV co-infection compared with HIV-mono-infected subjects (Bhattarai *et al.*, 2012a; Maidana-Giret *et al.*, 2009; Rydze *et al.*, 2012; Stapleton *et al.*, 2012b). Further evidence consistent with HPgV reducing inflammation comes from a recent report which found that HPgV infection was associated with reduced mortality in individuals infected with ebolavirus (Lauck *et al.*, 2015). Finally, higher concentrations of HPgV RNA are found in naive T-cells or non-proliferating cells (Chivero *et al.*, 2014). Taken together, the data suggest that activation reduces HPgV replication, and that HPgV infection interferes with activation and proliferation of lymphocytes. During *in vitro* replication, this would reduce the proportion of cells harbouring virus within a cell culture over time, potentially contributing to the difficulty in detecting HPgV replication in cell culture systems.

In addition to T- and B-lymphocytes, HPgV RNA is found in highly purified monocytes and NK-cell preparations (Chivero *et al.*, 2014). As HPgV RNA is present in both lymphoid and myeloid cells, either wide tropism occurs or perhaps the primary target cell is a progenitor haematopoietic stem cell (HSC) that subsequently differentiates, carrying the HPgV genome. To examine this, we infected peripheral blood CD34$^+$ cells, but this did not result in improved replication compared with PBMC cultures (E. T. Chivero, N. Bhattarai, E. L. Mohr & J. T. Stapleton, unpublished observation). However, this does not exclude the possibility of an HSC target cell, as the properties of peripherally derived stem cells differ considerably from bone-marrow-derived HSCs. Table 2 summarizes HPgV RNA localization studies of different cell types. Potential mechanisms by which HPgV transmits its RNA to cells are summarized in Fig. 2.

HPgV negative-strand RNA (a marker of replication) has not been reproducibly found in PBMCs, presumably due to the very low viral RNA copy number in PBMCs (Chivero *et al.*, 2014; Mellor *et al.*, 1998). However, several lines of evidence strongly support HPgV replication in PBMCs. Specifically, PBMCs removed from infected individuals produce viral RNA over many weeks despite washing and/or treatment with trypsin (George *et al.*, 2003; Rydze *et al.*, 2012). Secondly, uninfected PBMCs inoculated with viral particles for 1 h and then treated with trypsin to remove virus adherent to the outside of cells demonstrate a subsequent increase in HPgV RNA over time, and release more viral RNA into culture supernatants than used in the initial inocula (Xiang *et al.*, 2000; Chivero *et al.*, 2014). The fact that HPgV negative-strand RNA is preferentially found in bone marrow and spleen rather than PBMCs (Radkowski *et al.*, 2000; Tucker *et al.*, 2000), and the finding of HPgV RNA in multiple lineages of peripheral blood white cells (references in Table 1), reinforce the possibility that a HSC precursor may serve as the primary target of HPgV infection, and that the virus persists and can be produced in cells during and following maturation into B- and T-lymphocytes, NK-cells and monocytes, as suggested in Fig. 2. If this model is true and HPgV replicates in stem cells without cytopathic effect, HPgV may be a useful platform for developing viral vectors for gene delivery similar to current adenoviral vectors. Nevertheless, until there is an animal model or efficient *in vitro* replication system, it will be difficult to further elucidate the cellular receptor(s) and target cell(s) for HPgV replication or test the use of HPgV as a gene therapy vector.

Host immune responses in chronic HPgV infection

T-cell activation

HIV infection results in chronic activation of T-cells, thus promoting activation-induced CD4^{+} T-cell death with subsequent immune dysfunction and progression to AIDS. Consequently, T-cells in HIV-infected individuals have increased expression of activation markers and lower CD4^{+} T-cell counts (Hunt *et al.*, 2003). In contrast, HPgV infection is associated with significantly lower expression of surface markers of activation on T-cells (CD38, CD69 and CD25) in acutely HIV-infected individuals (Maidana-Giret *et al.*, 2009) and chronically HIV-infected individuals compared with those without HPgV, independent of HIV treatment status (Bhattarai *et al.*, 2012a; Stapleton *et al.*, 2012b). HPgV replication *ex vivo* was reduced in activated PBMCs compared with cells without activation (Rydze *et al.*, 2012), suggesting an interaction between HPgV and T-cell proliferation. Consistent with this hypothesis, HPgV viraemia was associated with reduced CD4^{+} T-cell expansion in HIV-infected individuals receiving recombinant IL-2 therapy (Stapleton *et al.*, 2009). The CD4^{+} T-cell count did not increase significantly in subjects with HPgV infection, whilst it increased by >1000 cells mm^{-3} in those without HPgV. In addition, the proportion of naive CD8^{+} and CD4^{+} T-cells is increased relative to memory and effector cells in individuals with persistent HPgV infection compared with HPgV-negative controls (Stapleton *et al.*, 2012b). As IL-2 is required for proliferation and differentiation of T-cells, and HPgV inhibits T-cell activation and IL-2 production, we hypothesize that the CD8^{+} T-cell cytotoxic functions that control viral infections are reduced in those with HPgV infection, contributing to HPgV persistence and high serum viral loads (typically >10^7 copies ml^{-1}) (Tillmann *et al.*, 2001). This is consistent with a beneficial effect in HIV infection, as HIV mediates disease by inducing chronic immune activation leading to lethal immune pathology (Welsh & Waggoner, 2013). This may be mediated in part by the finding that HPgV inhibits T-cell receptor signalling (Bhattarai *et al.*, 2012b, 2013); however, the effects of HPgV on negative signalling molecules such as programmed death-1, 2B4 (CD244) or cytotoxic T-lymphocyte antigen-4 have not been studied.

HPgV is also associated with an increase in the number of double-negative (DN) T-cells (CD4^{−}CD8^{−}CD3^{+}) (Bhattarai *et al.*, 2012a). DN T-cells are associated with reduced immune activation and high levels of immune-suppressive cytokines, including TGF-β and IL-10, in HIV infection (Petitjean *et al.*, 2012). In simian immunodeficiency virus (SIV) infection of non-human primates, DN T-cells are capable of performing CD4^{+} T-cell-like helper functions whilst remaining refractory to SIV infection and are associated with a lack of clinical disease progression (Milush *et al.*, 2011; Sundaravaradan *et al.*, 2012, 2013). Thus, the increased numbers of DN T-cells in HPgV infection may play important roles in reducing immune activation and maintenance of immune homeostasis, contributing to improved survival in HIV co-infection.

Although little is known regarding HPgV persistence and T-cell escape mechanisms, data related to HCV persistence may be relevant. Key factors in HCV persistence include the emergence of viral neutralization and T-cell escape mutations, and reduced virus-specific CD4^{+} T-cell function (reviewed by Rehermann, 2013). CD4^{+} T-cell help is critical in HCV

persistence, as depletion of CD4$^+$ T-cells from chimpanzees that had recovered from HCV infection abrogated the protective CD8$^+$ T-cell-mediated immune response upon HCV rechallenge (Grakoui *et al.*, 2003). In contrast to HCV, HPgV does not have a hypervariable region in the envelope proteins and neutralizing antibodies directed against viral structural proteins are not detected in chronically infected individuals. Thus, neutralizing antibody escape does not appear to be a strategy utilized by HPgV (Thomas *et al.*, 1998). The critical roles of T-cell escape mutations and impaired CD4$^+$ T-cell help during HPgV infection, and how these features mediate viral persistence, have not been studied.

B-cell function and antibody production

As noted above, antibodies against HPgV structural proteins are generally not detected during viraemia and most individuals develop conformation-dependent antibodies to E2 at or shortly following viral clearance (Gutierrez *et al.*, 1997; Tacke *et al.*, 1997; Tanaka *et al.*, 1998a). However, anti-E2 antibodies are occasionally found during active viraemia and some of these individuals may be in the act of clearing viraemia (Schwarze-Zander *et al.*, 2006; Tan *et al.*, 1999; Williams *et al.*, 2004). Some recent reports describe the detection of anti-HPgV peptide antibodies (Fernández *et al.*, 2013; Gómara *et al.*, 2011); however, no clear relationship between peptide-reactive antibodies in actively HPgV-infected or convalescent subjects has been reported. As anti-E2 antibody is usually inversely related to HPgV viraemia (Gutierrez *et al.*, 1997; Tacke *et al.*, 1997; Tanaka *et al.*, 1998a), it is possible that HPgV suppresses the production of antibodies, contributing to persistent infection. There are some data suggesting that HPgV may reduce B-cell activation in addition to T-cell activation. Specifically, subjects with HPgV and HIV co-infection had reduced activation marker (CD86) expression on B-cells when compared with HIV-infected subjects without HPgV co-infection (Stapleton *et al.*, 2013). To examine this further, PBMCs were isolated from HPgV-positive (*n*=9) and HPgV-negative control subjects (*n*=8), stimulated with soluble CD40 ligand (sCD40L) and activation was measured on B-cells (CD3$^-$CD19$^+$) by measuring surface CD86 expression. CD40–CD40L interaction is required for germinal centre formation, isotype switching, immunoglobulin production and induction of memory B-cells (Durie *et al.*, 1994; Foy *et al.*, 1994). We found a significant reduction in activation as measured by CD86 surface expression on B-cells following sCD40L stimulation (*P*=0.04; Fig. 3). Further research is required to understand specific B-cell pathways suppressed by HPgV.

HPgV and NK-cells

Viral infection of NK-cells is relatively uncommon, although a few examples have been described. NK-cells are permissive for vaccinia infection *in vitro* (Kirwan *et al.*, 2006; Rajagopalan & Long, 2000; Sánchez-Puig *et al.*, 2004) and TTV genomes are present in NK-cells *ex vivo* (Maggi *et al.*, 2001; Okamoto, 2009; Takahashi *et al.*, 2002; Zhong *et al.*, 2002). We found HPgV RNA in highly purified NK-cells obtained from four of five subjects (mean 42 genome equivalents per 10^4 cells) (Chivero *et al.*, 2014). Viral RNA was shown to be taken up by NK-cells obtained from donors not infected with HPgV, and viral RNA increased and was released into culture supernatants by PBMCs in these studies (Chivero *et al.*, 2014). As noted earlier, HPgV RNA is present in serum extracellular microvesicles and these may be involved in infection of various PBMCs (Bhattarai *et al.*, 2013).

One clinical study suggested that HPgV infection may modulate NK-cell activation in addition to its effect on T-cells (Stapleton *et al.*, 2013). This study found lower levels of the activation marker CD69 were detected on peripheral CD56^{bright} NK-cells in HPgV/HIV co-infected individuals compared with HIV mono-infected individuals (Stapleton *et al.*, 2013). CD56^{bright} NK-cells are major cytokine producers (IFN-γ and TNF-α); thus the reduction suggests that HPgV may impair NK-cell function. To date, no studies examining the relationship between HPgV infection and NK function have been reported. As NK-cells act as rheostats modulating antiviral T-cells, interference with NK-cell function may also contribute to viral persistence (Welsh & Waggoner, 2013). In humans infected with the closely related HCV, NK-cell production of IFN-γ and TNF-α is suppressed (Ahlenstiel *et al.*, 2010; Oliviero *et al.*, 2009; Peppa *et al.*, 2010), whilst cytotoxicity and degranulation is increased (Ahlenstiel *et al.*, 2010; De Maria *et al.*, 2007). Further study to examine the effect of HPgV infection on NK-cell function is needed, and these studies may identify critical and novel host immunomodulatory mechanisms pertinent to viral persistence.

HPgV: an ancient and successful human virus

Although HPgV was discovered only ~20 years ago (Leary *et al.*, 1996; Simons *et al.*, 1995), several lines of evidence suggest that it is an ancient virus that is well-adapted to growth in the human host. Genetically divergent isolates of HPgV have been isolated from different parts of the world with distribution extending to highly isolated populations, such as indigenous tribes in Papua New Guinea and Central and South America (Simmonds & Smith, 1999). The geographical distribution of HPgV genotypes reflects that of ancient human migrations, suggesting HPgV co-migration and viral diversification within the human hosts (Pavesi, 2001; Sharp & Simmonds, 2011). Two examples of this are the finding of isolates in Southeast Asia (genotype 3) that are most closely related to those of African origin, consistent with a major route of ancient human migrations from Africa to southeastern parts of the Asian continent (Pavesi, 2001), and the finding of genotype 3 viruses in indigenous (native) populations of South Americans, with a mixture of the Western European and North American genotype 2 and genotype 3 viruses in mixed-race South American populations (Loureiro *et al.*, 2002; Tanaka *et al.*, 1998b).

The lack of a causal relationship between HPgV infection and disease raises the possibility that HPgV is a symbiont or commensal of humans. If so, HPgV may provide some benefit to humans. There are considerable data suggesting a beneficial effect of HPgV infection on survival in HIV-positive subjects (Heringlake *et al.*, 1998; Nunnari *et al.*, 2003; Tillmann *et al.*, 2001; Toyoda *et al.*, 1998; Vahidnia *et al.*, 2012; Williams *et al.*, 2004; Xiang *et al.*, 2001; Zhang *et al.*, 2006). It is unlikely that the relationship between HPgV infection and immune activation was selected for by HIV, as HIV was only introduced into humans during the past century (Zhu *et al.*, 1998). Mechanisms purported to contribute to slower HIV disease progression include the findings of reduced surface expression of the chemokine receptors CCR5 and CXCR4, entry co-receptors for HIV (Nattermann *et al.*, 2003; Schwarze-Zander *et al.*, 2007; Xiang *et al.*, 2001), and high plasma levels of the ligands for these receptors, i.e. macrophage inflammatory protein (MIP)-1*a*, MIP-1*b*, RANTES (CCR5) and stromal-derived factor-1 (CXCR4) (Xiang *et al.*, 2004), in chronic HPgV viraemic subjects compared with those without HPgV infection. Additionally, the relationship

between HPgV infection and reduced immune activation (Bhattarai *et al.*, 2012a; Maidana-Giret *et al.*, 2009) suggests that this virus has anti-inflammatory effects that may be generally beneficial to humans. In contrast, this subtle reduction in immune function may contribute to the observed association between HPgV and non-Hodgkin's lymphoma by reducing immune surveillance mechanisms (Chang *et al.*, 2014; Civardi *et al.*, 1998; De Renzo *et al.*, 2002; Ellenrieder *et al.*, 1998; Giannoulis *et al.*, 2004; Keresztes *et al.*, 2003; Krajden *et al.*, 2010; Michaelis *et al.*, 2003; Nakamura *et al.*, 1997).

The association between HPgV infection and survival outcomes in HIV-infected individuals suggests that HPgV has a symbiotic relationship; however, due to the lack of an animal model, this has been difficult to assess directly. The application of next-generation sequencing has identified pegiviruses closely related to HPgV in Old World primates and numerous other species, raising the possibility that an animal infection model may be feasible (Kapoor *et al.*, 2013; Sibley *et al.*, 2014). An animal model would allow direct testing of the hypothesis that chronic infection is beneficial.

Conclusions and future directions

The mechanisms by which HPgV infects diverse blood cell types and persists in humans remain enigmatic, and require further clarification. First, although HPgV RNA is found in many cell types, negative-strand RNA is only found consistently in bone marrow and spleen (Radkowski *et al.*, 2000; Tucker *et al.*, 2000). Replication at these sites suggests the possibility that the primary permissive cell type is a HSC. Second, the mechanisms of HPgV persistence and clearance are not understood. Whilst HPgV effects on B-cells have not been studied in detail, failure to mount an antibody response to HPgV non-structural proteins and delayed development of E2 antibodies suggests impaired B-cell function. Furthermore, CD8$^+$ T-cells play a critical role in the clearance of many viral infections. As HPgV infection inhibits CD8$^+$ T-cell activation, further characterization may provide insight into persistence. In addition, host genetic factors that may contribute to viral persistence are also unknown and warrant further studies. Third, the effects of HPgV on immune cells may be shared amongst other viruses and characterization of these may provide novel insights into mechanisms used by other viruses to evade host immune responses. Finally, as HPgV is a non-cytopathic virus that persistently infects humans without illness, and appears to replicate in spleen and bone marrow tissues, HPgV may provide novel approaches to gene therapy.

Acknowledgments

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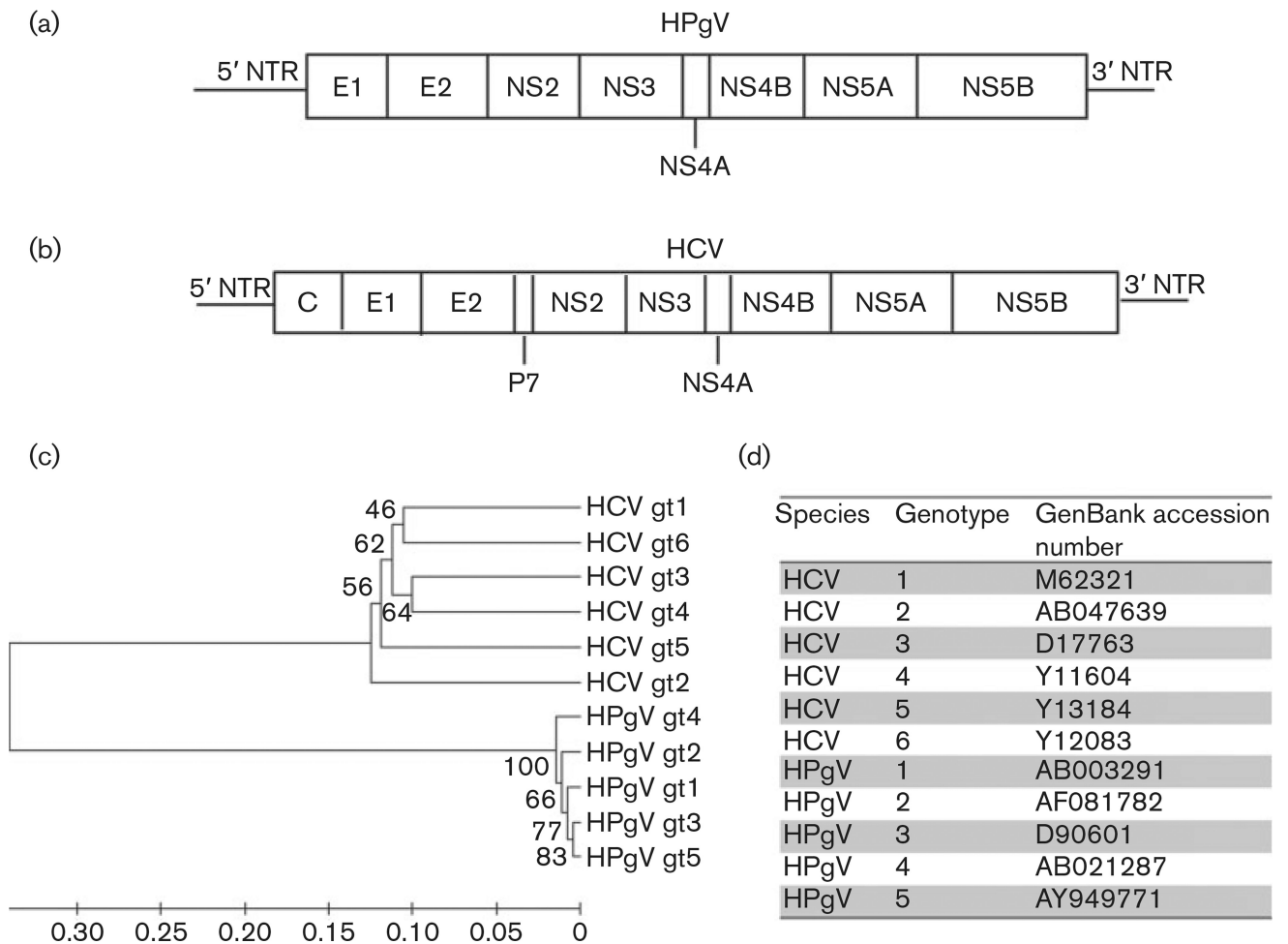
(a)

HPgV

| 5′ NTR | E1 | E2 | NS2 | NS3 | | NS4B | NS5A | NS5B | 3′ NTR |

NS4A

(b)

HCV

| 5′ NTR | C | E1 | E2 | | NS2 | NS3 | | NS4B | NS5A | NS5B | 3′ NTR |

P7   NS4A

(c)   (d)

46	HCV gt1
62	HCV gt6
56	HCV gt3
64	HCV gt4
	HCV gt5
	HCV gt2
	HPgV gt4
100	HPgV gt2
66	HPgV gt1
77	HPgV gt3
83	HPgV gt5

0.30 0.25 0.20 0.15 0.10 0.05 0

Species	Genotype	GenBank accession number
HCV	1	M62321
HCV	2	AB047639
HCV	3	D17763
HCV	4	Y11604
HCV	5	Y13184
HCV	6	Y12083
HPgV	1	AB003291
HPgV	2	AF081782
HPgV	3	D90601
HPgV	4	AB021287
HPgV	5	AY949771

Fig. 1.
HPgV and HCV genome organization and phylogenetic relationships. (a, b) Whilst HPgV (a) is within the genus *Pegivirus* and HCV (b) is within the genus *Hepacivirus* of the family *Flaviviridae*, both viruses share many features, including a ssRNA positive-sense genome with 59 and 39 NTRs. Both viruses are capable of causing persistent human infection. (c, d) HPgV and HCV phylogenetic trees (unweighted pair group method with arithmetic mean) (c) were reconstructed in MEGA6 using alignments of isolates with GenBank numbers shown in (d). The original tree without bootstrapping is shown. gt, Genotype.
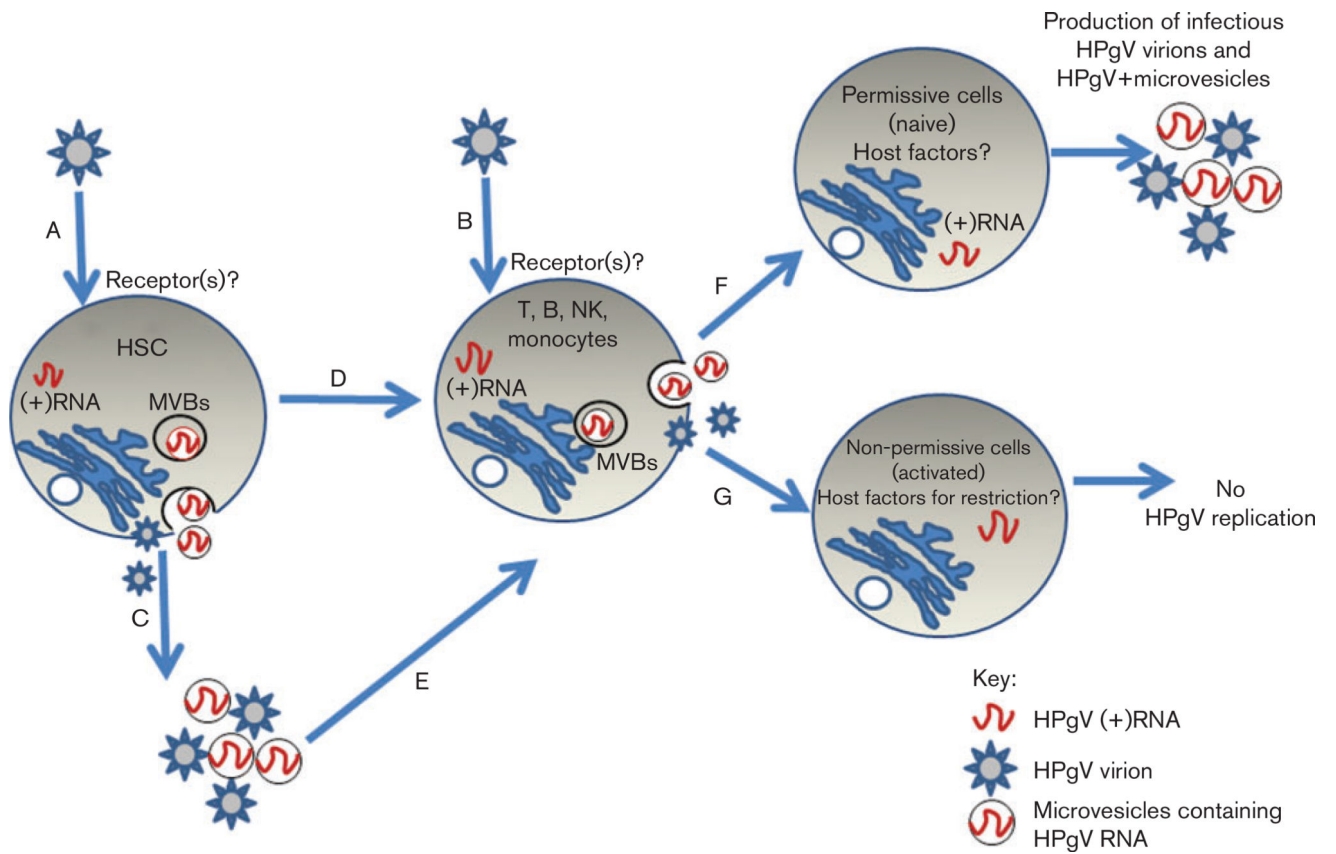
Fig. 2.
Model for HPgV tropism. HPgV may infect HSCs in spleen and/or bone marrow due to expression of a specific receptor that is not present in differentiated cells (A) or lymphocytes expressing yet unidentified common receptors (B). If HSCs are infected, HPgV genomes are passaged during differentiation (D) with the production of low-level virus. Alternatively, infected HSCs produce exosomes that deliver RNA to multiple cell types (C, E) that produce low-level virus. Host factors either promote (in resting cells F) or restrict (in activated cells; G) viral replication within each specific cell type. MVBs, multivesicular bodies.
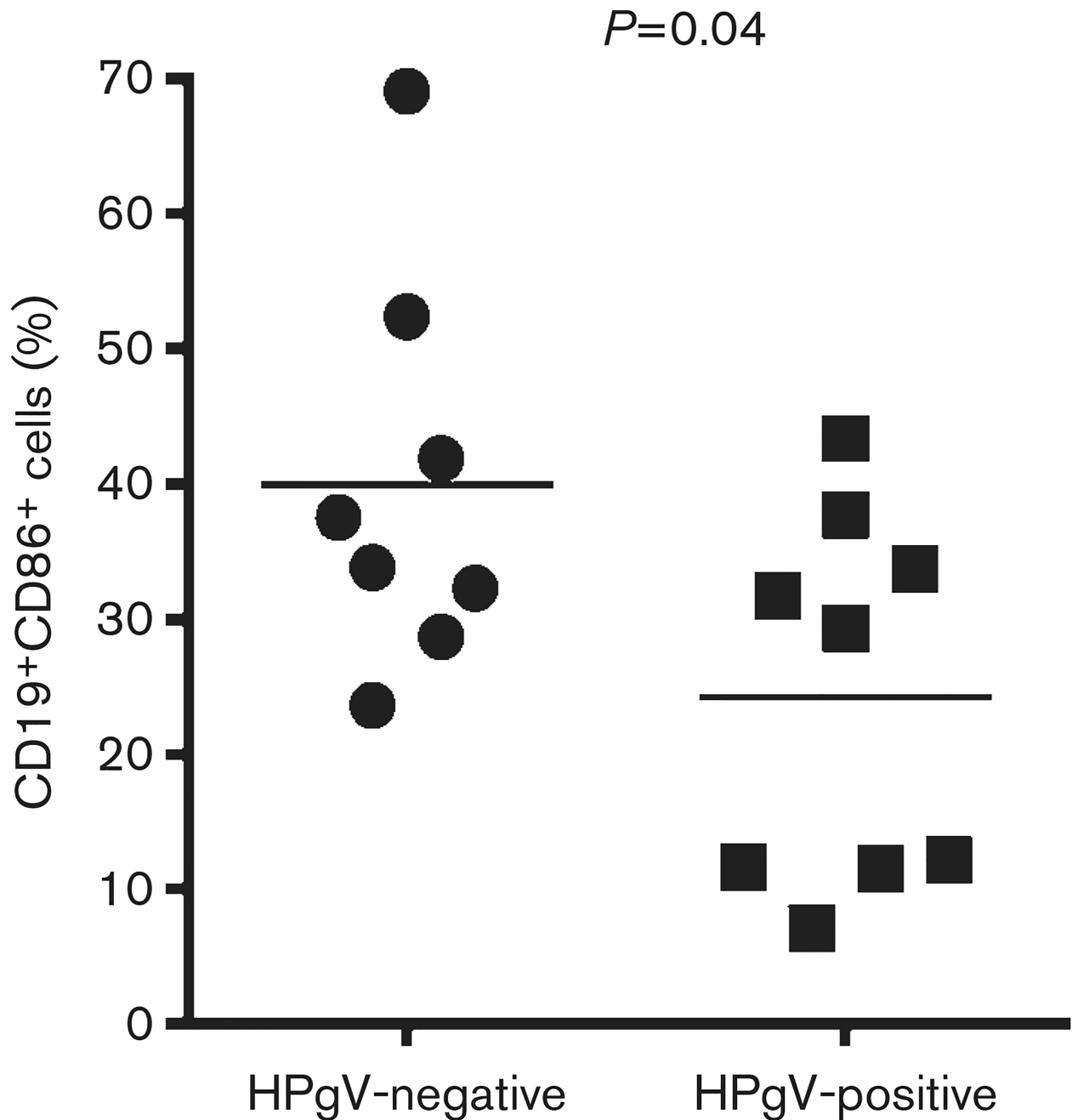
Fig. 3.

HPgV inhibits sCD40L–mediated B-cell activation (CD86 expression). PBMCs isolated from HPgV-positive subjects (n=9) or HPgV-negative subjects (n = 8) were stimulated *ex vivo* with sCD40L (5 ng ml^{-1}) overnight. Expression of the B-cell activation marker CD86 was quantified on CD19$^+$ CD3$^-$ B-cells by flow cytometry. HPgV viraemic subjects had a significantly lower percentage of cells expressing the activation marker CD86 (P=0.04) compared with HPgV-negative subjects.

Table 1

Selected studies examining replication of HPgV

Reference	Cell/tissue type	Positive-strand RNA	Negative-strand RNA
Mellor *et al.* (1998)	PBMCs	11/20	0/20
Mellor *et al.* (1998)	Hepatocytes	8/13	0/13
Laskus *et al.* (1997)	Liver	6/10	0/10
Laras *et al.* (1999)	Hepatocytes	6/12	0/12
Tucker *et al.* (2000)	Spleen	3/3	3/3
Tucker *et al.* (2000)	Bone marrow	2/2	2/2
Radkowski *et al.* (2000)	Bone marrow	9/48	4/5

Table 2

Selected studies examining HPgV RNA in different cell types

Reference	PBMCs	Bone marrow	CD4+ T-cells	CD8+ T-cells	B-cells	Monocytes	NK-cells
Chivero *et al.* (2014)	14/14	nt	13/13	13/13	7/10	4/5	4/5
Kisiel *et al.*. (2013)	11/23	18/23	NT	NT	NT	NT	NT
Ruiz *et al.* (2010)	1/1	NT	1/1	1/1	1/1	NT	NT
George *et al.* (2006)	9/9	NT	9/9	9/9	9/9	NT	NT
Radkowski *et al.* (2000)	NT	9/48	NT	NT	NT	NT	NT
Fogeda *et al.* (1999)	4/4	NT	NT	NT	NT	NT	NT

HPgV viral RNA was detected by reverse transcription-PCR.

NT, Not tested.