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Reflection paper on viral safety of plasma-derived medicinal products with respect to hepatitis E virus

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1. Introduction

Hepatitis E virus (HEV) is a causative agent of hepatitis in many countries and of emerging concern in industrialised countries. HEV is a non-enveloped, single-stranded, positive-sense RNA virus and a member of the family Hepeviridae. In developing countries, HEV (genotypes 1 and 2) is a major cause of acute hepatitis, transmitted by the faecal–oral route and associated with contamination of drinking water. In industrialised countries, HEV (genotypes 3 and 4) has been found to be more prevalent in the human population than originally believed. HEV genotypes 3 and 4 infect not only humans but also animals such as swine, wild boar and deer. Zoonotic transmission of HEV genotypes 3 and 4 to humans can occur by consumption of undercooked pork and wild boar products or by contact with infected animals. Genotypes 3 and 4 are generally less pathogenic than genotypes 1 and 2, although serious infections have been reported also with genotypes 3 and 4. Chronic infection with HEV genotype 3 is an emerging concern among transplant recipients and may also occur in persons with HIV and certain haematologic disorders.

HEV infection is widespread and blood/plasma donors are often asymptomatic. Therefore, there is a risk for viraemic blood donations. HEV has been recognised as a transfusion transmissible agent since 2004 and transfusion-related cases have been documented in several countries (United Kingdom, France, Japan, Saudi Arabia, People's Republic of China). Recent analysis of blood and plasma donations has identified HEV-infected donors in Europe and USA. Consequently, HEV-RNA has been detected in plasma pools used for production of medicinal products.

The published reports on frequency of viraemic blood donations and studies on plasma pools indicate that plasma pools used as starting material for manufacture of medicinal products can be contaminated with HEV. In addition there have been cases with post donation information, indicating that HEV-affected donations have entered plasma pools for fractionation.

This raises questions about the safety of plasma-derived medicinal products. The Ph. Eur. monograph for human plasma pooled and treated for virus inactivation (1646) was revised to include a test for HEV RNA (implementation date 1 January 2015). A WHO International Standard for HEV RNA has been established promoting the standardisation of HEV assays by nucleic acid amplification technology (NAT). Manufacture of other plasma-derived products includes process steps for inactivation/removal of non-enveloped viruses. Their effectiveness against HEV is currently under investigation. HEV is difficult to grow in cell culture and current information about susceptibility of HEV to virus inactivation/removal steps used in the manufacture of plasma-derived medicinal products is limited.

An EMA Workshop on Viral safety of plasma-derived medicinal products with respect to hepatitis E virus was held on 28-29 October 2014. The purpose of the workshop was to obtain further information on the safety of plasma-derived medicinal products with respect to HEV and to provide the basis for deciding what further action may be needed. Key questions that were addressed were:

- *Clinical experience with HEV infections and transfusion-associated infections: How serious are HEV infections and which patient populations may be particularly at risk?*
- *HEV detection and epidemiology of HEV in blood/plasma donations*
- *Do serum antibodies against HEV significantly neutralise?*
- *Latest experience from studies on inactivation/removal of HEV: Which steps are effective to remove / inactivate HEV? Which model viruses can be used to assess that? Do we need more virus validation data?*
- *Risk assessment for plasma-derived medical products and implication for warning statements: Do we need risk assessments and/or warning statements?*

- *NAT testing will be required in the Ph. Eur. for SD plasma. Should this also be required for any other products?*

2. Discussion

2.1. Transfusion-associated infections and clinical experience with HEV-infections

In industrialised countries, HEV-infection with genotypes 3 and 4 can lead to an acute self-limited hepatitis. Most infections are asymptomatic or mild. This is indicated by the large sero-prevalence of antibodies whereas only few cases of hepatitis E are notified per year in European countries where reporting is practiced. It seems therefore that only few infections with genotype 3 (0.1 to 1%) lead to acute hepatitis. Nevertheless, given the wide distribution of HEV, hepatitis E is now the most common form of acute enteric hepatitis in Western Europe and more clinical cases of endemic hepatitis E than cases of hepatitis A are diagnosed per year.

Clinical signs of hepatitis caused by genotypes 3 and 4 of HEV are indistinguishable from infections with genotypes 1 and 2. Hepatitis is commonly associated with jaundice, anorexia, lethargy, fever, abdominal pain, and vomiting. Infection may also be associated with pruritus, weight loss, and headaches. Acute hepatitis E is more frequently observed in older persons (more than 40 years old) and more than two thirds of the affected patients are men. In contrast to infections with genotypes 1 and 2, infection of immunocompetent persons with HEV genotypes 3 and 4 is mostly mild or asymptomatic and severe cases are rarely observed. Similar to hepatitis A, patients with pre-existing chronic/advanced liver disease are at risk for developing liver failure after infection with HEV genotypes 1 and 2 in developing countries. Experience with genotype 3 infection in developed countries is more limited although cases of acute hepatitis with genotype 3 have been described. HEV infection was retrospectively found in 3-13% of cases of drug-associated liver injury. It seems that HEV infection is underdiagnosed as clinicians often do not consider HEV testing of patients who have not travelled to developing countries.

Hepatitis E virus infection with genotypes 1 and 2 can lead to high mortality among pregnant women in developing countries. However, no serious infections of pregnant women with genotype 3 have been observed, so far.

The knowledge about extrahepatic clinical manifestation of HEV infections with genotypes 3 and 4 is “still emerging”. Cases of arthritis, pancreatitis, bilateral brachial neuritis and encephalitis have been described and potential association with Guillain-Barré Syndrome was discussed at the 2014 EMA workshop.

Chronic hepatitis E has been defined as persistent viraemia for more than 3 months. Infection of immunosuppressed persons with HEV genotype 3 can result in chronic hepatitis. Chronic HEV infection may occur in transplant recipients, in case of haematological malignancy and in HIV-infected patients. HEV infection takes a chronic course in about 60% of solid organ transplant recipients infected with HEV. Persistent viraemia in transplant recipients can resolve spontaneously or can be treated by reduction of immunosuppression or by treatment with ribavirin. However, in some cases, infection may rapidly progress to liver fibrosis/cirrhosis in immunosuppressed patients. In one study, cirrhosis was observed in 9.4% of HEV RNA-positive solid organ recipients. In general, reduction of immunosuppression can help to clear the virus (e.g. kidney transplants). However, it may not be an option for specific transplant recipients (e.g. heart, liver) or it can be dangerous at haematopoietic stem cell transplantation because of Graft-Versus-Host-Disease (GVHD) risk.

Most HEV RNA positive blood/plasma donors do not have clinical symptoms at donation and often do not develop symptoms after donation. Therefore they are not recognised as HEV-infected at donation or thereafter. Thirty-nine transfusion-transmitted infections (TTI) with HEV have been described so far in the scientific literature. Identification of TTI may be difficult and results from antibody-testing have to be interpreted with caution. The time until seroconversion may be prolonged in immunocompromised patients. Transfusion of anti-HEV positive plasma or administration of immunoglobulins can lead to the detection of antibodies to HEV. In addition, re-activation of pre-existing chronic infections as well as re-infections of patients with an initial IgG positive status have been observed. Other possible sources of infection such as contaminated food have to be considered. Therefore detection of HEV-RNA and sequence analysis is desired in order to confirm TTI.

In France, 19 cases of post-transfusion hepatitis were declared between 2006 and October 2014. Among these cases, 14 cases were declared between 2012 and 2014 (ANSM, 2014). The clinical course of TTI with HEV ranged from mild symptoms with elevated liver enzyme values to acute cytolytic hepatitis. The assigned grades of severity were Grade 1 (non-severe) or Grade 2 (severe, not life-threatening). Most TTIs in France were observed with immunosuppressed patients. All the categories of blood components were involved in the transmission: Fresh frozen plasma (FFP) (8/19), SD-plasma (5 cases), quarantine-FFP (1 case), amotosalen-treated FFP (2 cases), red blood cell concentrates (7/19), platelet concentrates (4/19), standard platelet concentrates (2 cases), and apheresis platelet concentrates (2 cases). The HEV RNA concentrations in donations ranged from $10^{1.08}$-$10^{4.83}$ IU/ml. In one case there was evidence for transmission by SD-plasma containing 41.4 IU/ml HEV RNA. In a recent study from UK (Hewitt et al, 2014), there was a 42% transmission rate from viraemic blood components. The median viraemic concentrations of donations associated with transmission was above 10^4 IU/ml while the median concentration of donations not associated with HEV transmission was around 100 IU/ml. However, transmission cases from patients with low viraemia such as slightly above 100 IU/ml have been described. An estimate of 450 transfusion-transmitted infections per year by blood components in the UK was given.

In conclusion, HEV genotype 3 is considered mainly a threat for immunocompromised people (e.g. transplant recipients) and patients with underlying liver impairment or disease. The clinical symptoms of transfusion-transmitted infections seem similar to those from the oral route. Blood or plasma donations from patients with low viraemic HEV RNA concentrations such as 100 IU/ml may be infectious.

2.2. HEV detection and epidemiology of HEV in blood/plasma donations

Sero-prevalence has been studied in various populations including blood donors from several countries in Europe. Depending on the individual study and region, the IgG-prevalence in Europe ranged from 1% to 52% in South Western France. In central Italy, the overall anti-HEV IgG prevalence was 48.9% using a sensitive assay. In studies investigating sero-conversion, incidence ranged from 0.2 % infections per person year in the UK to 3.2% in South Western France. Interpretation and comparison of the various sero-epidemiological studies is difficult. A main reason is that different antibody assays show substantial variability in sensitivity and specificity. In addition, batch to batch variability of antibody assays has been reported. A WHO standard for HEV RNA is available. However, no international standard for HEV-antibodies is yet available, although an anti-HEV containing human serum has been developed as reference reagent.

Despite of the current issues with standardisation of antibody assays, it can be concluded from the many sero-epidemiological studies that the prevalence of HEV genotype 3 infection in the general

population and blood/plasma donors in developed countries is high. The sero-prevalence generally increases with age, irrespective of gender. Prevalence of HEV genotype 3 infection in pigs (and wild boar) is immense and zoonotic infection by raw or undercooked pork meat and offal or by contact to pigs is considered the main transmission route responsible for the wide distribution of HEV genotype 3 in the human population. Sero-prevalence in vegetarians is lower than in non-vegetarians. Cases of oral transmission by contaminated shellfish and fruits have been described.

Analysis of birth cohorts by sero-epidemiological studies indicates that HEV has been present in the European population for a long time. Studies from UK and the Netherlands show that HEV incidence fluctuated in the past and continues to fluctuate. An increase or re-emergence of HEV infections has been observed in the recent years in the Netherlands.

Progress has been made in developing sensitive HEV NAT assays and a WHO standard for HEV RNA is available. There are now many studies on HEV-RNA in blood donations. HEV RNA was detected in 1 of 1595 up to 1:14250 blood/plasma donations depending on the assays and region. Plasma pools for fractionation are composed of more than 1,000- 10,000 individual donations. Therefore, there is a high probability that such pools contain viraemic donations. Viraemic RNA titers from serologic window phase donations are usually low or moderate with not more than 10^6 IU per ml and will be diluted to very low concentration in plasma pools for fractionation. However, peak concentrations exceeding 10^7 IU HEV RNA per ml have been observed in single donations and there is a risk that such donations enter plasma pools for fractionation. HEV RNA has been detected in plasma pools from Europe as well as from USA. In a study of 75 plasma pools, HEV-RNA was detected in 8 pools. HEV concentrations were between 100 and 1000 genome equivalents per ml, (Baylis et al., 2012).

In conclusion, infections of blood donors with HEV genotype 3 are widespread in Europe and, given the lack of plasma donor screening, there is a high probability of viraemic donations entering plasma pools. Although the viraemic loads are frequently low or moderate, peak concentrations of up to 10^7 IU HEV-RNA have been observed in single donations.

2.3. Do serum antibodies against HEV significantly neutralise?

HEV-antibodies can be found in plasma for about 10 years after infection. However, antibody titres decline with time and IgG antibody-status may change from positive to negative. This raises questions about long-term immunity. There is no licensed vaccine in Europe. A vaccine (Hecolin) produced from recombinant *E. coli* has been licensed in China. With this vaccine, over 87% protection from disease has been described in a 4 years observation period for healthy subjects aged 16–65 years. However, protection from (sub-clinical) infection was more limited. Infection with HEV may develop after re-exposure to the virus. This risk for re-infection might be higher in immunosuppressed patients. In a study of solid organ transplantation patients from Toulouse region in France, 3 of 6 of HEV-infections were re-infections of seropositive patients. This indicates a limited protection by serum antibodies.

HEV particles from blood and in vitro cell cultures have been found associated with lipids while HEV particles from faeces show the typical appearance of “non-enveloped” virus particles. The particle-associated lipids seem to protect the virions from antibody-neutralisation. *In vitro* neutralisation of HEV derived from serum is poor and HEV can efficiently replicate in cell culture despite the presence of HEV antibodies. Virions from faeces are somewhat more susceptible towards antibody-neutralisation than serum-derived virus particles. Pre-treatment of virus particles with chloroform or detergent increased the *in vitro* neutralization capacity of antibodies. However, the reduction capacity remained limited and residual infectious virus was recovered.

Low levels of HEV-specific antibodies may be found in plasma pools. In a study from France, anti-HEV IgG was detected in nearly all minipools consisting of 96 test samples. Antibody concentrations ranged from 0.3 to 10.6 IU/ml. Five cases of HEV-transmission by SD-plasma have been reported in France Unfortunately, the antibody-concentrations from the implicated product batches or plasma pools were not reported. However, given the general anti-HEV sero-prevalence in France, it seems unlikely that none of the five implicated batches contained HEV antibody positive donations. One transmission case from Canada has been implicated by several sero-conversions in recipients of a specific SD plasma batch.

In summary, the data presented indicate that the neutralisation capacity of serum antibodies against HEV is limited. Antibodies might contribute towards reduction of HEV infectivity in product intermediates. However, it is not possible to rely on neutralizing antibodies in plasma pools or product intermediates preventing transmission of HEV by plasma-derived medicinal products.

2.4. Studies on inactivation/removal of HEV during manufacture of plasma-derived products

2.4.1 Viruses used in validation studies

HEV

HEV is a small (27-33 nm) non-enveloped RNA virus, the only representative of the hepevirus genus in the family *hepeviridae*. HEV isolates have been obtained from human plasma or human faeces, faeces from pigs or wild boar, or liver homogenates from pigs or wild boar. Unfortunately, HEV does not grow well in cell culture and establishing a suitable *in vitro* cell culture system has been difficult. Nevertheless, some cell culture systems have been developed and sufficiently high HEV titres have been achieved for investigation of virus removal/inactivation. Okamoto and co-workers adapted a genotype 3 from human faeces (JE03-1760F) to replicate to high titres in two human cell lines, A549 lung cells and PLC/PRF/5 hepatoma cells (Tanaka et al., 2007). The HEV genotype 3 strain Kernow-C1 was isolated from a chronically-infected patient and has been adapted to growth in human hepatoma cell line HepG2/C3A and a recombinant cDNA clone can be used for transfection of cells (Shukla et al., 2011, Shukla et al., 2012).

Infectivity assays are necessary for investigation of virus inactivation procedures. The propagation and detection of HEV in cell culture is hampered by the generally poor susceptibility of cultured cells to HEV, requiring relatively high virus titres for infection. This reduces the sensitivity of studies to determine the HEV reduction capacity of the manufacturing process of plasma-derived products. A classical cytopathic effect-based infectivity assay is not available for HEV and infected cells must be stained by immunological methods. Alternative read-outs for the infectivity assay such as production of HEV RNA (or antigen) can be used. However, care has to be taken that a positive read-out represents *de novo* produced virus.

Alternatively, NAT can be used for investigation of the HEV reduction capacity of manufacturing steps in cases where the mechanism for virus reduction is partitioning (virus removal), e.g. for manufacturing steps such as nanofiltration, precipitation/depth filtration, or chromatography. NAT assays are highly sensitive, thus improving the dynamic range for demonstration of logarithmic reduction factors. When using NAT for study of virus partitioning, it has to be kept in mind that NAT detects virus particle-associated RNA as well as free RNA. Therefore, care should be taken to minimize free viral nucleic in virus spike preparations. Detection of free nucleic acids can be reduced by enzymatic pre-treatment of samples.

The physical form of HEV in plasma, where the virus is present as a lipid-associated form, is different from its physical form in faeces where the virus is not lipid-associated. Similarly to plasma derived HEV, cell culture-derived HEV is lipid-associated. The difference in physical form of the different HEV spikes available should be taken into account when selecting the most appropriate virus spike for use in validation studies. For instance, the efficacy of a 35N nanofiltration step could be affected by an upstream ethanol or S/D treatment which may remove HEV associated lipids and thereby reduce the size of the virus particles. Also, partitioning during cold ethanol fractionation or adsorption to ligands may depend on whether HEV is lipid associated or not, depending on the fractionation process. Therefore, the HEV spike should be carefully selected and a pre-treatment of virus spike according to the specific manufacturing procedure should be considered.

Model viruses

Estimates of the virus reduction factors for HEV could be obtained from viral validation studies carried out with other non-enveloped viruses having similar characteristics/size. The reduction capacity of manufacturing steps for plasma derivatives has been validated using several non-enveloped model viruses such as non-enveloped RNA viruses hepatitis A Virus (HAV) or encephalomyocarditis virus (EMCV) and the small non-enveloped DNA viruses such as canine parvovirus (CPV), porcine parvovirus (PPV) or minute virus of mice (MVM). When interpreting reduction data from HAV, it should be kept in mind that, similarly to HEV, HAV particles from serum or cell culture can be associated with lipids.

In theory, there is a high probability that HEV will be removed/inactivated if effective removal/inactivation of a broad variety of non-enveloped model viruses has been demonstrated. However, given several peculiar physical properties of HEV in its different physical forms, and lack of data, accurate extrapolations from model viruses are currently not always readily possible. An important issue seems whether or not lipid association of HEV may play a role in the reduction capacity of the production step. The available data suggest that no single model virus or single virus preparation seems appropriate for all different manufacturing steps that may contribute to HEV reduction.

Feline calicivirus (FCV), murine norovirus (MNV), and cutthroat trout virus (CTV) have been suggested as specific model viruses for HEV. There are suitable cell culture systems for these viruses in order to study virus inactivation. However FCV was more susceptible than HEV toward inactivation at low pH or at high temperature and, therefore, cannot be considered as a suitable model for HEV inactivation in this respect. Experience with MNV and CTV seems yet too limited to conclude how accurate these model viruses reflect inactivation of HEV.

2.4.2 HEV reduction by specific manufacturing steps

The limited data available on this subject and the implications for further reduction studies are discussed below.

Precipitation

Reduction of non-enveloped viruses such as picornaviruses or parvoviruses has been demonstrated for several cold ethanol fractionation steps. Some well-controlled fractionation steps have been found effective for reduction of non-enveloped model viruses while others showed only moderate or non-significant virus reduction capacity. The reduction capacity depends on the specific manufacturing step and process conditions and, therefore, product-specific studies are needed.

Variable results have been reported so far when comparing reduction of model viruses with reduction of HEV. There have been cases where HEV reduction was comparable to reduction of model viruses while, in other cases, reduction differed markedly. Interpretation and comparison of data is further

complicated by the observation that different forms of HEV spike (e.g. serum derived or lipid-associated HEV particles versus HEV particles from faeces or pre-treated HEV particles) showed different partitioning. No clear partition of lipid-associated HEV particles was observed at the initial plasma fractionation steps. In summary, it seems difficult to draw general conclusions on the efficacy of specific fractionation steps for HEV reduction at this point and the relevance of data from model viruses needs to be further clarified.

In conclusion, additional research is welcomed. Product-specific investigation of selected plasma fractionation steps for HEV reduction is recommended in cases where effective reduction by other manufacturing steps has not been demonstrated. The HEV-spike should be selected according to the specific manufacturing step and pre-treatment of virus spike might be considered. As plasma-derived virus particles seem associated with lipids, non-treated virus preparations from blood or cell culture should be used for initial fractionation steps from plasma, while preparations pre-treated according to the specific manufacturing process might be considered for later steps. Virus partitioning at precipitation steps can by studied by NAT assays.

Pasteurisation

Pasteurisation is a heating procedure for 10 hours at 60°C in liquid phase. Pasteurisation has been demonstrated to inactivate effectively HAV in many cases. The actual efficacy of inactivation depends on the specific manufacturing conditions (e.g. the stabilisers present). Some heat resistant cell culture-adapted HAV-strains have been described where inactivation at pasteurisation of albumin was limited to 2-3 $\log_{10}$ while other HAV-strains show robust inactivation of more than 4 $\log_{10}$.

Inactivation of HEV by pasteurisation has been investigated in few studies, so far. Inactivation in albumin was limited to 2-3 $\log_{10}$ while effective inactivation was observed in control experiments using buffer instead of albumin. It seems therefore that albumin has a stabilizing effect on HEV. Few studies on pasteurisation of HEV in coagulation factors or other plasma proteins have been performed so far. Inactivation varied from 1.3 $\log_{10}$ in case of pasteurisation of an alpha-1 antitrypsin preparation to more than 4 $\log_{10}$ at pasteurisation of a FVIII product intermediate. These differences could be explained by the different composition (stabilisers) of the products.

The reported HEV sensitivity to pasteurisation is similar to that of the most heat-resistant HAV strains. However, it has to be considered that only few studies have been performed using such heat-resistant HAV-strains and it seems, therefore, not possible to extrapolate existing validation data from HAV towards HEV-inactivation. A heat-stable model virus such as an animal parvovirus could be selected as worst-case model for HEV. However, with this approach, there is a risk of underestimating HEV inactivation at pasteurisation.

In conclusion, more data on the effect of pasteurisation on inactivation of HEV is desired. Where further investigation of pasteurisation with respect to HEV reduction is required, a product-specific study with HEV itself should be performed. Infectivity assays are essential for such studies. The HEV spike preparation can be selected according to the specific manufacturing process.

Dry heat treatment

Dry heat treatment is the key elimination step for non-enveloped viruses in the manufacture of many complex or intermediately-purified plasma-derived medicinal products which are not processed through a parvovirus-removing nanofilter. Extrapolation of HAV inactivation data from validation of dry heat-treatment to HEV seems not possible as HAV shows significant reduction at lyophilisation. However, such an effect has not been observed with HEV. A relatively heat-stable model virus such as an animal parvovirus or murine norovirus could be considered as a worst-case scenario. However, the experience is still too limited to conclude how accurately these model viruses reflect inactivation of HEV by dry

heat treatment. Therefore, product-specific studies with HEV seem necessary. If a HEV spike is used it should be determined whether the lipid-associated form or the non-lipid associated form is most representative for the physical form of the virus at the stage of dry heat treatment. Studies should consider robust conditions, e.g. low residual moisture during dry-heat treatment.

Nanofiltration

Virus reduction by nanofiltration is based on the retention of viruses based on their particle size. Different types of filter membranes or hollow fibers are used. It is not always possible to define a unique pore size of a specific filter. Virus filters have been developed for reduction of small non-enveloped viruses such as parvoviruses. The particle size of parvoviruses is between 18 and 26nm. These filters are sometimes called small virus filters or small pore size filters. Product specific validation of these filters usually includes a parvovirus and a picornavirus such as HAV or EMCV. The particle size of non-lipid associated HEV has been specified between 27 and 33nm while the size of picornaviruses is similar or slightly smaller.

Considering the particle sizes of HEV and picornaviruses, it seems therefore reasonable to consider picornaviruses as a (worst case) model for HEV at virus filtration. No HEV-specific validation studies are required for virus filters suitable for removal of parvovirus and virus reduction data from HAV or EMCV can be considered appropriate. Effective reduction of HEV has been experimentally confirmed (Yunoki et al, 2008) and others. It seems reasonable to postulate effective reduction of HEV in cases where effective reduction of a picornavirus or a parvovirus has been demonstrated.

The so-called “medium pore size virus filters”, have been designed for removal of large or medium-sized virus particles. Retention of HEV in buffer matrix was low or moderate. However, studies presented at the workshop indicated significant reduction (ranging from 3 to 4 $\log_{10}$) when virus-spiked product intermediates were applied to the filters. One of the presented studies showed increased reduction of cell-culture derived virus spike while reduction of faeces-derived or detergent-treated virus was more limited. This study implies that the size of the lipid associated particles would be greater than that of the “naked” virus particles. However it seems difficult to predict to what extent the nature of virus particles will influence the retention at a specific manufacturing process step.

Virus filters designed for reduction of medium-sized virus particles might contribute to virus safety by moderate reduction capacity for HEV. However, it is not possible to predict HEV reduction and product specific studies seem necessary. As for other process steps, where virus removal is the mechanism of virus reduction, such studies might be performed using NAT assay and consideration should be given to the appropriate spike preparation. Considering the limited reduction capacity and the potential influence of the nature of spike preparation and matrix, the use of model viruses seems not appropriate.

Low pH

HEV is stable at low pH, as can also be deduced from its route of infection. No or limited HEV inactivation was observed in IVIG after incubation at pH 4.2 and HEV was stable at pH 2.5 for 5 hours. Stability of HEV at low pH seems somewhat similar to that of animal parvoviruses such as CPV. CPV seems to be a model virus for HEV with respect to lack of sensitivity to low pH. HEV is much more stable to low pH than the calicivirus FCV which was investigated as a potential model virus for HEV.

In summary, there will be no gain in further investigation of the effect of low pH incubation on HEV inactivation as no or very limited contribution of such steps is expected during manufacture of plasma derived medicinal products.

Chromatography

In general, for chromatography steps, the achieved reduction factors can vary amongst the viruses, even within the same family. The results obtained with model viruses can therefore not be readily extrapolated to HEV. The mechanisms of partitioning and process parameters influencing virus reduction (robustness) should be understood. Product-specific studies with an appropriate HEV spike would be necessary if HEV reduction by chromatography steps is to be demonstrated. As for other partitioning steps, NAT studies could be performed.

2.5. Risk assessment for plasma-derived medical products

Risk assessments are essential for evaluating the safety of plasma-derived medicinal products. The general principles of virus risk assessments have been outlined in Chapter 9 of Guideline EMA/CHMP/706271/2010 (EMA 2011). The following considerations might be helpful for performing risk assessments with respect to HEV.

Frequency of viraemic plasma donations and virus loads.

Virus RNA concentrations from viraemic blood or plasma donations have been recently analysed in multiple studies. Viraemia is usually low or moderate with maximum titres below 10^6 IU/ml. However, some donations with more than 10^6 IU /ml have been identified and the maximum concentrations reported so far were up to 10^7 IU/ml. The frequency of viraemic donations ranged from less than 1:1000 to more than 1:14,000 depending on the individual donor population and the sensitivity of the NAT assay. However, considering that current plasma pools for fractionation can be composed of more than 10,000 donations, there is a risk that plasma pools include viraemic donations. In a worst case scenario a donation with 10^7 IU/ml would be diluted in a pool of 10^4 donations to a concentration of 1000 IU/ml.

Virus Inactivation / removal by manufacturing process.

Effective steps for inactivation/removal of HEV are considered a key factor for the virus safety of plasma-derivatives. The Guideline on plasma-derived medicinal products (EMA, 2011) requests at least one effective step with a reduction capacity in the order of 4 $\log_{10}$ or more for removal or inactivation of non-enveloped viruses. For virus filtration steps using small virus filters that have been demonstrated to remove effectively parvoviruses and/or picornaviruses (e.g. HAV, EMCV) it seems reasonable to consider similar reduction capacity for HEV. Currently, it seems more difficult to extrapolate model virus data from other manufacturing steps such as heating steps, other virus filters, precipitations or chromatographic steps and HEV-specific studies might be necessary in these cases. The specific aspects of virus inactivation/removal by individual manufacturing steps have been discussed above.

Neutralising antibodies

The *in vitro* neutralising capacity of serum antibodies against HEV is very limited. Depending on the specific product intermediate and physical state of virus particles, antibodies might moderately contribute towards reduction of infectious virus particles. However this would have to be confirmed by product-specific investigations using appropriate HEV spike preparations.

Infectious dose

Experience from transfusion-transmitted infections (TTI) was reviewed at the workshop. All kinds of blood components for transfusion (i.e. plasma, platelet concentrates, red blood cell concentrates) have

transmitted HEV. Plasma seemed the most risky component, probably because the viral load is highest in plasma. However, no information is available on the partitioning of HEV into the different components from a single blood donation. Blood components with high viraemic titres had higher probabilities for HEV transmission than low titre components. A median RNA concentration of TTIs above 10^4 IU/ml is reported. However, there is a broad variability and HEV RNA titres in blood donations or blood components from individual TTI cases ranged from more than 10^6 IU/ml down to about 100 IU/ml (Hewitt et al., 2014). Considering a volume of ca 200ml of a transfused blood product this would indicate total virus loads of at least 20,000 IU HEV RNA. The lowest TTI-associated total RNA load reported so far was of 7056 IU HEV RNA from an apheresis platelet concentrate (Huzly et al., 2013).

Although, the infectious dose represents a significant factor for risk assessment, it has to be kept in mind that it can be associated with a considerable variability depending on the individual scenario. The overall experience with transfusion-transmitted HEV infection is still limited.

Experience with transmission of HEV by plasma derived medicinal products

HEV has been in the donor population for a long time. A serologic study from Japan implied that HEV might have been transmitted in the past via coagulation factors which have not been subjected to virus inactivation/removal while there was no signal for transmission to patients receiving only virus-inactivated coagulation factors (Toyoda et al., 2007). There have been no specific case reports of HEV transmission via plasma-derived medicinal products (except S/D plasma). This lack of transmission reports is reassuring. Nevertheless, it should be kept in mind that hepatitis E can be overlooked unless specific diagnosis has been performed. Clarification of suspected transmission cases has been difficult in cases where the plasma pools tested negative for HEV RNA and where it was not possible to retest all individual donations contributing to the pool.

3. Conclusion

HEV genotype 3 has been observed in blood/plasma donations from asymptomatic donors in developed countries. There is a risk that such donations enter the manufacturing process of plasma-derived medicinal products. Infections with genotype 3 are often asymptomatic or mild. As far as patient population is concerned, HEV genotype 3 is considered a threat for immune compromised people (e.g. transplant recipients) and patients with underlying liver impairment or disease. The clinical presentation of HEV-infection can be diverse and is not yet completely known, although it is well established that HEV can lead to prolonged or chronic infection in immune deficient patients.

Infections of humans and pigs with HEV genotype 3 are widespread in Europe. Some fluctuations of incidence have been observed in the past and it is difficult to predict the future epidemiology. However, considering the widespread distribution of the zoonotic virus in pigs and the absence of stringent animal health measures to reduce HEV in pigs, it cannot be expected that the epidemiological situation will significantly improve in the near future. Given the lack of blood/plasma donor screening, there is a high probability that viraemic donations enter plasma pools. Although the viraemic loads are frequently low or moderate, peak concentrations of up to 10^7 IU HEV-RNA have been observed in single donations.

In-process testing of plasma pools for HEV-RNA using a mini-pool testing strategy (see Vollmer et al, 2012) might be helpful to screen out donations with high virus concentrations. Similarly to screening for HAV RNA, this could be considered as an additional safety measure contributing to the safety margin of plasma-derived medicinal products. However, a recommendation for a general HEV RNA

screening of plasma pools for fractionation is currently not considered. Products complying with the Guideline on plasma-derived medicinal products contain at least one manufacturing step effective against non-enveloped viruses. It seems currently more important to obtain further assurance that steps effective against non-enveloped viruses are robust HEV inactivation/removal steps, and studies on this issue are strongly encouraged. HEV-RNA screening might be considered for specific plasma-derived medicinal products where the HEV reduction capacity is expected to be very low or limited. This is in line with the revised Ph. Eur. monograph for human plasma pooled and treated for virus inactivation (1646), which from January 2015 includes a test for HEV RNA.

Concerning warning statements in product information, a general warning that the possibility of transmitting infective agents cannot be totally excluded is included in the Guideline on the warning on transmissible agents in summary of product characteristics (SmPCs) and package leaflets for plasma-derived medicinal products (EMA, 2011). In addition, specific reference is made to viruses that have been transmitted in the past by plasma-derived medicinal products and information is included on whether or not the measures in place for a specific product are effective for the non-enveloped hepatitis A and parvovirus B19 viruses. No HEV transmission cases have been reported so far with the currently produced plasma-derived medicinal products, with the exception of SD-plasma. However, it should be kept in mind that HEV transmission could go undetected. Nevertheless, given the lack of reported HEV transmissions and the currently incomplete information on effectiveness of inactivation/removal steps for HEV, it is not considered necessary or useful to introduce a specific reference to HEV in the warning statements. However, one exception might be SD plasma because there is no effective inactivation/removal step for HEV and HEV transmissions have been reported.

The current Ph. Eur. requirement for HEV RNA testing of plasma pools for SD plasma is expected to reduce the risk for HEV transmissions. However, the experience with SD plasma from HEV-RNA tested pools is as yet limited. Therefore, it is recommended to continue careful surveillance of SD plasma with respect to potential HEV transmission.

Recognising the clear evidence for contamination of plasma donations and pools with HEV, manufacturers are advised to perform preliminary risk assessments for their plasma-derived medicinal products on the basis of the available information on HEV. Consideration should also be given to whether the product concerned is likely to be administered to risk groups (e.g. transplant patients, and patients with immunodeficiency or hepatic disease). These preliminary steps will allow manufacturers to establish a priority order for further investigation of their products.

Robust inactivation/removal of HEV is the key factor towards the HEV-safety of plasma-derived medicinal products and manufacturers are advised to assure that their manufacturing processes are effective against HEV. It is recognised that extrapolation of virus reduction data from model viruses for HEV might be difficult in several cases. Specific studies with HEV seem necessary for heat-treatments, precipitations, chromatographic methods and virus filters with size exclusion in the range 30-50nm. It is recognised that infectivity assays with HEV are technically difficult and these systems are not yet ready to fulfil all formal requirements for validation studies. However, manufacturers are strongly encouraged to perform additional research or investigational studies with HEV on their key steps for inactivation/removal in the cases where data from model viruses cannot be extrapolated.

Albumin manufactured according to European Pharmacopoeia specifications and purified by established Cohn or Kistler/Nitschmann fractionation processes has an excellent virus safety record and no virus-specific risk assessments are expected according to Guideline (EMA/CHMP/BWP/706271/2010 (EMA 2011). Nevertheless, considering the limited inactivation of HEV at pasteurisation, the limited available

data on HEV-reduction during fractionation and the specificities of individual manufacturing processes, manufacturer should investigate their fractionation process with respect to HEV reduction.

A risk assessment should be performed when sufficient data is available for each product. If the outcome of this risk assessment should indicate that HEV may not be sufficiently inactivated/removed, additional measures such as improvement of virus inactivation/removal methodology or HEV testing should be considered.

The viral safety of plasma-derived medicinal products with respect to hepatitis E virus will be kept under review as further information becomes available.

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Appendix: summaries of individual presentations from the EMA Workshop on Viral safety of plasma-derived medicinal products with respect to hepatitis E virus, 28-29 October 2014

Clinical Experience with Hepatitis E Virus (Harry Dalton, University of Exeter Medical School, Cornwall, UK)

H. Dalton reviewed the clinical experience with HEV infections. Acute hepatitis E, caused by HEV genotypes 1 and 2, represents a major health issue in developing countries with high mortalities of 25% in pregnant woman and 70% in patients with chronic liver disease. In developed countries hepatitis E, caused by genotypes 3 and 4, is often asymptomatic or associated with mild symptoms. However, sporadic cases of severe hepatitis have been observed and the few studies with genotype 3 also indicate a higher risk for patients with underlying liver disease. Serious infections of pregnant woman with genotype 3 and 4 have not been observed so far. Genotype 3 may establish chronic infection in immunocompromised patients and re-infections have been observed. Hepatitis E infections have been under-diagnosed because physicians have not always been aware about HEV in developed countries. It has been found that HEV was overlooked in some cases that had been wrongly diagnosed as drug-associated liver injury. Chronic infection of immunocompromised patients, such as transplant patients and HIV infected patients, has now been repeatedly described. The knowledge about the full clinical spectrum of disease is still emerging. H. Dalton highlighted association of HEV with several extrahepatic manifestations of disease such as monoclonal gammopathy of uncertain significance, encephalitis, Bell's Palsy, ataxia/proximal myopathy, bilateral brachial neuritis, and Guillian-Barré-Syndrome.

HEV Experience from the Netherlands (Hans L Zaaijer, Sanquin and Academic Medical Centre – Clinical Virology, Amsterdam NL)

H. Zaaijer presented experience from diagnostic HEV-testing in the Netherlands (NL) from 2009-2014. Most HEV-infections were autochthonous and an average diagnostic laboratory in the Netherlands detects now more clinical cases of endemic hepatitis E than hepatitis A. In a first study on 5239 donors, 27% were found IgG positive. HEV RNA was detected in 1:3000 donations. However, recent experience from monthly analysis of screening of donations for SD-plasma showed even higher frequency of HEV RNA positive donations (up to 1:611). This and the different distribution of RNA or antibody positive donations among age-groups of donors indicated fluctuations of HEV incidence in NL with a recent increase. Pigs are still considered the predominant source for human infections. So far, there are no governmental actions against HEV-positive blood donations. H. Zaaijer pointed out that the main source of endemic HEV infection for patients probably is contaminated food or water, and it would be more important to uncover and eliminate this source of HEV. There seems a negligible significance of HEV genotype 3 infection for immune competent babies, children, pregnant women and adults <30 years, while HEV genotype 3 is considered a threat for immune compromised children and adults.

Transfusion Transmission: Hepatitis E Virus (Richard Tedder, Hepatitis E Study Group, Transfusion Microbiology Service, NHBSTT and Blood Borne Virus Unit, PHE, Colindale, UK)

R. Tedder presented the recent studies from UK investigating HEV in blood donations and transmission cases. In a study on 9382 minipools (consisting of 24 donations) from blood donations collected in 2013, 0.03% of donations were HEV RNA positive and 79 donors could be identified. The median viral RNA load of viraemic donations was 3.9 x 10^3 IU/ml (ranging from 50 to 2.37 x 10^6 IU/ml). A look back could be completed on 43 of 60 recipients and, in 18 cases (42%), transmission could be confirmed by identity of the virus sequences from donor and recipient. HEV genotype 3 (mainly clade2) was found in all cases. All kinds of blood components (plasma, red blood cells, platelets) were involved. The median viraemic concentrations of donations associated with transmission was above 10^4 IU/ml while the median concentration of donations not associated with HEV transmission was around 100IU/ml. However, transmission cases from patients with low viraemia (between 100IU/ml and 1000IU/ml) were observed. There was only one clinical case of mild post transfusion hepatitis. Ten recipients developed prolonged or persistent infection. An estimate of 450 transfusion-transmitted infections per year by blood components in UK was given. However, zoonotic HEV transmission via food was estimated to result in 100,000 infections per year in England.

HEV Infections Associated with Transfusion/Blood-derived Products/Organ Transplants- Situation and Cases in France (Wahiba Oualikene-Gonin, ANSM, France).

An overview about the HEV cases from the French hemovigilance database was presented. Nineteen cases of post-transfusion hepatitis E have been registered between 2006 and October 2014. Among these cases, 14 cases were declared between 2012 and 2014. All the categories of blood products were involved in the transmission: (FFP (8/19) = FFP-SD (N = 5), FFP-Quar (N = 1), FFP-IA (N = 2); RBC (7/19); Platelets (4/19) = MPC (2), PCA (2)). Viral RNA load of donor ranged from $10^{1.08}$-$10^{4.83}$ IU /ml. The clinical course of transfusion transmitted HEV-infections ranged from mild symptoms with elevated liver enzyme values to acute cytolytic hepatitis. The assigned grades of severity were Grade 1 (non-severe) or Grade 2 (severe, not life-threatening). Most transfusion transmitted infections (TTIs) in France were observed in immunosuppressed patients. One recipient, a solid organ transplant patient, developed a chronic HEV-infection. In addition, two cases of suspected transmission via plasma-derivatives were presented. However, it was not possible to confirm these cases by sequence analysis, as the affected plasma pools tested negative for HEV-RNA and it was not possible to test all individual plasma donations. One transmission case in 2013 by a kidney graft could be confirmed by sequence analysis.

Hepatitis E in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) and organ transplantation (Annemiek van der Eijk, Department of Viroscience, Erasmus MC, Rotterdam, NL)

A cross-sectional study was performed of all living adult solid organ transplant (SOT) recipients (n=1188) for whom serum or EDTA-plasma samples were available in the Erasmus Medical Center biobank. In 12 (1%) patients, hepatitis E virus infection was identified; in 10 patients, chronic infection developed. In a retrospective study from Erasmus Medical Centre, 8 infections were found in 328 recipients (2006-2011) of allogenic hematopoietic stem cell transplantation and 5 recipients developed chronic infection. All infections were by genotype 3. Five of 8 patients were misdiagnosed with graft versus host disease (GVHD), and 3 with drug induced liver disease. Three patients had positive HEV

IgG status before transplantation, which did not protect them. Four patients died with HEV viraemia and signs of ongoing hepatitis and there have been cases with rapid development of cirrhosis. The 4 surviving patients cleared HEV after a median period of 6.3 months. One patient was diagnosed with HEV reactivation after a preceding infection prior to allogenic HSCT. This patient was treated with ribavirin and cleared HEV infection up until this moment. Histopathology showed fibrosis in the periportal area of the liver, inflammation, and necrotic hepatocytes (councilman bodies). In conclusion HEV should be always included in the differential diagnosis of transplant patients presenting with liver enzyme abnormalities. Although the symptoms of graft rejection or graft versus host disease (GVHD) and HEV-infections are similar, the effects of modulating immunosuppression are contrary.

Hepatitis E Virus in solid organ transplant patients (Nassim Kamar, Toulouse University Hospital France)

Whereas chronic HEV infection has been defined in the literature by detection of HEV in serum or stool 6 months after diagnosis, N Kamar proposed to define chronic HEV infection by persisting HEV replication beyond 3 months after infection. A multicentre study involving 85 recipients of solid organ grafts was presented. 29 patients cleared the virus within the 6 months after diagnosis and 56 developed chronic hepatitis. The main clinical symptom was fatigue. In 8 cases, there was a progression towards cirrhosis. In contrast to chronic HCV infections, more rapid progression towards cirrhosis in 2-3 years has been observed. Chronic infection is usually observed in highly immunosuppressed patients. The use of tacrolimus versus cyclosporin A and low platelet counts could be associated with a risk of developing chronic infection. *S*ome immunosuppressive drugs (cyclosporin, tacrolimus) promote virus replication *in vitro* while other (mycophenolic acid) inhibit virus replication. Patients who developed a chronic infection had lower serum concentrations of IL-1 receptor antagonist and IL-2 receptor compared to those with resolving hepatitis. A considerable quasispecies heterogenicity of viral RNA sequences has been observed in chronically infected patients. Chronic infections of solid organ recipients can be managed by reduction of immunosuppression or by treatment with ribavirin. Treatment with pegylated interferon is another option but should not be done after heart, kidney or lung transplantation because of the risk for graft rejection. Two cases have been observed where ribavirin treatment failed to clear the virus. Three cases of re-infections of patients with a former positive antibody status have been identified. One of these re-infections resulted in chronic infection.

HEV in Europe and Latin America (José M. Echevarria, National Centre of Microbiology, Spain)

In Europe, the anti HEV seroprevalence ranged from 1.1 to 14% depending on the geographic region and the individual study. The different performance of available anti HEV assays seems to be, at least in part, responsible for the various outcomes. The prevalence of HEV in pigs is immense and zoonotic transmission by pig meat is considered the main source of infection. Epidemiology of hepatitis E virus infection in Latin America is more complex. In Chile, Argentina, Brazil, and Bolivia, infections were by genotype 3 while in Caribbean regions infections were due to genotype 1. Seroprevalence ranged from 5 to 20% but was found up to 30-70% in some studies. Isolated Amazonian population showed a distinct pattern of seropositivity with 30% in the age group of 21 to 30 years.

Detection of HEV infections and epidemiology in Italy (Anna Rita Ciccaglione, Istituto Superiore di Sanità, Italy)

The Italian national surveillance system for acute viral hepatitis (SEIEVA) collects data from Local Health Units covering 72.6% of the Italian population. Between 2007 and 2011, 49.4% of 6761 notified acute viral hepatitis cases were attributed to HAV and only 1.2% to HEV. Travel to endemic countries (India, Bangladesh) and consumption of seafood were the most prominent risk factors. However, HEV seems to be under-diagnosed or under-reported. Serum samples from 84 patients with non-A to non-C acute hepatitis were tested for HEV infection and 38 of them (33.3%) could be attributed to HEV. Genotypes 1 and 3 were identified in positive serum samples. In 2014, a study on 313 blood donors from Abruzzo (a rural region of central Italy located in the Apennines mountains) found two HEV genotype 3-RNA positive donors and 153 (48.9%) anti-HEV IgG-positive donors. In this study, the only risk factor independently-associated with anti-HEV IgG positivity was the consumption of raw dried pork-liver sausage. In another study conducted in 2013 on blood donors from Lazio, central Italy, anti-HEV IgG prevalence was found to be much lower (9%).

Hepatitis E Virus Assay Standardization (Sally A. Baylis, Paul-Ehrlich-Institut, Langen, Germany)

In 2009, the Paul-Ehrlich-Institut started to develop an HEV RNA standard on behalf of the WHO. The 1st WHO International Standard (IS) for HEV RNA (code number (6329/10) was established in October 2011 and was assigned a unitage of 250,000 international units (IU)/ml based on the collaborative study data. The standard has been derived from a HEV genotype 3a RNA-positive plasma donation from Japan. The PEI is currently developing a WHO international reference panel representing all four HEV genotypes. A secondary standard for HEV RNA has been prepared for the Biologicals Standardization Programme (BSP127) of the European Directorate for the Quality of Medicines and HealthCare to support the implementation of HEV NAT testing for S/D-treated plasma; there are now at least 10 commercially-available NAT assays and several of them have a CE mark according to Directive 98/79/EC on in vitro diagnostic medical devices.

Performance of IgG tests is very variable and also batch to batch variability has been observed. The consequences are a lack of comparability of results from different assays, significant discrepancies in performance and poor concordance between assay results. The serological WHO international reference reagent (95/584) was prepared at NIBSC and established by the ECBS in 1997. It is a lyophilized preparation of pooled sera from a patient in the US who developed acute hepatitis following travel to India. It was not established as an IS because the number of laboratories able to participate in the collaborative study was limited (n=7). There is a need for a validation of all existing serology tests and the poor performance of existing assays has led to underestimation of the seroprevalence of HEV, a matter which was highlighted at the consensus workshop on HEV at NIH in 2012 as well as by the WHO SAGE working group on the HEV vaccine in 2014.

Investigation about potential HEV-transmission through SD-plasma and HEV epidemiology in Canadian blood donors (Anton Andonov, Public Health Agency of Canada, Canada)

Anton Andonov presented a study indicating HEV transmission via SD Plasma. A serological follow up of 17 patients treated with 40 litres of SD-plasma for thrombotic thrombocytopenic purpura showed anti-HEV IgG/IgM in two cases who also became viraemic one month post exposure while no markers of HEV infection were observed in patients treated with cryo-poor plasma. None of the patients demonstrated any clinical signs of viral hepatitis during the 6-month period of observation. HEV

seroprevalence in Canadian swine herds is high ranging from 38% to 88%. The number of laboratory confirmed human HEV cases reported from 2006 to 2013 in Canada fluctuated between 10 and 41 per year. The majority of these were travel related. During the same period of observation only a dozen of autochthonous HEV cases belonging to genotype 3a have been confirmed both by serology and PCR. A recent study on 14,000 blood donors found anti- HEV IgG in 5.9% which is lower than the seroprevalence seen in other surveys of blood donors in North America. Seroprevalence was significantly higher in older age groups and males. None of 14,000 blood donors were viraemic for HEV (threshold of detection 250 IU/ml).

SD plasma and neutralization of HEV antibodies (Jaques Izopet, University of Toulouse, France)

In France, 558 testing pools (96 donations) for SD plasma corresponding to 53,234 plasma donations from Nov 2012 to Dec 2013 were tested for HEV-RNA (sensitivity 23 IU/ml) and antibodies. Twenty-two pools were HEV RNA positive indicating that HEV RNA was detected in 1 of 2200 donations. The median viral RNA titre of positive donations was $10^{4.4}$ IU/ml with individual values ranging from 468 IU/ml to 5 x 10^6 IU/ml. Frequency of positive donations was higher in South France than in North France and higher in males than in females. Nearly all 96-pools were positive for anti HEV with concentrations ranging from 0.3 to 10.6 U/ml. The proportion of subgenotypes characterized in France corresponded to that observed in pig populations. Preclinical trials with candidate vaccine in rhesus macaques indicated that a level of more than 100 IU/ml anti-HEV correlated with 50% reduction in infection. Experience with HEV vaccine from China indicates that protection against HEV infection by immunity is not absolute in immunocompetent individuals. With this vaccine, over 87% protection from disease has been demonstrated for healthy subjects aged 16–65 years in a 4 years observation period. However, protection from (sub-clinical) infection was more limited. In a prospective study of 263 solid-organ recipients at Toulouse University Hospital, six HEV infections were found in a 1 year follow up. Three of them were re-infections as patients had a positive IgG status at the beginning. Re-infection resolved spontaneously in two cases while one case showed chronic infection. The experience with solid organ transplant recipients shows that serum antibodies do not protect immunocompromised patients. An *in vitro* antibody neutralisation assay has been developed. The neutralisation capacity of antibody positive plasma depended on the virus spike preparation. A limited neutralisation capacity (1-2.2 $\log_{10}$) could be measured using plasma with 50 IU/ml anti-HEV and HEV-spike without lipid-associated virus particles while virus reduction was always below 1 $\log_{10}$ using lipid-associated virus spike.

HEV reduction in Virus Inactivation / Virus Elimination steps of plasma products manufacturing processes (Benoît Flan, LFB, France)

An infectivity assay has been developed at the Laboratoire de Virologie, Toulouse (J. Izopet) using HepG2/C3A cells and an adapted HEV genotype 3f isolate. The read out is *de novo* production of viral RNA. With this system, a virus stocks with 10^8 – 10^{10} HEV RNA copies/ml – corresponding to 5 $\log_{10}$ $TCID_{50}$ / ml could be obtained. A study on pasteurisation (at 58 ± 1°C) of a 20% albumin using cell-culture derived virus spike showed 2$\log_{10}$ inactivation after 10 hours. Inactivation kinetics was similar to delayed inactivation of some heat-resistant HAV strains reported in the literature (Farcet et al. Transfusion 2012 52:181-7). Pasteurisation of HEV in an intermediate from alpha-antitrypsin production showed only 1.3 $\log_{10}$ reduction indicating that the specific matrix or composition of stabilisers can influence HEV inactivation. Cell culture derived virus spike in PBS was significantly removed (≥4.55 log10 reduction of infectivity) by Pall DV50 filters while reduction was lower (3 $\log_{10}$)

when a faeces-derived virus spike or an NP40 treated virus spike was used. This indicated that the lipid-association of virus particles can influence particles size and virus retention; in these latter conditions HEV reduction was higher than HAV (CHCl3 treated) reduction (1.4 $\log_{10}$). Product-specific investigation of HEV–reduction at Planova 35N filtration of von Willebrand factor using a detergent-treated spike showed more than 2.5 $\log_{10}$ reduction of HEV infectivity and Planova 35N filtration of an immunoglobulin intermediate using ethanol treated virus spike showed 3 $\log_{10}$ reduction of HEV RNA. In vitro neutralisation experiments were performed using intravenous immunoglobulin (IVIG) preparations. There was no inactivation of cell culture-supernatant derived virus while faeces derived virus was moderately neutralised (1.8$\log_{10}$ and 2$\log_{10}$ reduction of infectivity). Virus stocks were also pre-treated with ethanol in order to simulate potential HEV-contaminants from IVIG production. There was a combined effect of ethanol-treatment and neutralisation leading to 2.8$\log_{10}$ overall reduction of infectivity. In addition to virus reduction data, a review of viraemic titres from blood/plasma donation was presented and TTI were reviewed in order to define an infectious dose for HEV (correspondence between HEV RNA and infectivity) for the HEV risk assessment of plasma-derived medicinal products.

Hepatitis E Virus: Baxter inactivation / removal data. Thomas R. Kreil, Baxter BioScience, Austria

RNA transcripts from the recombinant Kernow-C1 clone were used to transfect HepG2/C3A cells and supernatants from transfected cells could be used to infect fresh HepG2 cells. Read out for infectivity assay was by immunofluorescence analysis. When investigating the suitability of HEV RNA as read out, there was a virtual increase in HEV-concentration from inoculated CHO cells which are not permissive for HEV replication. This increase probably represented desorption of virus particles from inoculated cells. Therefore, NAT read-outs should be interpreted with care. An alternative virus spike was HEV from an infected pig liver homogenate. Partitioning steps were investigated by NAT using both virus spikes. 3.6 and 4.1 $\log_{10}$ reduction could be demonstrated for FVIII immuno-affinity chromatography and HEV was removed to below the limit of detection (up to >4.2 $\log_{10}$) for a Cohn II+III extraction step from IgG-purification. Reduction at the fractionation step was comparable to that of HAV and FCV. At pasteurisation of albumin, HEV inactivation was at least 3 $\log_{10}$. Treatment of virus stocks with solvent/detergent (SD) and C18 column chromatography to remove SD reagents resulted in reduction of infectivity by less than 1 $\log_{10}$. As expected, HEV was removed to below the detection limit by Planova 20N filtration.

HEV Reduction by Selected Manufacturing Steps of CSL Behring's Plasma-derived Products (Albrecht Gröner, CSL Behring, Germany)

An *in vivo* assay has been developed at the Friedrich-Loeffler-Institut – Federal Research Institute for Animal Health, Germany. In an inactivation study for VWF/FVIII intermediate (pasteurisation of stabilised aqueous solution of VWF/FVIII for 10 h at 60°C), samples of the intermediate spiked with a filtered liver homogenate from a wild boar with HEV genotype 3 prior to pasteurisation were pasteurised and inoculated into piglets. Read outs for infection of piglets were the time course of HEV RNA in faeces and detection of HEV RNA in bile after termination of study. This study showed inactivation of HEV in the order of at least 4 $\log_{10}$. Combined precipitation and adsorption steps from the VWF/FVIII manufacturing process were studied using NAT demonstrating an overall removal capacity of 3 $\log_{10}$. In summary, the overall reduction capacity was found comparable to that indicated by studies with HAV or B19V/CPV. Furthermore, cell culture derived cutthroat trout virus (CTV) was spiked into Ig-Matrix and infectivity could be removed below the detection limit by Pall DV20 filtration.

Plasma Products HEV Program Update (Rodrigo Gajardo, Grifols, Spain)

A double/complementary approach to study HEV removal/inactivation in different plasma derivatives production processes steps was presented i.e. investigation of new virus models for HEV and development and application of an HEV infectivity assay. The new HEV infectivity assay was described. The calicivirus murine norovirus (MNV) was investigated as a model for HEV inactivation by dry-heat treatment. Inactivation kinetics were slower than that of HAV, however, MNV was inactivated (around 4-5 $\log_{10}$) after treating of FVIII/vWF at 48-72h at 80°C. In addition inactivation of HEV was studied by infectivity assay. A mean reduction factor of 3.7 $\log_{10}$ (2 runs, residual infectivity detected) could be achieved using a detergent pretreated virus spike, with similar inactivation kinetics. HEV removal at partitioning steps (precipitation/depth filtration) from IVIG production were also studied using HEV infectivity assay and an overall reduction capacity of 6 $\log_{10}$ could be observed. Nanofiltration of another IVIG product using filters designed for parvovirus removal were found effective for removal of HEV by infectivity ($\geq$ 5.4 $\log_{10}$). Finally a comparison of HEV removal/inactivation results with other non-enveloped viruses was made showing similar results.

Experiences of HEV elimination during the manufacturing process steps and the suitable model viruses (Mikihiro Yunoki, Japan Blood Products Organization, Japan)

Evidence indicating that anti-HEV IgG / IgM may have no or only weak neutralising activity against HEV infection was reviewed. Adsorption experiments of HEV to protein G indicated that lipids may be attached to viral particles and inhibit (interfere with) IgG binding. Maternal antibodies failed to protect against mother to piglets infection and transfusion transmitted infection (TTI) with anti HEV containing donation has been observed in Japan.

Hepatitis E virus isolates in albumin solutions were inactivated slowly at 60°C for 10 h and the $\log_{10}$ reduction factor (LRF) varied from 1·0 to >3·0. Heat stability of HEV depended on the concentration of albumin. Non-detergent treated HEV spike from serum was found to be more resistant. The virus was slowly inactivated in a freeze dried fibrinogen containing stabilisers and the LRFs were 2·0 and 3·0, respectively, after 72 h at 60° C, but inactivated to below the detection limit within 24 h at 80 °C with an LRF of more than 4·0. Studies on partitioning at ethanol fractionation steps showed different behaviour of virus spikes according to their origin (serum, faeces) or pretreatment. It was found difficult to predict HEV-reduction from data with EMCV or CPV and reduction of HEV was more limited than that of model viruses. HEV was stable at 5hours incubation at pH 3.0 or pH2.5. Filtration experiments showed effective removal at Planova 20N filtration while reduction at Planova 35N filtration was limited to about 3 $\log_{10}$.

PPTA perspective on risk assessment for plasma-derived medicinal products and implications for warning statements (Ilka von Hoegen, Plasma Protein Therapeutics Association)

I. von Hoegen summarised PPTA's point of view. PPTA member companies have demonstrated HEV reduction capacity of manufacturing process steps such as virus filtration/nanofiltration, heat (pasteurisation, dry-heat) treatment, and partitioning steps. No HEV transmission by plasma-derived medicinal products (PDMPs) has been reported with virus-inactivated products and a recent study (Modrow et al. Vox Sang 100:351-8, 2011) failed to detect HEV RNA in different coagulation factor concentrates. Toyoda and collaborators have reported a suspected HEV transmission by non-virus inactivated coagulation factor concentrate in Japan. The warning statements in the Guideline on the

warning on transmissible agents in summary of product characteristics (SmPCs) and package leaflets for PDMPs (EMA/CHMP/BWP/360642/2010 rev. 1) make specific reference to viruses that have been transmitted in the past by PDMPs but do not, for instance, make a specific reference to vCJD. PPTA does not consider the addition of a warning statement on HEV as justified as no HEV transmission has been reported for "state-of-the-art" PDMPs. The warning statement should indicate the remaining potential risk of transmitting infective agents by PDMPs, i.e. the general statement in the SmPC and package leaflet, "the possibility of transmitting infective agents cannot be totally excluded. This also applies to unknown or emerging viruses and other pathogens," is considered appropriate and sufficient.

Risk assessment for plasma-derived medical products and implication for warning statements, IPFA Perspective (Françoise Rossi, International Plasma Fractionation association).

F. Rossi pointed out that HEV is not an emerging virus and the virus has been in the donor population for a long time. Infections are most of the time inapparent and there has been no report of transmission associated with the use of plasma-derivatives. An overall risk analysis shows that blood/plasma donations can contain HEV RNA. However viraemia is usually low or moderate and due to exposure to HEV in a significant part of the donor population, plasma pools also contain HEV antibodies which may contribute to the safety of plasma products through neutralisation. There is some indication that neutralization can contribute to HEV reduction in the context of IgG. However, virus particles associated with lipids are non neutralisable. Experimental data, reported so far, indicate significant removal/inactivation of HEV during manufacture of plasma-derived medicinal products and product-specific evaluation was not generally recommended. Only for the few products with lower safety margin, a scientific evaluation could/would be beneficial. When performing theoretical risk assessments, care should be taken not to overestimate the risk and data are available which indicate a minimum infectious dose in the order of 10,000 IU HEV RNA. Specific warning statements for HEV were not recommended for the SmPC as the objective is to inform on "established/proven risk only".

In conclusion, available information and risk assessment for plasma-derived medicinal products according to the current state of knowledge support the safety regarding the HEV transmission risk Implementation of additional regulatory measures (such as pool NAT testing or product-specific validation studies) will not contribute to improving safety for patients.