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Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission

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ABSTRACT

The aim of the present study was to investigate whether the use of porcine blood(products) in food could be a risk for a hepatitis E virus (HEV) infection. HEV RNA was detected in 33/36 batches of (non-heated) liquid products and in 7/24 spray dried powder products. Contamination levels varied among the products, but were highest in liquid whole blood, plasma and fibrinogen reaching levels of 2.2×10^2 to 2.8×10^2 HEV genome copies per 0.2 g. Sequence analyses revealed genotype 3 strains, of which two were 100% (493 nt) identical to recently diagnosed HEV cases, although no direct epidemiological link was established. The industry provided information on processing of blood products in (ready-to-eat)-meat. From this, it was concluded that blood products as an ingredient of processed meat may not be sufficiently heated prior to consumption, and therefore could be a vehicle for transmission.

1. Introduction

The incidence of hepatitis E virus (HEV) infection in the Netherlands has increased recently and is high as compared to other European countries (Adlhoch et al., 2016). In 2013 and 2014, HEV RNA was detected in 1 per 762 blood donations (Hogema et al., 2016) and the HEV strains detected belong to genotype 3 (gt3). HEV gt3 is abundantly present in domestic pigs, which indicates that these animals probably are a major zoonotic HEV reservoir. The main transmission route(s) still need to be determined, but consumption of raw or undercooked porcine meat might be of great importance. Domestic pigs and wild boars show high infection rates in Europe with viral sequences that are closely related to those detected in human hepatitis E patients (Van der Poel et al., 2001, Van der Poel, 2014). Apart from a high seroprevalence of HEV antibodies in pigs, also viremia has been demonstrated in pigs at slaughter age (Grierson et al., 2015; Rutjes et al., 2014). It has long been considered plausible that the persistence of viremia in pigs up to the time of slaughter could provide a potential vehicle for zoonotic transmission to humans in relation to meat products. Food-borne transmission of HEV via consumption of raw and undercooked liver, meat, or sausages from domestic pigs has been documented in several studies and HEV RNA has been detected in porcine liver, pork and pork products by several groups as recently been reviewed (Pavio et al., 2015). The presence of infectious HEV was demonstrated in pork liver sausage and livers (Berto et al., 2013; Feagins et al., 2007).

Blood is a rich source of iron and proteins of high nutritional value and functional quality. The maximal utilization of animal blood, coupled with recent advances in blood collection and processing techniques, have led to a myriad of blood protein ingredients becoming available for use in human foods and dietary supplements (Ofori and Hsieh, 2012). Blood proteins are used as ingredients in meat industry, mainly as a binder of water and fat, but also as natural color enhancers and emulsifiers. Products like fibrinogen are used as a fresh meat cold-set binder to produce restructured fresh meat, whereas spray-dried plasma powder is used as a hot-set binder due to its ability to form gels upon heating to bind water and fat from meat.

Blood taken from a healthy animal is essentially sterile, and both manufacturers and processors have instituted measures concerning bacteria to guarantee the safety of these blood proteins to be used in food industry. Porcine blood has been evaluated for its microbial quality (Ramos-Clamont et al., 2003) and the use of fibrinogen and

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thrombin in food has been evaluated (EFSA, 2005, 2015), however, viral zoonotic threats, like hepatitis E virus (HEV), might have been overlooked.

The aim of the present study was to investigate whether the use of porcine blood (products) in food can be an additional risk. Unlike the situation for HEV gt1, pregnant women are not considered a risk group for HEV gt3 infection with serious consequences. Older men with no international travel history are considered the most at risk for clinically overt HEV gt3 infection. In addition, HEV gt 3 infections are increasingly recognized to cause persistent hepatitis E infections in immunocompromised patients, with an increased risk of progression to cirrhosis (Clemente-Casares et al., 2016; Tedder et al., 2017). For this, blood products were analyzed by quantitative RT-qPCR for the presence of HEV RNA. Subsequently, the obtained data were combined with an inventory of applications and processing characteristics of blood products in meat industry to provide data for risk assessment. In addition, sequences detected in blood products were compared to those recently identified in Dutch patients.

2. Materials and methods

2.1. Virus preparations

A porcine fecal sample was selected as source for virus preparation after testing positive for HEV RNA in RT-qPCR and being typed as genotype 3c (Acc No. MF185108) using methods as described in this paper. The fecal sample was suspended in 10 mM phosphate buffer saline, pH 7.4, to obtain a final of 50% suspension (w/v), then vortexed and centrifuged at 4000*g* for 20 min at 4 °C. The supernatant was added to two volumes of 30% (w/v) polyethylene glycol and 0.9 M NaCl, mixed, and stored at 4 °C for overnight virus precipitation. After centrifugation (4000 g for 20 min at 4 °C), the obtained pellet was resuspended PBS, aliquoted and stored in − 80 °C. The titer of the virus stock was estimated by RT-qPCR using serial-dilutions of the WHO HEV genotype 3a standard (PEI code 6329/10) at the ViroScience group of the ErasmusMC Rotterdam (Dr. S. Pas)(Pas et al., 2012). Based on this method the titer of the HEV virus was estimated at 1.2×10^7 IU/ml. Murine norovirus (MuNoV) was kindly provided by Dr. H. Virgin IV, Washington University, St. Louis, Missouri and estimated by cell culture method at 4×10^7 /ml $TCID_{50}$ by Dr. E. Duizer, RIVM, the Netherlands (Tuladhar et al., 2012).

2.2. Sampling

A selection was made for porcine blood products intended to be used in food, including both liquid and spray dried powder products. Sampling occurred on three separate points in time, October 2015, April 2016 and August 2016. Six batches of whole blood and stabilized hemoglobin and eight batches of hemoglobin, plasma, and fibrinogen were sampled, as well as eight batches of plasma, hemoglobin and stabilized hemoglobin spray dried powder products. The size of the batches varied between 25 kg and 1000 kg. One sample of about 500 g was taken from each batch. Frozen whole blood, frozen fibrinogen and plasma powder of bovine origin were collected and served as control material for the in-house-validation study after they had tested negative for the presence of HEV RNA in the RT-qPCR.

2.3. Viral and RNA extraction

2.3.1. Liquid blood products

For virus extraction from liquid blood products, 0.2 g blood product was mixed with 1.8 ml TGBE buffer (100 mM Tris, 50 mM Glycine, 1% (w/v) beef extract, pH 9.5 buffer) and 10 µl of MuNoV (4×10^4 TCID50), and incubated at room temperature for 20 min. Subsequently, the mixture was clarified by centrifugation (10,000 *g* for 20 min at 4 °C) and the supernatant was transferred into a new tube. For RNA extraction, 2 ml of Nuclisense lysis buffer (BioMérieux) was added and mixed with the supernatant by rotation for 10 min at room temperature. Subsequently, 50 µl of magnetic silica (Nuclisense Magnetic Extraction Reagents kit, BioMérieux) was added to the buffer and the buffer was mixed well by vortexing briefly. After an incubation period of 10 min at room temperature, the total mixture was used as input for the extraction of nucleic acids using reagents from the Nuclisense Magnetic Extraction Reagents kit (BioMérieux) according to the manufacturer's instruction. RNA was tested directly or stored frozen at − 80 °C until testing.

2.3.2. Blood product powders

Virus extraction from hemoglobin containing powders was performed in the same way as described for the liquid blood products, except that 0.2 g of powder was pre-wetted with 300 µl ethanol (100%) prior to mixing with 1.8 ml TGBE buffer. Virus extraction from plasma powder was performed as described above, except that 0.2 g of powder was pre-wetted with 300 µl ethanol (100%) prior to mixing with 1.8 ml Nuclisense lysis buffer (BioMérieux). All solubilized powders were clarified by centrifugation (10,000 g for 20 min at 4 °C) and supernatants were added to 2 ml of Nuclisense lysis buffer (BioMérieux) for RNA extraction. RNA was tested directly or stored frozen at − 80 °C until testing.

2.4. Detection of MuNoV and HEV RNA

All RT-qPCR reactions were performed in a CFX96 real time PCR detection system (BioRad). RNA of MuNoV and HEV was detected by RT-qPCRs using oligonucleotides as described previously (Baert et al., 2008; Jothikumar et al., 2006) after in-house optimization for the CFX96 platform. The RNA Ultrasense one-step qRT-PCR system kit (ThermoFisher) was used with 5 µl of nucleic acid preparation in a total reaction volume of 25 µl.

Each series of virus extractions consisted of a negative extraction control sample in between each set of three samples that was run through all stages of the analytical process. Water controls and positive target RNA template controls were included in each PCR run. Each sample was also tested for inhibition of the reaction in separate reaction well using a HEV reaction mix with the ssRNA HEV-standard used as external control (Diez-Valcarce et al., 2011) and using the PrfAP probe for detection of the control (Diez-Valcarce et al., 2011).

2.5. Calculation for process control extraction efficiency and efficiency of RT-PCR detection

An equal amount of MuNoV as spiked to each sample (4×10^4 TCID50) was also subjected to RNA extraction as a 100% control. Viral extraction efficiency for each sample was calculated using the process control virus RNA standard curve (ISO 15216-1, 2017), setting the minimal recovery to be ≥ 1%. Inhibition of RT-PCR detection was determined by subtracting the Cq value for the external control (EC) ssRNA HEV standard when added to water from the Cq value for the EC-ssRNA HEV standard when added to a RNA sample, allowing the result of the subtraction to be maximally 2.

2.6. Quantification of HEV RNA

To develop the dsDNA standard a synthetic oligo was ordered at Metabion, Germany, stretching from 5261 to 5330 based on GenBank accession no. M73218, having a *Bam*HI restriction site added at the 3′of the probe binding site. This oligo was amplified with JHEVF and JHEVR primers (Jothikumar et al., 2006), subsequently ligated into pGEM-T-easy. After Midiprep, DNA was linearized using *Apa*I, quantified by A260 measurement, diluted in TE and stored in single use aliquots. Each RNA sample was run against serial dilution of dsDNA HEV standard (10^1 to 10^5 genome copies/µl). Results were expressed as genome