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ORIGINAL PAPER

Interlaboratory Validation of a Method for Hepatitis E Virus RNA Detection in Meat and Meat Products

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Abstract

Increasing numbers of hepatitis E cases are currently recognized in many European countries. The zoonotic hepatitis E virus (HEV) genotype 3 mainly circulates in domestic pigs and wild boars, and can be transmitted to humans via consumption of insufficiently heated meat or meat products produced from those animals. Here, a detailed protocol for detection of HEV RNA in meat products is provided, which is based on the method originally described by Szabo et al. (Intl J Food Microbiol 215:149–156, 2015). It consists of a TRI Reagent®/chloroform-based food matrix homogenization, a silica bead-based RNA extraction and a real-time RT-PCR-based RNA detection. The method was further validated in a ring trial with nine independent laboratories using pork liver sausage samples artificially contaminated with different amounts of HEV. The results indicate sufficient sensitivity, specificity, and accuracy of the method for its broad future use in survey studies, routine food control or outbreak investigations.

Keywords Hepatitis E virus · Meat products · Detection method · Validation · Ring trial

Introduction

During the last decade, the hepatitis E virus (HEV)—an *Orthohepevirus A* within the family *Hepeviridae*—has become one of the top priorities in the field of foodborne virus research (European Food Safety Authority 2016). An estimated number of 20 million infections and more than 3 million clinically relevant cases every year have been attributed to HEV (World Health Organization 2017). Currently, an increasing incidence of confirmed hepatitis E cases is documented in many high-income countries of Europe (Aspinall et al. 2017). The genotypes 3 and 4 are zoonotic and widely distributed in porcine hosts (Johne et al. 2014; Pavio et al. 2017). Although domestic pigs and wild boars are highly susceptible to HEV infection (Meng et al. 1997), these animals do not develop any signs of acute illness (van der Poel et al. 2001), which hinders the exclusion of meat or organs from infected animals for food production during meat inspection. In humans, HEV can cause an acute

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but self-limiting inflammation of the liver. In some patients, the disease can progress to acute liver failure or fatality, e.g., in pregnant women infected with HEV genotype 1 or in patients with underlying chronic liver disease (Peron et al. 2011; Purcell and Emerson 2008). HEV genotype 3 was also suggested to cause a wide range of neurological illness including Guillain–Barré syndrome (van den Berg et al. 2014). In addition, chronic HEV genotype 3 infections, which may progress to liver cirrhosis, were repeatedly described in immunosuppressed transplant patients (Gerolami et al. 2008; Kamar et al. 2008; Schlosser et al. 2012).

Consumption of faecally contaminated drinking water represents a common way of human HEV infections in many low-income countries. This may result in large outbreaks, especially after disastrous flooding or in refugee camps (Wedemeyer et al. 2012; CDC 2013). In contrast, in industrialized countries mainly sporadic hepatitis E cases are recognized. Thereby, infections may be acquired by traveling to low-income countries, or by transfusion or transplantation (Vollmer et al. 2012; Bajpai and Gupta 2011). But more importantly, consumption of raw or inadequately cooked meat or meat products from pigs or wild boars can result in HEV infection of humans (European Food Safety Authority 2017). This transmission route was confirmed by case/control studies and single case molecular epidemiological reports (Meng 2011). The presence of the viral genome—a single-stranded RNA—has been confirmed for pig liver or other pork products at retail in several countries including the US (Feagins et al. 2007), the UK (Berto et al. 2012), and Germany (Wenzel et al. 2011; Szabo et al. 2015). In fact, viral RNA isolated from hepatitis E patients and from commercially purchased pork liver showed very high sequence identities to each other (Wenzel et al. 2011; Yazaki et al. 2003).

The constantly growing incidence of HEV infections in many European countries not only raises public health concerns but also represents a challenge for interdisciplinary research as well as for food production industry. At a scientific workshop on foodborne viruses jointly held by the Food Standards Agency (FSA) and the European Food Safety Authority (EFSA) in February 2016, development of standard and ISO methods for detection of HEV in meat and meat products ranked as one of the top five priorities for further research (European Food Safety Authority 2016). So far, only a few methods for HEV detection in pork products have been published (Berto et al. 2013; Bouwknegt et al. 2007; Colson et al. 2010; Di Bartolo et al. 2012; Martin-Latil et al. 2014). Depending on the applied protocols and the food matrix used, the reported HEV detection rates differed remarkably. Szabo et al. (2015) compared several techniques for sample homogenization and virus extraction followed by HEV-specific real-time RT-PCR using artificially contaminated raw sausage samples. A TRI Reagent®/chloroform-based method was selected and optimized, resulting in an efficient and sensitive technique for HEV-RNA detection in meat products. The method was also shown to be suitable for HEV detection in raw sausages and liver sausages from German retail (Szabo et al. 2015).

This study describes a detailed protocol for an HEV-RNA detection method according to that developed by Szabo et al. (2015) and its validation through a collaborative trial organized by the working group "Viruses in Food" according to § 64 of the German food and feed code of law (legislative text available at http://www.gesetze-im-internet.de/lfgb/). For the trial, pork liver sausage samples artificially contaminated with different levels of HEV were distributed to 9 independent laboratories which analyzed the specimens using the same protocol. The results indicate a sufficient sensitivity, specificity, and accuracy of the method.

Materials and Methods

Method for HEV Detection in Meat and Meat Products

The three-step procedure, as described by Szabo et al. (2015), includes (1) food sample homogenization followed by (2) RNA extraction/purification and (3) specific detection of HEV genomes. It has been demonstrated to have a limit of detection (LOD) of 7.03×10^3 genome equivalents (GE)/g or 3.2×10^4 GE/g for raw sausages (salami) or liver sausages (spreadable sausages containing pig liver, see details below in ring trial description), respectively (Szabo et al. 2015), and includes a set of controls to verify high quality performance. The following three sections describe the procedure steps in more detail as they have been performed by the laboratories during the validation ring trail.

Food Sample Homogenization

Depending on the food matrix and its fat content, 2–5 g of meat or meat product is minced and transferred to a stomacher filter bag (Interscience, St Nom la Bretèche, France). During the ring trial, 2 g portions of a liver sausage were used (see below). After spiking the test sample with 10 µl of the process control virus MS2 (DSM 13,767 or ATCC 15,597-B1; 5×10^7 PFU/ml), a short incubation of 3–5 min at room temperature is performed and 7 ml of TRI Reagent® (ThermoFisher Scientific, Dreieich, Germany) is added. Homogenization is achieved by using a stomacher (Interscience, St. Nom la Bretêche, France) for 2 min at room temperature and the highest velocity. Afterwards, the fluid, which passed the stomacher bag's filter, is carefully transferred to a clean 50 ml conical tube. The following centrifugation (10,000×*g* for 20 min; alternatively 5000×*g*

for 40 min, both at 4 °C) clears the sample a second time from remaining solid pieces and fat. A recovery rate control (consisting of nuclease-free water spiked with a known concentration of MS2 phage; see below) and a negative process control (comprising TRI Reagent® only, without MS2 phage) were prepared and treated identically as subsequently described.

RNA Extraction/Purification

While the food matrix pellet and remaining fat is discarded, the supernatant (around 6–7 ml fluid) is transferred to a new 50 ml conical tube and 0.2 ml chloroform per 1 ml sample solution is added. The mixture is thoroughly vortexed for 15 s and incubated for 2–15 min at room temperature. The following centrifugation (10,000×*g* for 15 min; alternatively 5000×*g* for 30 min, both at 4 °C) separates two different phases. The upper RNA-containing aqueous phase (approximately 3.5–4 ml) which usually appears colorless or slightly yellow is transferred into a clean 50 ml conical tube without removing the thin and barely visible white layer in between the two phases. The lower, red phenol phase is discarded. Viral RNA is extracted and purified from 1 ml aqueous solution using the NucliSens Kit and the fully automated NucliSENS®easyMag system (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions which utilizes an enhanced magnetic silica-based extraction technology. Depending on available laboratory equipment, silica-based RNA extraction can also be performed semi-automated (e.g., NucliSENS®MiniMag, BioMérieux) or manually using a magnetic separator. Two additional samples containing either HEV or MS2 phage, respectively, are also included in this procedure to create positive controls for the specific real-time RT-PCRs. RNA of each sample or control is eluted in 60 µl elution buffer, immediately transferred to a clean 1.5 ml tube and chilled on ice.

Detection of Viral RNA

Detection of HEV genomes is done by performing a one-step real-time RT-PCR according to Jothikumar et al. (2006) using HEV-specific primers in combination with a TaqMan probe, all listed in Table 1. The QuantiTect^{TM}Probe RT-PCR Kit (QIAGEN, Hilden, Germany) is used. 20 µl reactions are prepared for each sample/control according to the manufacturer's instructions and 5 µl extracted RNA solution is added as template. The cycling conditions are listed in Table 2. Collection of fluorescence data occurs at the end of the 72 °C elongation step of each cycle. Detection of process control virus RNA (bacteriophage MS2) is carried out in separate reactions using different primers and probes for all samples and controls (Table 1). However, cycling conditions are the same as for the HEV-specific real-time RT-PCR (Table 2), allowing side by side runs.

Test Controls

To verify successful and efficient method performance, different controls were carried along each run. The use of bacteriophage MS2 as an appropriate internal control for reverse transcription-PCR assays was demonstrated by Dreier et al. (2005). In addition, this virus has been successfully used as complete process control for norovirus detection (Scherer et al. 2010). In this case, detection of its single-stranded RNA indicates appropriate sample treatment during matrix homogenization, RNA extraction and PCR-based RNA

Table 2 Cycling conditions applied in real-time RT-PCRs to detect HEV genomes and MS2 phage RNA

Reverse transcription	50 °C	30 min
Initial denaturation	95 °C	15 min
50 cycles of:		
Denaturation	94 °C	10 s
Annealing (primer and probe)	55 °C	20 s
Elongation (fluorescence data collection)	72 °C	1 min

Table 1 Oligonucleotides used for specific HEV- or bacteriophage MS2 genome detection by real-time RT-PCRs

Virus	Primer/probe		Sequence (5′–3′)	Final concen-tration	References
HEV	JVHEV-F	Sense	GGT GGT TTC TGG GGT GAC	500 nmol/l	Jothikumar et al. (2006)
	JVHEV-R	Anti-sense	AGG GGT TGG TTG GAT GAA	500 nmol/l	
	JVHEVP	Probe	[FAM][a]-TGATTCTCAGCCCTTCGC-[BHQ1][a]	100 nmol/l	
Phage MS2	MS2-TM2-F	Sense	TGC TCG CGG ATA CCC G	250 nmol/l	Dreier et al. (2005)
	MS2-TM2-R	Anti-sense	AAC TTG CGT TCT CGA GCG AT	250 nmol/l	
	MS2-TM2FAM	Probe	[FAM][a]-ACCTCGGGTTTCCGTCTTGCTCGT -[BHQ1][a]	125 nmol/l	

[a][FAM = 6-Carboxyfluorescein $\lambda = 520$ nm, BHQ1 = Black Hole Quencher1]

detection, thereby controlling extraction efficiency as well as the possible presence of PCR inhibitors.

Negative Process Control

To exclude laboratory contamination throughout sample treatment, one sample containing TRI Reagent® only is carried along the entire procedure—from matrix homogenization to real-time RT-PCR.

Recovery Rate Control

A food matrix-independent specimen consisting of nuclease-free water only is spiked with 10 µl bacteriophage MS2 particles (5×10^7 PFU/ml) and is treated as any regular food sample. By comparing the phage MS2-specific RT-PCR C_q value of the recovery rate control with that of each regular sample, the MS2 recovery rate (in %) is calculated by the formula $2^{-\Delta C_q} \times 100$. Thereby, ΔC_q is defined as the difference between the MS2-specific C_q value of a regular sample and that of the recovery rate control (Schmittgen and Livak 2008).

RT-PCR Controls

An HEV RNA-positive as well as a negative (nuclease-free water only) control are carried along with the negative process and the recovery rate control as well as all regular test samples. In the ring trial, RNA of a genotype 3c-positive liver homogenate derived from a wild boar (Schielke et al. 2009) was used as positive control; however, the freely available HEV RNA WHO standard (IU/ml; PEI No. 6329/10) may also be used for this purpose.

Ring Trial for Method Validation

Food Matrix and Artificial Contamination

German liver sausages purchased at German retail were used as food matrix for the ring trial, representing a spreadable, cooked sausage mainly consisting of pork meat (46%), pork liver (28%), fat, salt, and spices. The spreadable sausages were mixed and thereafter aliquoted into 2 g portions in 50 ml conical tubes. Prior to artificial contamination, the absence of HEV genomes was confirmed by testing 5 aliquots of the mixed sausages using the method described above. The sausage aliquots were thereafter spiked with an HEV genotype 3c-positive liver homogenate derived from a wild boar (Schielke et al. 2009). Quantification by real-time RT-PCR using the HEV RNA WHO standard (IU/ml; PEI No. 6329/10) resulted in a concentration of 3.2×10^6 IU/g for the liver homogenate. Two HEV contamination levels were prepared by inoculation of sausage aliquots with 100 µl of either undiluted (contamination level D_1: 1.62×10^5 IU/g) or 1:5 diluted (contamination level D_2: 3.23×10^4 IU/g) liver homogenate (Table 3). Additional sausage aliquots without HEV inoculation served as negative food controls (contamination level D_0).

Collaborative Trial Procedure

In total, 9 independent laboratories from Germany and Switzerland participated in the collaborative trial. Each laboratory received a set of 12 blind samples (coded by numbers) consisting of 4 HEV-negative samples (contamination level D_0), 4 specimens artificially contaminated above the LOD (contamination level D_1) and 4 specimens artificially contaminated at the LOD (contamination level D_2) (Table 3). The following main reagents used in the detection procedure were also provided to ensure consistency: TRI Reagent®, stomacher bag's, NucliSENS® lysis buffer and extraction reagents including magnetic silica, QuantiTect™Probe RT-PCR Kit, primers and probes. The packages were sent cooled at approximately 4 °C using cooling packages with an overnight courier service. If an immediate processing after arrival was not possible, participants were allowed to store the samples for up to 1 day at 4 °C until analysis. All laboratories had to follow the detection protocol as described above. As mentioned there, variability regarding the centrifugation conditions, the distinct system for magnetic bead extraction and the type of real-time PCR machine was allowed. Each sample was analyzed in a single reaction. A typical plate layout for real-time RT-PCR is presented in

Table 3 Composition of liver sausage sample sets distributed within the ring trial for validation of the method for detection of HEV genomes in meat products

	Contamination dose [D]	Number of samples distributed per lab	Total number of samples distributed
[D_0]	not contaminated	4	36
[D_1]	1.62×10^5 IU/g (above LOD)	4	36
[D_2]	3.23×10^4 IU/g (at LOD)	4	36
		Σ: 12	Σ: 108

IU international units, *LOD* limit of detection

Supplementary Figure S1. Participating laboratories had to report all C_q values of the HEV-specific PCR and those of the MS2-specific PCR as well as the resulting calculated recovery rates. Samples with MS2 recovery rates of $\geq 0.01\%$ were considered as valid. Samples showing typical exponential amplification curves and C_q values < 45 in the HEV-specific PCR were considered positive.

Statistical Data Analysis

In order to assess the method performance, all ring trial results of each laboratory were included into data analysis. The relative sensitivity [in %] was calculated as follows: (number of samples assessed as HEV-positive $[D_1 + D_2]$/ total number of all HEV-contaminated samples $[D_1 + D_2]$) $\times$ 100. The relative specificity [in %] was calculated as follows: (number of samples assessed as HEV-negative/total number of all not contaminated samples $[D_0]$) $\times$ 100. The relative accuracy [in %] was calculated as follows: (number of samples assessed correctly $[D_0 - D_2]$/total number of samples $[D_0 - D_2]$) $\times$ 100.

Results

Pre-testing of Artificially Contaminated Liver Sausage Samples

For the use in the method validation collaborative trial, liver sausage samples were artificially contaminated with two dilutions of an HEV-containing wild boar liver homogenate. Three randomly selected samples of each contamination level were tested prior to the ring trial using the described detection protocol. As evident from Fig. 1, all specimens contaminated above the LOD (contamination level D_1: 1.62×10^5 IU/g) were tested positive with C_q values between 32 and 35. From the three specimens contaminated at the LOD (contamination level D_2: 3.23×10^4 IU/g), two samples were tested positive with C_q values between 35 and 36, and one sample was negative.

Collaborative Trial

A total of nine laboratories took part in the method validation ring trial. For RNA extraction, six laboratories used the fully automated NucliSENS®easyMag system, two laboratories used the semi-automated NucliSENS®MiniMag and one laboratory applied the manual method utilizing a magnetic separator. For real-time RT-PCR, seven different PCR machines were used as listed in Supplementary Table S2. All laboratories provided valid results, which were fully incorporated into the data analysis. The reported C_q values for the individual samples returned by each laboratory are listed in Supplementary Figure S3.

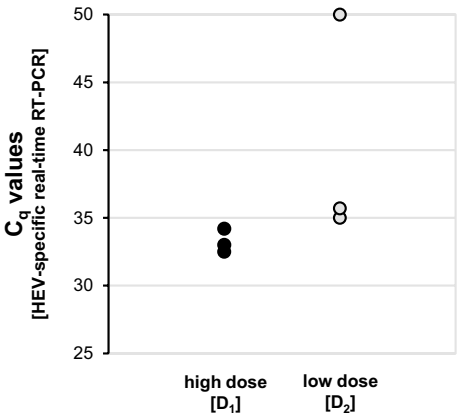

Fig. 1 Pre-testing of artificially contaminated liver sausage samples for use in the interlaboratory ring trial. Liver sausages verified as HEV RNA-negative were spiked with either a high $[D_1]$ or a low $[D_2]$ amount of an HEV-containing liver homogenate obtained from an infected wild boar. For each contamination level, three randomly selected samples were analyzed using the method also applied in the ring trial. The C_q values obtained by the HEV-specific real-time RT-PCR are shown

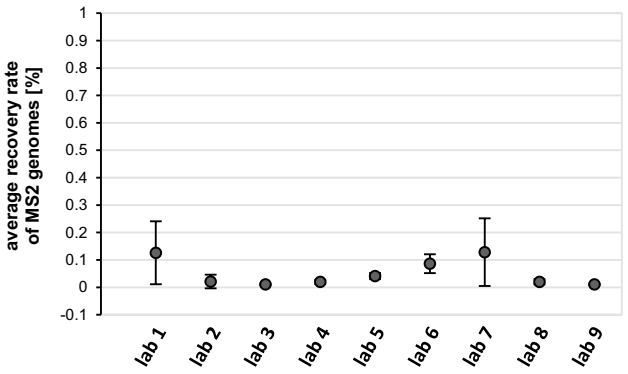

Fig. 2 Average recovery rates of the process control (bacteriophage MS2) for all ring trial liver sausage samples as reported by the participating laboratories. Each sample was spiked by the laboratories with bacteriophage MS2 particles prior to analysis and the resulting C_q values were used for calculation of recovery rates. The MS2 recovery rates (in %) $\pm$ standard deviation calculated for each participating laboratory ($n = 9$) are shown

The reported MS2 recovery rates for the samples ranged between 0.01% and 0.16%. Figure 2 indicates that the range of recovery rates mainly differed depending on the analyzing laboratory. By applying the defined threshold of a recovery rate $\geq 0.01\%$, all of the samples were considered as valid.

Figure 3 summarizes the sample results (as C_q values) provided by the participating laboratories. One out of 36 non-contaminated liver sausage samples (contamination level D_0) was returned as false-positive; even though the

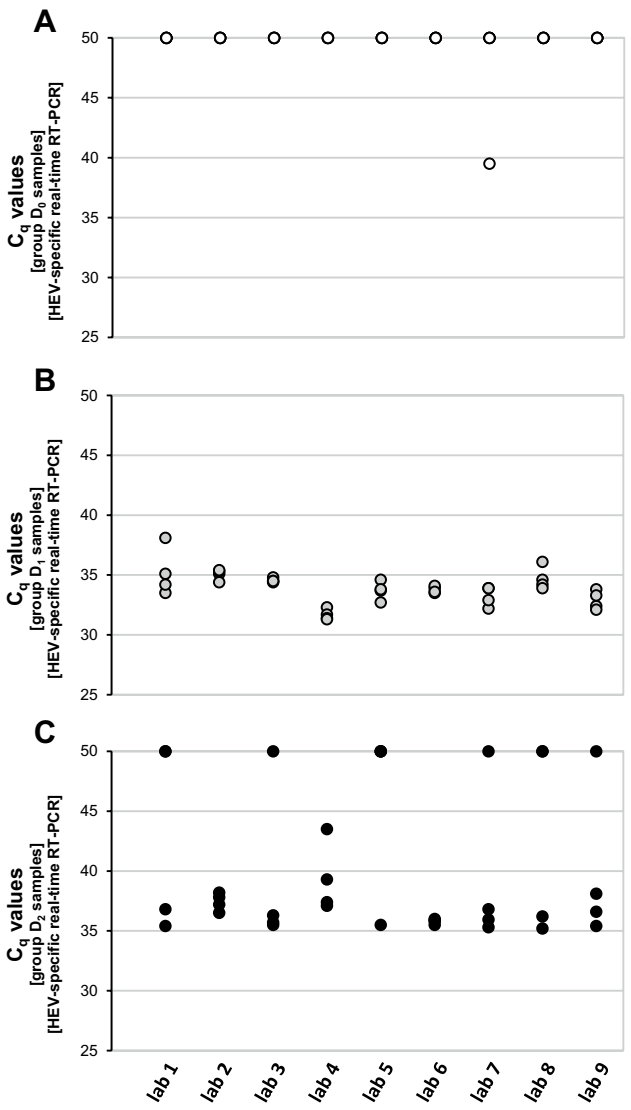

Fig. 3 Results of the HEV-specific real-time RT-PCR for the ring trial liver sausage samples as reported by the participating laboratories. The results are grouped into non-contaminated [D_0] samples (**A**) and samples artificially contaminated with either a high amount [D_1] of HEV (**B**) or a low amount [D_2] of HEV (**C**). The C_q values obtained by the HEV-specific real-time RT-PCR are shown

corresponding C_q value of 39.5 was rather high. All specimens spiked with a dose above LOD (contamination level D_1) were reported as positive with C_q values between 31 and 38. For samples spiked with a HEV dose right at LOD (contamination level D_2), a total of 10 out of 36 samples was reported false-negative. The remaining samples of this contamination level showed C_q values between 35 and 43.

Determination of Method Performance Parameters

The data were used to determine method performance parameters. This resulted in a relative specificity of 97.2% for the method. The relative sensitivity and accuracy were calculated as 86% and 90%, respectively.

Discussion

Detection methods for HEV in meat products are needed for screening of food during food control programs or in outbreak situations. For comparability of results and consistency of testing quality, a standardized and/or validated method is desirable, but not available so far. In this study, an interlaboratory ring trial for validation of such a method was performed. The method originally described by Szabo et al. (2015) was chosen for several reasons. First, this method contains an efficient lysis step, which guarantees the release of virus nucleic acids from infected cells present in the food as shown by detection of released pig DNA from raw sausages (Szabo et al. 2015). Second, the method was shown to have the best HEV recovery rate when compared to three other published methods (Szabo et al. 2015). Third, the method showed a relatively high HEV RNA detection rate in sausages derived from retail (Szabo et al. 2015). A general disadvantage of the method is its inability to differentiate between infectious and non-infectious HEV due to exclusive detection of RNA. However, there is currently no method available for routine testing of HEV infectivity in food (Cook et al. 2017).

For the validation ring trail, artificially contaminated liver sausages were chosen as sample matrix. Consumption of liver sausages has been described as the cause of several hepatitis E cases in the past (Colson et al. 2010; Renou et al. 2014; Kubacki et al. 2017) and HEV RNA has been repeatedly detected in sausages containing pig liver (Moor et al. 2018; Pavio et al. 2017). However, liver sausages represent a difficult food matrix in terms of ingredients and the resulting consequences for the detection of viral RNA. High contents of fat and protein lead to a relatively high detection limit of 3.2×10^4 HEV GE/g (Szabo et al. 2015). A recently published modification of the method was reported to result in a lower detection limit of 1.56×10^3 HEV GE/g in liver sausages from Switzerland; however, the data cannot be directly compared as the composition and consistency of the used liver sausages were different (Moor et al. 2018). Accordingly, a detection limit of 7.03×10^3 HEV GE/g was determined for raw sausages tested with our method (Szabo et al. 2015). Therefore, the matrix chosen for validation is relevant for two reasons. First, it represents a well-known HEV transmission vehicle and second, the method is expected to be applicable to other meat products, in some cases also with higher sensitivity.

The bacteriophage MS2 was used as a process control virus as it represents a genetically unrelated RNA virus with a similar capsid structure and it was already used as PCR

and extraction control for other foodborne viruses (Dreier et al. 2005; Scherer et al. 2010). However, the results from the ring trial indicate very low recovery rates for this virus using the applied method. By setting the threshold for consideration as a valid sample to ≥ 0.01% MS2 recovery rate, all samples could be further analyzed. However, this very low recovery rate indicates a different behavior between MS2 and HEV during the laboratory procedure. Hence, in the future, the use of other viruses might be considered as a suitable process control. In addition, slight modifications of the described method have recently shown to improve the recovery rates of both MS2 and HEV (Moor et al. 2018).

All of the nine participating laboratories provided results which could be used for the validation study. This indicates that the method is applicable for routine use in diagnostic laboratories. Also, the different machines used for RNA extraction and real-time RT-PCR did not seem to have an effect on the detection rate, although no systematic study was done here. Only one laboratory provided a false-positive result for a non-contaminated sample, which might be explained by a laboratory contamination. All of the samples contaminated above the LOD were identified as positive by the laboratories. In case of the samples contaminated with a dose at the LOD, about one-third of the laboratories documented a false-negative result, which is comparable with the detection rate previously determined for these samples by the laboratory providing the materials. Therefore, the determined values of 97.2% for relative specificity, 86% for relative sensitivity, and 90% for accuracy represent acceptable values for this validation study.

In conclusion, the detection method could be successfully validated in a ring trial, in which 9 different laboratories participated successfully. The method may therefore be used for testing of meat products for the presence of HEV RNA in survey studies, routine food control or outbreak investigations. A further standardization of the method may be attempted in the future.

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