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Research Paper

Examining the Effect of Organic Acids on Inactivation of Hepatitis E Virus

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ABSTRACT

Infection with hepatitis E virus genotype 3 (HEV-3) is an emerging cause of illness in developed countries. In North America and Europe, HEV-3 has been increasingly detected in swine, and exposure to pigs and pork products is considered the primary source of infection. We have previously demonstrated the prevalence of the HEV-3 genome in commercial pork products in Canada. In this study, we investigated the application of citric acid and acetic acid to inactivate HEV-3 on food and on food contact surfaces. For this purpose, plastic, stainless steel, and pork pâté surfaces were inoculated with HEV-3 and were treated with acetic acid or citric acid at 1, 3, or 5%. The infectivity of posttreatment viral particles was determined by cell culture. A greater than 2-log reduction in viral infectivity was observed on plastic and stainless steel treated with the organic acids, but the treatment was less effective on HEV infectivity on pork pâté (average reductions of 0.47 log citric acid and 0.63 log acetic acid). Therefore, we conclude that citric acid and acetic acid have potential application to control HEV-3 on food contact surfaces but are not suitable for food.

HIGHLIGHTS

- Citric acid reduces HEV-3c infectivity by a maximum of 2.5 log on stainless steel and plastic.
- Acetic acid reduces HEV-3c infectivity by a maximum of 2.4 log on stainless steel and plastic.
- Citric acid and acetic acid reduce HEV-3c infectivity by a maximum of 0.7 log on pork pâté.

Key words: Acetic acid; Citric acid; Hepatitis E virus; Inactivation; Infectivity assay

Hepatitis E virus (HEV) is a single-stranded, positive-sense RNA virus that belongs to the *Orthohepevirus* genus, within the *Hepeviridae* family *(30)*. The genus is divided into species *Orthohepevirus A* to *Orthohepevirus D*. To date, eight genotypes within the *Orthohepevirus A* species have been identified that infect a range of mammals, including humans, pigs, wild boars, rabbits, deer, and camels *(30)*. Hepatitis E virus genotype 3 (HEV-3) has emerged recently in many developed countries as a zoonotic pathogen, with infection associated with the consumption of raw or undercooked pork products *(26, 31)*.

Infection with HEV-3 causes subclinical or acute self-limiting hepatitis *(3, 21)*. However, chronic hepatitis is increasingly reported in immunocompromised patients, including organ transplant recipients, patients with hematological malignancy, and those with human immunodeficiency virus infection *(15, 33)*. Besides manifesting as typical hepatitis, HEV infection can cause extrahepatic manifestations, such as neurological manifestations, kidney injury, and hematological disorders *(7, 16, 19)*.

There is ample evidence that HEV-3 is endemic in domestic swine and wild boar populations in Europe and the Americas *(10, 31, 35)*. Therefore, exposure to wild or domestic swine and consumption of raw or undercooked pork or game meat are considered risk factors for HEV infection *(11)*. The prevalence of contaminated pork products varies from less than 1% to more than 50%, depending on the region, recovery methods, and tested commodity *(27)*. In our previous study, we reported that 47% of the Canadian pork pâté tested were positive for HEV RNA *(22)*. Because of this high prevalence, inactivation of HEV in ready-to-eat pork products should be considered to prevent foodborne HEV infection.

HEV is resistant to drying and is stable for hours to days on steel and plastic at room temperature *(37)*. Previously, we investigated the application of high pressure processing (HPP) for the inactivation of HEV-3 in artificially contaminated pork pâté *(23)*. HPP treatment resulted in minimal reduction in HEV infectivity (0.5-log reduction). Consequently, we sought other strategies for the

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control of HEV in the production of ready-to-eat pork products without a major effect on their sensory properties. Organic acids, such as acetic acid and citric acid, can be used under specific conditions as food additives or as processing aids in Canada *(12),* the United States *(32),* and the European Union *(9)*. Because of their perceived low toxicity and acceptance by consumers, organic acids are widely used in the food industry as antimicrobials, especially in meat and meat products. Organic acids have also been approved for use in meat decontamination in the United States (U.S. Department of Agriculture, Food Safety and Inspection Service, 1996), and it has been shown that treatment with an organic acid significantly reduces microbial populations in meat products *(17)*. However, there is no information on whether treatment with organic acids would reduce HEV infectivity on production surfaces or contaminated pork products.

The purpose of this study was to examine the effectiveness of organic acids, particularly citric acid and acetic acid, in the inactivation of HEV on food contact surfaces and pork products as a risk mitigation strategy. The potential virucidal mechanism of action of organic acids probably dependent on their ability to permeate through the viral envelope or capsid and damage the viral genome. The effect of citric acid and acetic acid on inactivation of surrogates of human norovirus has been examined previously *(20, 25)*. These viruses belong to the *Caliciviridae* family and are nonenveloped. Nonenveloped viruses are generally less sensitive to antimicrobial agents compared with their enveloped counterparts. However, HEV-3 has been shown to be quasienveloped *(8)*; thus, its resistance to many antimicrobial agents, including acetic acid and citric acid, might be different from that of nonenveloped foodborne viruses.

Citric acid is a weak organic acid in many kinds of citrus fruits and is produced by fermentation in large quantities as a food acidifier or chelating agent. Acetic acid is used as a preservative to reduce microbial contamination of certain food commodities, including pork products *(9, 12)*. In this study, we applied citric acid and acetic acid, at different concentrations allowed in the food industry, to artificially inoculated food contact surfaces (stainless steel and plastic), as well as to a high-risk food commodity for HEV contamination, pork pâté. The potential virucidal effect of the acids was assessed by performing infectivity assays on the extracted viruses.

MATERIALS AND METHODS

Cells and viruses. A549/D3 human lung carcinoma cells were kindly provided by Dr. R. Johne (German Federal Institute for Risk Assessment, Berlin) as two cell lines, with and without persistent infection with HEV-3c strain 47832c. Both A549/D3 cell lines were cultured in growth medium composed of minimum essential media (Gibco, Waltham, MA), supplemented with 1% nonessential amino acids, 1% glutamine, 0.5% gentamicin, and 10% fetal bovine serum (FBS; Gibco).

For the infectivity assay, a cell density of 1.0×10^5 A549/D3 cells was seeded in 500 μL of growth medium per well of a 24-well plate. The plate was incubated for 5 days at 37°C and 5% CO_2 until a confluent cell monolayer formed.

HEV-3 47832c stocks were prepared for experiments by freeze-thawing confluent persistently infected A549/D3 cells for three freeze-thaw cycles *(37)*. HEV supernatants were pooled together and centrifuged at $2,000 \times g$ for 10 min at 4°C to remove cell debris. The remaining supernatants were concentrated by ultracentrifugation at 157, $500 \times g$ for 2 h at 4°C using a Beckman LE-80 ultracentrifuge (Beckman Coulter Life Sciences, Brea, CA). The supernatant was removed and the pellet was resuspended in sterile RNase/DNase free water and incubated at 4°C overnight. The pellet was mixed by pipetting and then centrifuged at $2,000 \times g$ for 5 min at 4°C. The supernatant was collected and the viral stock solution was stored at –80°C until required for experiments. The concentration of viral genomes was determined by digital droplet reverse transcriptase PCR (ddRT-PCR) as described below.

HEV inoculation and treatment with organic acids: surfaces. High-density polyethylene (HDPE) and stainless steel (grade 304) coupons were used for the surface experiments. Both surfaces were prepared by disinfecting with 70% ethanol and were allowed to air dry in a biosafety cabinet. An area (5 by 5 cm) was then demarcated on each surface using tape.

Surfaces were prepared in triplicate for each acid treatment, in addition to triplicate untreated control (inoculated but untreated) surfaces and one negative control (uninoculated and untreated). All surfaces were inoculated with 100 μL of HEV-3 virus containing approximately 1×10^7 RNA copies spread over the demarcated area (5 by 5 cm) and dried for 30 min in a biosafety cabinet. The treated triplicate surfaces were then treated with 100 μL of the respective organic acid, using the end of a pipette tip to minimize contact and the potential for removal of the virus dried on the surface during application of the treatments. The surfaces were then dried for 10 min in a biosafety cabinet at ambient conditions (22°C; relative humidity, 30 to 40%). The following treatments were tested on both HDPE and stainless steel: 1, 3, and 5% citric acid and 1, 3, and 5% acetic acid.

HEV inoculation and treatment with organic acids: pork pâté. Commercial pork pâté was obtained from a local grocery store (containing 8% sodium and 27% fat). Triplicate samples were prepared with 2 g of pork pâté per sample. Two additional 2-g samples were weighed for a positive control (inoculated but untreated) and an uninoculated, untreated negative control (altogether five samples per treatment). The triplicate samples and the positive control were inoculated with 250 μL of HEV-3 virus containing approximately 1×10^7 RNA copies spread over the entire surface area and dried for 10 min in a biosafety cabinet. The triplicate samples were treated with 100 μL of the organic acid applied over the entire surface area and dried for 10 min in a biosafety cabinet.

HEV extraction: surfaces. The International Organization for Standardization (ISO) 15216-1:2017 method for surfaces *(13)* was used for viral extraction following treatment. All surfaces were swabbed five times using sterile cotton swabs moistened in 500 μL of dilution media (identical to growth media but with 0% FBS). The samples were then used in the HEV infectivity assay as described below.

HEV extraction: pork pâté. The modified ISO 15216-1:2017 method for viral extraction of bivalve molluscan shellfish *(13)* was employed as described previously *(23)*. Following treatments, each pâté sample was placed in a stomacher bag with 16 mL of Tris glycine beef extract and incubated on a rocking

TABLE 1. *pH of the organic acids used in this study*[a]

Sample[b]	pH
CA 1%	2.31
CA 3%	2.13
CA 5%	2.06
AA 1%	2.44
AA 3%	2.41
AA 5%	2.38

[a] The results are demonstrated as the average of three measurements.
[b] CA, citric acid; AA, acetic acid.

plate for 20 min at room temperature. The resulting suspension was centrifuged at 10,000 × *g* for 30 min at 4°C. The pH of the supernatant was adjusted to 7.0 ± 0.5 using 12 N HCl, and 5× polyethylene glycol 8000 (PEG)/NaCl to the volume of 25% of the weight of the sample was added to each tube. Samples in tubes were inverted to mix and incubated on ice on a rocking plate for 1 h. The tubes were centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was discarded. The pellet containing the virus particles was resuspended with 500 µL of phosphate-buffered saline (PBS) and stored at −80°C until required for the infectivity assay.

Infectivity assay. The HEV infectivity assay developed by Johne et al. *(14, 28),* with slight modifications, was used to quantify the infectious HEV after treatment with organic acids. The growth medium was removed, and each well was washed twice with 200 µL of PBS. Cells were infected using 100 µL of each of the viral samples, in duplicate, for 1 h at room temperature. In addition, cells were infected with 100 µL of the 1×10^7 RNA copies of virus stock and 100 µL of dilution media, in duplicate, as the positive and negative controls, respectively. After 1 h, the virus samples and controls were removed and the cells were washed twice with 200 µL of PBS. Next, 500 µL of growth medium containing 5% FBS was added to each well, and cells were incubated at 34.5°C and 5% CO_2 for 7 days. Growth medium was replaced with 500 µL of fresh growth medium with 5% FBS, and the cells were incubated for an additional 7 days in the same conditions, for a total of 14 days. Growth medium was collected and stored at −80°C. The cells remaining in the plate were lysed for RNA extraction. The RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) protocol "Purifying Total RNA from Animal Cells with Spin Technology" was used to isolate RNA from the A549/D3 cells. The recovered RNA was quantified using Bio-Rad ddRT-PCR technology (Bio-Rad Laboratories, Mississauga, Ontario, Canada) as previously described *(22, 23)* for determination of intracellular HEV RNA. The One-Step ddRT-PCR Kit for Probes (Bio-Rad) was employed according to manufacturer's instructions. Primers and probes used to quantify HEV ORF2 were (5′–3′) JV (forward)—GGTGGTTTCTGGGGTGAC and JV (reverse)—AGGGGTTGGTTGGATGAA. The probe sequence was (FAM)-TGATTCTCAGCCCTTCGC-(BHQ-1). The thermocycling conditions were 60°C for 30 min, 95°C for 5 min, 40 cycles of 94°C for 30 s (Ramp = 2°C/sec), 55°C for 1 min (Ramp = 2°C/sec), 65°C for 30 s (Ramp = 2°C/sec), and 98°C for 10 min. ddPCR results were analyzed using the QX200 Droplet Digital system (Bio-Rad Laboratories Ltd.). Approximately 20,000 droplets are generated per sample, and data from at least 12,000 droplets are used in concentration calculations. QuantaSoft software applies Poisson statistics in order to quantify the concentration of RNA and gives a result in copies/µL of the final ddPCR reaction.

TABLE 2. *Recovery efficiency of the tested matrices*

Matrix	Recovery rate (%)[a]
Plastic	9.97 ± 1.68
Stainless steel	8.85 ± 2.04
Pork pâté	3.7 ± 0.62

[a] The recovery rate was calculated by comparison of the absolute genome copy number of the virus recovered to the absolute genome copy number of the virus in the inoculum. The results are the average of three independent experiments obtained from untreated matrices.

Calculation of recovery rate. The recovery rates of viral extraction were calculated for the untreated samples by comparison of the absolute genome copy number of the virus recovered to the absolute genome copy number of the virus in the inoculum:

$$\text{Recovery rate } (\%) = (\text{Obtained genome copies}/\text{Inoculated genome copies}) \times 100$$

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA). Multiple unpaired *t* test was used to determine whether there was a significant difference ($P < 0.05$) between treatments.

RESULTS

Virucidal effect of citric acid and acetic acid on HEV-3 on surfaces. To examine whether citric acid or acetic acid could be used as a risk mitigation strategy against HEV, common food contact surfaces such as stainless steel and HDPE were artificially inoculated with HEV-3c and were then subjected to treatment with 1, 3, and 5% citric acid or acetic acid at ambient temperature (5% is the highest concentration permitted in the meat industry). We measured the pH of 1 to 5% acetic acid and citric acid in triplicate, and the result is shown in Table 1. As demonstrated, the pH range of 1 to 5% citric acid (pH 2.31 to 2.06) is slightly lower than the pH range of 1 to 5% acetic acid (pH 2.44 to 2.38).

Following treatment with organic acids, the virus was extracted using the ISO 15216 method for surfaces and was introduced to A549/D3 cells for the infectivity assay. The average recovery efficiency for each surface is demonstrated in Table 2 and is 9.97% ± 1.68% for plastic and 8.85% ± 2.04% for stainless steel. Previously, we demonstrated that the amount of harvested HEV RNA postinfectivity assay directly correlates with the amount of HEV-3c inoculum *(24)*. Consequently, we examined HEV replication by measuring the HEV RNA production at 14 days postinfection (dpi). The obtained results were compared with the inoculated but untreated control samples and were demonstrated as log reductions.

As shown in Figure 1A, treatment of HEV-3c with 1, 3, and 5% citric acid on HDPE resulted in an average 2.30- to 2.48-log reduction compared with the untreated control. Similar treatment with acetic acid led to an approximately

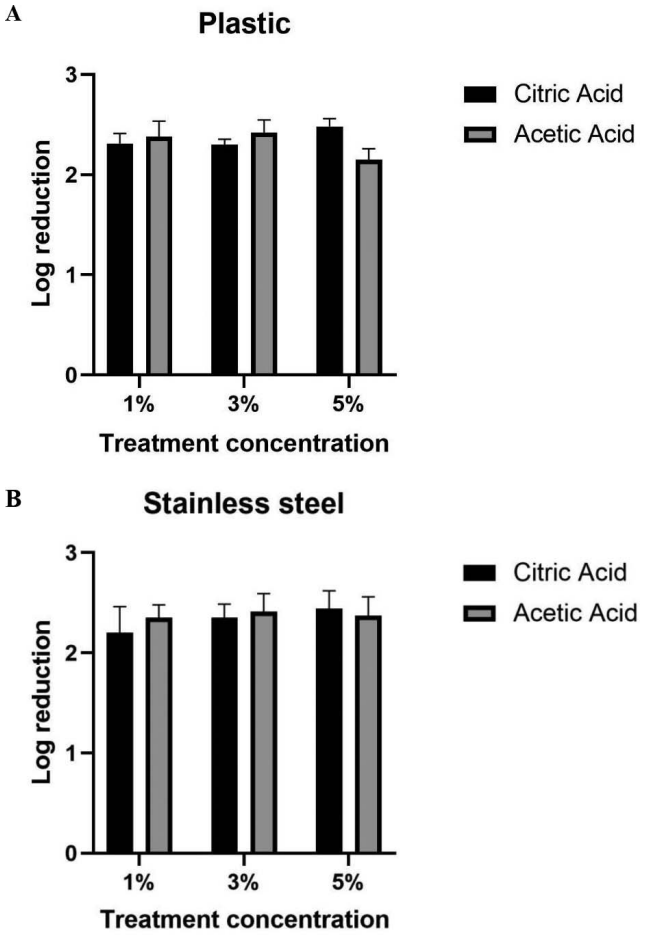

FIGURE 1. *Log reduction compared with untreated samples following treatments with citric acid or acetic acid on (A) plastic (HDPE) or (B) stainless steel. The ISO 15216 method was employed for virus recovery, and viral inactivation was calculated following the infectivity assay and determination of viral genome copy numbers using ddRT-PCR at 14 dpi. The data represent the average of three independent experiments. Error bars represent the standard deviation. * P < 0.05, calculated by* t *test.*

2.15- to 2.42-log reduction. In addition, treatment of HEV-3c with 1, 3, and 5% citric acid on stainless steel resulted in an average 2.20- to 2.44-log reduction compared with the untreated control, whereas similar treatment with acetic acid caused a 2.35- to 2.41-log reduction (Fig. 1B). No statistically significant differences were observed between treatment conditions and surfaces.

Examining the virucidal effect of citric acid and acetic acid on HEV-3 in pork pâté. Next, we examined whether similar inactivation results could be achieved in a food commodity at high risk of HEV contamination, pork pâté. Therefore, pork pâté was artificially inoculated with HEV-3c and was then subjected to treatment with 1, 3, and 5% citric acid and acetic acid at ambient temperature. Following treatment with organic acids, the virus was extracted using the modified ISO 15216 method and was introduced to A549/D3 cells for the infectivity assay. The average viral extraction efficiency for the mock-treated samples was 3.7% ± 0.62%. The obtained results were

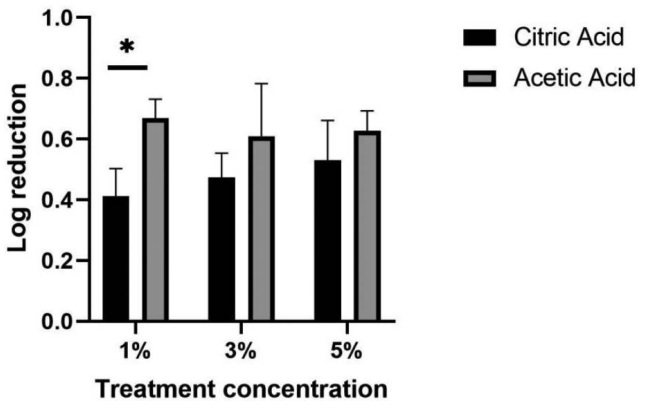

FIGURE 2. *Log reduction compared with untreated samples following treatments with citric acid or acetic acid on pork pâté. The adapted ISO 15216 method was employed for virus recovery, and viral inactivation was calculated following the infectivity assay and determination of viral genome copy numbers using ddRT-PCR at 14 dpi. The data represent the average of three independent experiments. Error bars represent the standard deviation. * P < 0.05, calculated by* t *test.*

compared with the inoculated but untreated positive control samples and were demonstrated as log reductions.

As shown in Figure 2, treatment with organic acids on pork pâté led to considerably lower inactivation, with an average reduction of 0.41 to 0.53 log with 1 to 5% citric acid, whereas treatment with 1 to 5% acetic acid resulted in a 0.61- to 0.67-log reduction compared with the untreated control. Overall, treatment with acetic acid resulted in slightly higher inactivation compared with treatment with citric acid; however, the difference was only statistically significant with the 1% treatment.

DISCUSSION

In the meat processing industry, organic acids are considered suitable antimicrobials for pathogen control because of their low cost and quick action *(24, 29)*. The allowed concentration of these acids should be such that they are efficient in pathogen inactivation while retaining acceptable sensory qualities of the treated meat portion *(34)*. However, the inactivation potential of organic acids has not been studied against HEV or against foodborne viruses in meat or meat products. One study reported a less than 1-log reduction in murine norovirus (MNV) infectivity following treatment with different concentrations of vinegar (acetic acid) on green laver *(25)*. Herein, we observed a greater than 2-log reduction in viral infectivity on surfaces following treatment with citric acid and acetic acid at concentrations of 1% or higher. This might indicate that in the absence of a food matrix, HEV is more susceptible to inactivation by organic acids compared with MNV.

It has been reported that citric acid has the potential to block human norovirus from binding to histo–blood group antigens *(20)*. Another study demonstrated complete inactivation of feline calicivirus by 50% citric acid; however, this concentration is 10 times higher than the maximum allowed use in the food industry *(36)*. In contrast, treatment of norovirus surrogates with natural products that

contain up to 5% citric acid resulted in a less than 1-log reduction in infectivity (reviewed in Bosch et al. *(2)*). Moreover, treatment of norovirus surrogates such as Tulane virus and MNV with organic acids such as acetic acid in solution did not result in significant inactivation *(18)*.

Herein, the increase in concentration of organic acids from 1 to 5% did not lead to a statistically significant increase in viral inactivation. This is consistent with a previous observation of treatment of norovirus surrogates with organic acid in solution *(18)*. It has also been shown that the inhibition of bacterial strains with organic acids was not solely dependent on pH or on their concentration *(1)*. The mechanism of action of organic acids against bacteria is likely quite different from that against viruses. Although it is believed that the intracellular accumulation of anions is a primary reason for inhibition of bacteria by organic acids (reviewed in Beier *(1)*), the damage to the viral capsid or envelope is considered the main reason for viral inactivation by organic acids *(20, 25)*.

In this study, the reactions with organic acids were never stopped on the tested surfaces. However, following the incubation time (10 min, precisely), the virus was extracted using cotton swabs moistened in minimum essential media containing sodium bicarbonate, sodium phosphate dibasic, and sodium phosphate monobasic, which are expected to neutralize the organic acids. However, we cannot rule out the potential effect of residual acid on viral recovery efficiency.

It has been shown that when HEV leaves the host cell through the noncytolytic budding process, it acquires the lipid envelope, whereas the particles that accumulate inside the infected cells are nonenveloped *(8)*. In this work, we prepared the viral stock by freeze-thawing the infected cells and thereby obtained both enveloped and nonenveloped particles. Consequently, the viral stock used for the experiments was a mixture of enveloped and nonenveloped viruses that is considered quasienveloped. It is believed that enveloped particles are less infectious compared with nonenveloped particles *(8)*; however, it is not known whether non–lipid-rich environments (plastic and stainless steel) would be more hostile to the overall viral population compared with lipid-rich ones (pâté). Nevertheless, because of the complexity of the matrix and the employed method, the recovery rate for the pork pâté was lower compared with that for the plastic and stainless steel; however, it was still above the accepted limit for the ISO 15216 method, which is 1% *(13)*.

The advantage of this study is examination of the virucidal effect of organic acid in artificially contaminated pork products, as well as common food contact surfaces, rather than in viral suspensions in media or PBS. We have also observed that HEV-3c in pork pâté is protected against inactivation by organic acids compared with HEV-3c on surfaces. The protective effect of food against different inactivation strategies has been studied for various foodborne viruses and their surrogates (reviewed in Cook et al. *(4–6)*). The protective effect of food against inactivation of HEV-3c by HPP has been previously demonstrated by our group *(23)*. It can be speculated that food components in pork pâté, such as fat, protein, and salt, can interact with the viral capsid or organic acids, reducing the inactivation efficiency.

In summary, our data demonstrate that 1 to 5% citric acid and acetic acid show some efficacy in the inactivation of HEV on food contact surfaces; however, their inactivation potential is drastically reduced in a meat product. Therefore, they cannot be used for HEV-3 risk mitigation of HEV contamination in high-risk food commodities. The obtained data will help with establishing proper measures for prevention and control of foodborne transmission of HEV. It thus supports the risk assessment and future policy development.

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