Absence of zoonotic hepatitis E virus infection in Flemish dairy cows

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ABSTRACT

Recently, infectious HEV particles were discovered in milk and fecal samples of dairy cows in China. Given the recent increase of autochthonous HEV infections in Europe, we wanted to assess whether cows constitute an HEV reservoir in this region and hence may be responsible for the advance of HEV through consumption of cow produce. To verify the zoonotic risk cows potentially pose towards European consumers, we screened > 10% of dairy milk farms in Flanders, Belgium for the presence of HEV. A quarter of these housed both cows and pigs, the latter a well-known reservoir for HEV. Milk and fecal samples were analyzed for the presence of HEV RNA and HEV-specific antibodies.

Despite the fact that HEV is circulating amongst pig farms in Flanders and proof of active HEV infection in the pigs of at least one of the mixed farms included in our study, we could not detect any sign of active or past HEV infection in cows. The HEV prevalence in our study was 0%, with a 99.99% confidence interval (CI) for HEV RNA and anti-HEV antibody of [0%-2.30%] and [0%-4.23%] respectively. Our results suggest that, at least in Flanders, cows are not an HEV reservoir and hence do not pose a major health risk towards humans.

1. Introduction

Hepatitis E virus (HEV) is a worldwide underdiagnosed pathogen responsible for approximately 20 million infections per year (Kamar et al., 2014; Sayed et al., 2015). Worldwide it is the most common cause of acute hepatitis (Kamar et al., 2012) and almost 2 billion people, a third of the world’s population, is at risk of infection due to living in endemic areas (Pérez-Gracia et al., 2015). There has been a yearly increase in reported hepatitis E cases amongst Western European countries (ECDC, 2017; Pischke et al., 2014). This might reflect a true rise in the incidence of HEV in Europe, suggesting there has been a change in the risk of acquiring this virus (Cornelia et al., 2016).

HEV is a positive-sense single-stranded RNA virus that has a 7.2 kb genome containing 3 overlapping open reading frames (ORFs) (Okamoto, 2013). Hepatitis E virus isolates that can infect mammals are classified in the Orthohepevirus genus of the Hepeviridae family. The Orthohepevirus A species is subdivided into seven major genotypes. Genotypes 1 and 2 only infect humans. Genotypes 3 and 4 are zoonotic and can infect humans, pigs, rabbits, deer and mongoose. Genotypes 5 and 6 comprise strains isolated from wild boar, whereas isolates from camel are classified into genotype 7 (Sayed et al., 2015).

Genotypes 1 (subtypes a-e) and 2 (subtypes a-b) are endemic in many developing areas such as Asia, Africa and Mexico (Lu et al., 2006; Pérez-Gracia et al., 2015; Sayed et al., 2015). They are often associated with large outbreaks due to poor sanitation conditions and are estimated to account for 70,000 deaths annually (Kamar et al., 2014; Rein et al., 2012; Yugo and Meng, 2013). Nevertheless, not all infected patients develop overt clinical signs and in most patients HEV infection is
A self-limiting illness (Kamar et al., 2014; Sayed et al., 2015).

The zoonotic genotypes 3 (subtypes a-j) and 4 (subtypes a-g) are responsible for food-borne autochthonous cases reported in industrialized countries (Lu et al., 2006; Pérez-Gracia et al., 2015; Sayed et al., 2015). The transmission via consumption of raw or undercooked meat from infected animals has been well-documented in several studies (Colson et al., 2010; Masuda et al., 2005; Matsuda et al., 2003). In addition, HEV can also be transmitted by leafy green vegetables and field-grown strawberries contaminated by infected irrigation water and/or manure (Brassard et al., 2012; Kokkinos et al., 2012; Sayed et al., 2015). Although a significant proportion of people in industrialized countries is seropositive for HEV-antibodies, they usually have no history of symptomatic acute hepatitis. Most gt3 infections cause either no or only mild non-specific symptoms and resolve spontaneously (Kamar et al., 2014; Sayed et al., 2015). However, people with pre-existing liver disease or a chronic HEV infection have a poor prognosis. In addition, many patients cannot be treated with the standard therapy, ribavirin, due to contraindications and comorbidities. Finally, HEV infection is increasingly recognized as an important cause of extrahepatic complications, including renal and neurological disorders (Dalton et al., 2016).

HEV is the only member of the major hepatitis viruses that can infect humans, with an animal reservoir (Pérez-Gracia et al., 2015). The virus has been genetically identified in a variety of animals such as pigs, wild boar, deer, rabbit, mongoose, cattle and sheep, while serologic evidence of HEV infection has been reported for other animal species such as cat, dog and goat (Meng, 2013). A genetic similarity between HEV strains circulating in pig and humans as well as cross-species infection supports the likelihood that pigs are the main reservoir for HEV strains circulating in pig and humans as well as cross-species (Kamar et al., 2014; Sayed et al., 2015). Moreover, people with pre-existing liver disease or a chronic HEV infection have a poor prognosis. In addition, many patients cannot be treated with the standard therapy, ribavirin, due to contraindications and comorbidities. Finally, HEV infection is increasingly recognized as an important cause of extrahepatic complications, including renal and neurological disorders (Dalton et al., 2016).

2. Materials and methods

2.1. Sample collection

A total of 504 bulk milk samples, originating from 460 distinct dairy farms, were provided by the Flanders Milk Control Centre (MCC), a non-profit organization acknowledged by the Belgian government for the investigation of the quality and composition of raw milk produced by Flemish farms with intended use in the dairy industry. The samples were collected during the period July–September 2016. The 460 farms were randomly selected from the MCC database that comprises all Flemish dairy producers, based on stratification per province. The percentage of selected farms within each province matched the distribution of all dairy farms over that province (Antwerp: 19%; Flemish Brabant: 5%; Limburg: 10%; East Flanders: 28%; and West Flanders: 38%). Within each province, farms were randomly selected using the Excel RAND function. The selection covered about 10% of the total number of Flemish farms and comprised 120 mixed farms housing > 10 pigs in addition to dairy cows.

During the same period MCC additionally provided 1104 individual milk samples that originated from 7 dairy herds regionally divided over Flanders. All samples were blinded in order to preserve the privacy of the farms involved. The individual samples were composite samples, indicating they enclose a mixture of milk from the four quarters of the udder of the cow. Finally we collected 60 individual milk samples, 30 cow fecal samples and 80 pig fecal samples at a mixed farm in the province West-Flanders. The milk samples encompass 60 individual samples collected in 2016 at 2 different moments with a 3-month interval. The cow fecal samples were collected at the first time point and originated from the same 30 dairy cows. The pig fecal samples originated from fattening pigs and were collected at the same 2 time points. At the second sample collection point, the pig herd had been completely renewed.

2.2. Detection of HEV RNA

Detection of HEV RNA in milk samples was performed using the cobas® 6800 system (Roche Diagnostics, Vlizhoord, Belgium). The cobas® HEV assay is a real-time PCR test for the detection of hepatitis E virus RNA (genotypes 1–4) in human plasma. The assay was carried out according to manufacturer's instructions. The kit has a 95% limit of detection (LOD) of 18.6 IU/mL. Since preliminary experiments indicated that the milk matrix may interfere with the extraction process and hence impact the sensitivity of the test, different dilutions of an internal standard (range 1.6 × 10⁵–1.6 × 10⁻¹ IU/mL) were tested in a PBS solution containing 0, 1, 3, 10 and 100% milk. This validation experiment showed that a 10% milk matrix did not negatively influence the sensitivity of the Roche cobas® HEV assay (data not shown). Therefore all milk samples were diluted 1/10 in PBS (10% milk).

To detect HEV RNA in fecal cow and pig samples, a 10% (v/v) fecal suspension was prepared. The fecal cow samples were screened using the cobas® 6800 system (Roche Diagnostics, Pleasanton, CA, USA) as described above. The fecal pig samples were first pooled per 5 and then analyzed with an in-house assay. Briefly, total RNA was extracted from 1 ml suspensions using the NucliSens easyMAG system (Biomérieux, Craponne, France). Amplification was performed using a one-step real-time RT-qPCR on the LightCycler 480 (Roche Diagnostics) by means of the “Lightcycler Multiplex RNA Virus Master” (Roche Diagnostics). The following primers and probe targeting the ORF2/ORF3 overlapping region were used: forward primer HEVORF3-S: 5′-GGTGGTTTGCTGGGTGAC-3′, reverse primer HEVORF3-AS: 5′-AGGGTTGTTGGAAGA-3′ and probe: 5′-FAM-TGATTCTGACGCCTTGCCG-TAMRA-3′ (Abravanel et al., 2012; Jothikumar et al., 2006). For RT-PCR, the 10 μl of reaction mix, containing 0.4 μl of reverse primer (4 μmol/L), 0.2 μl of forward primer (2 μmol/L), 0.2 μl of probe (1.5 μmol/L), 4 μl RT-PCR reaction mix, 0.1 μl RT-Enzyme and 5.1 μl nuclease free H₂O, was combined with 10 μl of sample extract. The RNA was first reverse transcribed at 50 °C for 10 min, followed by a 30-second long denaturation step at 95 °C. The resulting cDNA was amplified in 45 cycles consisting of denaturation at 95 °C for 5 s and primer attachment/elongation at 60 °C for 30 s. The limit of quantification (LOQ) of this assay on undiluted samples is 40 IU/ml and equals the limit of detection (LOD).

2.3. Detection of anti-HEV IgG

A random selection of 245 bulk milk samples and the 30 individual milk samples collected at the second time point at the mixed farm were tested for the presence of HEV-specific IgG antibodies using an in-house adapted version of a commercial ELISA kit (Wantai Biologic Pharmacy Enterprise, Beijing, China). Samples were analyzed according to an...
adapted protocol in order to adjust the reactivity of the kit towards cow IgG and to minimize background. Briefly, the kit specimen diluent was replaced by an in-house sample diluent (1% casein in PBS), of which 100 μl was added to each well of the kit microwell plate that was precoated with HEV antigen. Next, 10μl of sample, negative control and positive control was added and the plate was incubated at 37°C for 30 min. Wells were washed 5 times with the kit’s wash buffer, which was supplemented with 1% casein. Between each wash cycle we introduced a 60-second soak period. After washing, 100 μl of HRP-conjugated rabbit anti-bovine IgG antibody (Sigma-Aldrich, St. Louis, MO, catalogue number A5295; 1/15,000 dilution) was added. This conjugate replaced the kit’s human-specific conjugate that was found not to be cross-reactive with bovine IgG. Due to cross-reactivity of the anti-bovine IgG conjugate with human IgG, both the kit negative and positive controls could be used. The plate was incubated at 37°C for 30 min. After a second washing step, 50 μl of the Chromogen A and Chromogen B solutions were added to each well followed by a 15-min incubation step at 37°C avoiding light. The reaction was stopped by adding 50 μl of the Stop solution. Absorbance was measured at 450 nm. The cut-off value was set at 0.335 OD and was calculated by the mean of the negative controls plus 3 standard deviations.

2.4. Sequencing and phylogenetic analysis of HEV RNA from positive pig fecal samples

The pig fecal samples that tested positive for HEV RNA were subjected to a nested PCR performed on the LightCycler 480 (Roche Diagnostics). For the first PCR step the “Lightcycler Multiplex RNA Virus Master” (Roche Diagnostics) has been used with following primers targeting ORF2: HEV Belgian ORF2-3156 forward primer: 5′-AATTATGCC-CAGTAYCGGRGTTG-3′ and HEV Belgian ORF2-3157 reverse primer: 5′-CCCTRTYCTYGCGTGMG-CATTCTC-3′. The 10 μl of reaction mix contained 0.4 μl of reverse primer (25 μmol/L), 0.2 μl of forward primer (25 μmol/L), 4 μl RT-PCR reaction mix, 0.1 μl RT-Enzyme and 5.3 μl nuclease free H2O, and was supplemented with 10 μl of sample extract. The RNA was first reverse transcribed at 50°C for 10 min, followed by a 1-minute long denaturation step at 95°C. The resulting cDNA was amplified in 40 cycles consisting of a denaturation step at 95°C for 5 s, a primer annealing step at 47°C for 45 s and an elongation step at 60°C for 45 s. Finally, a final extension was performed at 60°C for 7 min. For the second - nested - PCR the “Taq DNA Polymerase with ThermoPol® Buffer M0267L” (New England Biolabs Inc., Ipswich, UK) was used with the following primers: HEV Belgian ORF2-3158 forward primer (5′-GTWATGCTYTGACATGGCT-3′) and HEV Belgian ORF2-3159 reverse primer (5′-AGCCGACGAAACGCACTGTC-3′). The 50 μl reaction mix contained: 5 μl 10 × ThermoPol Reaction Buffer, 1 μl dNTPs (200 mmol/L), 1 μl forward primer (0.2 μmol/L), 1 μl reverse primer (0.2 μmol/L), 0.25 μl Taq DNA Polymerase (1.25 units), 10 μl of 1st-round PCR product, nuclease free H2O up to 50 μl. The 2nd round PCR protocol consisted of an initial denaturation at 95°C for 30 s followed by 30 cycles of a denaturation step at 95°C for 30 s, an annealing step at 47°C for 45 s and an extension step at 68°C for 40 s. Finally, final extension was performed at 68°C for 7 min. Subsequently the PCR product was run on a 1% agarose gel and the 348 base pair amplicon was cut and purified using the “Wizard® SV Gel andPCR Clean-Up System” (Promega, Leiden, The Netherlands). The PCR products were sent for sequencing (GATC Biotech, Germany).

The resulting sequences were imported in BioEdit (available from http://www.mbio.ncsu.edu/bioedit/bioedit.html) and aligned with Muscle (available from http://www.drive5.com/muscle/), together with 57 reference sequences. These sequences have been described in literature and were downloaded from GenBank (Thiry et al., 2014). A statistical selection of the best-fit model of nucleotide substitution was carried out using the Akaike information criterion (AIC) included in the program jModeltest (available from http://jmodeltest.org/login). A maximum likelihood phylogenetic tree was constructed using the program MEGA6 (available from http://www.megasoftware.net/).

2.5. Statistics

The power of the study was calculated using the Sas Power and Sample Size program (SAS, NC, USA) The analysis for the number of examined farms and screened bulk milk samples was based on the Exact Test for Binomial Proportion. The power of our study was calculated based on a null proportion of 0%, a binominal proportion of 5% and a nominal alpha of 0.01. An a priori calculation showed that a sample size of n = 460 (number of farms) and n = 245 (number of bulk milk samples tested for the presence of HEV-specific IgG antibodies) would result in a power > 0.999, which is sufficiently high. A 99.90% Wilson score confidence interval (CI) for proportions was calculated using the program R, version 3.3.2 (The R foundation for Statistical Computing).

3. Results

3.1. Screening for active HEV infection in Flemish dairy cows and pigs

A total of 504 bulk milk samples originating from 460 farms spread throughout Flanders were evaluated for the presence of HEV RNA. Apart from 1 invalid sample, all samples scored negative on RT-PCR.

![Fig. 1. Molecular and serological analysis of milk samples.](image-url)
investigate whether the pig herd was actively infected with HEV. A 3-months interval from one of the mixed farms, which enabled us to
isolates con
and 1 fecal sample contained both isolates. P-distances between the
isolate that belonged to gt3f, 3 fecal samples contained a gt3c isolate
(Fig. 2). This analysis showed that 5 of the fecal samples contained an
57 reference sequences available in Genbank covering genotypes 1 to 4
A phylogenetic tree was constructed using the obtained sequences and
alignment showed that essentially only 2 di
from 9 of these samples and the amplicons were sequenced. Sequence
negative, HEV RNA was readily detected in 15 (19%) pig fecal sus-
time point from the 30 dairy cows reared at that mixed farm scored
indicating that the true HEV RNA prevalence will be lower than 2.30%.
Despite the fact that HEV was actively circulating at this farm, anti-HEV
collected at the second time point at the mixed farm in West-Flanders.
vious exposure. In addition, we analyzed 30 individual milk samples
alyzed the milk samples for the presence of HEV-speci
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fication (data not shown).
Within the pool of tested farms, 121 farms were of mixed nature. We
were able to collect 80 pig fecal samples at two different moments with a
3-months interval from one of the mixed farms, which enabled us to
investigate whether the pig herd was actively infected with HEV. Although the 30 individual milk and fecal samples collected at the first
time point from the 30 dairy cows reared at that mixed farm scored negative, HEV RNA was readily detected in 15 (19%) pig fecal sus-
pensions.
Using nested RT-PCR targeting ORF2 we could amplify the virus
from 9 of these samples and the amplicons were sequenced. Sequence
alignment showed that essentially only 2 different strains were present. A phylogenetic tree was constructed using the obtained sequences and
57 reference sequences available in Genbank covering genotypes 1 to 4
(Fig. 2). This analysis showed that 5 of the fecal samples contained an
isolate that belonged to gt3f, 3 fecal samples contained a gt3c isolate and 1 fecal sample contained both isolates. P-distances between the
isolates confirmed this classification (data not shown).

3.2. Flemish dairy cows do not show signs of past HEV infection

In order to identify past HEV infection of the dairy cows, we ana-
yzed the milk samples for the presence of HEV-specific IgG. Analysis of
245 out of 504 bulk milk samples did not show any evidence of pre-
vious exposure. In addition, we analyzed 30 individual milk samples
collected at the second time point at the mixed farm in West-Flanders.
Despite the fact that HEV was actively circulating at this farm, anti-HEV
specific antibodies were not detected in any of the screened milk
samples. The 99.99% CI for the anti-HEV antibody prevalence was
[0.0–4.23%] indicating that the true anti-HEV antibody prevalence will be lower than 4.23%.

4. Discussion

Shortly after hepatitis E virus was first identified in the early 1980s, it was regarded as an important public health concern in developing
countries (Aggarwal, 2011; Ruggeri et al., 2013). Later studies reported
cases of HEV in people living in non-endemic countries (Ruggeri et al.,
2013). Most cases were travel-associated but also autochthonous in-
festions have been reported (Sayed et al., 2015). Since the early 1990s,
studies also showed the presence of anti-HEV antibodies in different animal species worldwide, clearly demonstrating hepatitis E is zoonotic
(Emerson and Purcell, 2003). In 1997 a swine HEV strain that geneti-
cally correlated to two human HEV strains was identified in the USA,
demonstrating that pigs are probably the main viral reservoir in in-
dustrialized countries (Meng et al., 1997). According to a study per-
formed in 2011, 21.7% of the 23 tested Belgian pig farms were design-
nated HEV positive (Honig et al., 2011).

In this study we evaluated whether HEV is circulating in Flemish
dairy cows by screening a substantial amount of Flemish milk samples.
Despite our comprehensive analysis, we could not detect HEV RNA or
anti-HEV antibodies in any of the screened milk samples even though we have evidence of an active HEV infection in one of the screened
mixed farms.

The discrepancy between our results and the results obtained by
Huang et al., who reported that 37.14% of investigated cows in China
were actively infected with HEV, remains unclear (Huang et al., 2016).
One possible factor could be the difference in HEV genotype between
the viruses that circulate in China and in Europe. In China, the most prevalent genotype is genotype 4 (Huang et al., 2016), while genotype
3f is predominant in Belgium (Thiry et al., 2014). The virus identified in
cows by Huang et al. was of genotype 4, subtype 4b. Subtype 4d was
detected in Yellow cattle by Yan et al. (Yan et al., 2016). In the latter
study, about 47% of cows had HEV-specific antibodies in their plasma.
It is conceivable that cows would only be susceptible to gt4 HEV, hence
explaining the absence of HEV infection in our cohort.

Alternatively, differences in housing and management could also
contribute. In order to minimize the potential spread of infectious dis-
eases, Belgian farmers should organize their pig stables such to prevent
direct contact with rodents, other mammals and raptors (FASFC -
Federal agency for the safety of the food chain, 2014). This is probably
different from normal practice at Chinese mixed farms, especially
smaller farms in rural areas (Huang et al., 2016). Nevertheless, it does
not exclude indirect contact between dairy cows and pigs. For example,
it is not unlikely that Flemish cows graze on pasture that is fertilized
with pig manure. Likewise, HEV could be transmitted from the pig
stable to the cows via contact with the farmer or his contaminated
workwear. In any case, the likelihood of direct and indirect contact
between pigs and cows at Belgian mixed farms is most-likely sub-
stantially lower than in China.

During the preparation of our manuscript, Baechlein and Becher
(2016) reported that retrospective analysis of 400 milk samples col-
lected in 2008 from German dairy cows and stored at ~20 °C did not
show the presence of HEV RNA. Our prospective study differs from the
German study at several aspects: 1) it included much more samples and
had higher coverage of dairy farms, 2) fresh samples were analyzed
thereby avoiding a potential negative impact of long-term storage and
3) we screened for HEV-specific antibodies as proof of past exposure.
Moreover, cows were screened on a farm with proven ongoing HEV
infection in the resident pig herd.

If genotype-dependent differences in species tropism play a role, con-
tinuous and more in-depth surveillance of the European pig and cow
population is mandatory. HEV of gt4b has been identified once in one
Belgian pig fattening farm (Honig et al., 2011). This was the first
 genotype 4b strain ever reported in swine in Europe. Since then, this
genotype has been detected in France (Bouamra et al., 2014; Colson
et al., 2012; Jeblaoui et al., 2013; Tessé et al., 2012), Italy (Garbugli
et al., 2013; Monne et al., 2015) and Denmark (Midgley et al., 2014).
If gt4 HEV would become more prevalent in Europe it may then increase
the risk of infection of the (dairy) cow population and consequently
increase the risk of transmission to humans.

In conclusion, we could not find any evidence for active or past HEV
infection in Flemish dairy cows. Our results suggest that, at least in
Flanders, cows are not a signi
cant reservoir of HEV and that milk and
derivative dairy products do not pose a major risk of infection towards
humans. Further research is needed to investigate if genotype-depen-
dent factors are responsible for the absence of HEV infection in Flemish
dairy cows.

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