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New Subcluster of HEV Genotype 3 Strains Linked to the First Confirmed Swiss Case of Foodborne Hepatitis E Infection

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Abstract

We describe the first confirmed foodborne Hepatitis E virus (HEV) infection in Switzerland linked to the consumption of a raw sausage ("mortadella di fegato cruda" type) containing pig liver and the appearance of a new "Swiss" subcluster of genotype 3 HEV strains.

Case Report

A 78-year-old male was hospitalized for 4 days in Lugano (canton Ticino, Southern Switzerland) in October 2016. The patient came to the Emergency Ward for extreme fatigue, severe jaundice and dark urine, without preceding fever or flu-like symptoms and without any abdominal pain. Laboratory tests at admission suggested an acute hepatitis (ALAT 3519 U/L, ASAT 2475 U/L, ALP 341 U/L, total bilirubin 117 µmol/L, GGT 578 U/L). Abdomen ultrasound and CT scan excluded colelithiasis, suggesting hepatitis. Serological tests for HBV, HCV, and CMV were negative, and acute hepatitis E was diagnosed based on both IgM and IgG positivity. During hospitalization, deambulation and appetite were normal, and no pain was claimed. After four days the symptoms improved. A patient's stool sample was collected within ten days from symptoms onset. Based on a questionnaire, the patient mentioned that he used to consume on a regular basis raw meat products (especially pork sausages from the mordadella di fegato type).

Food Products Available for Microbiological Testing

We analysed samples from two raw cured sausages produced with raw liver from pigs (mortadella di fegato cruda). Both samples were sold in the same butcher shop. The first sample was brought by the patient and analysed on November 07, 2016, whereas the second sample was officially taken by food safety authority and analysed on January 24, 2017

Microbiological Testing

The food products were processed as described by Szabo et al. [1]. Five grams of manually defatted mortadella were placed in an 80 ml sterile filter bag (Interscience by Axon Lab, Baden-Dättwil, Switzerland) and 10 µl of a Mengovirus stock as a process control (Mengovirus Extraction Control kit, bioMérieux, Geneva, Switzerland) were added and incubated at room temperature for 5 min. Thereafter, the sample was homogenized with 7 ml of TRI Reagent® (Lucerna-Chem AG, Luzern, Switzerland) using a blender (MiniMix®, Interscience by Axon Lab, Baden-Dättwil, Switzerland) for 2 min. The rinse fluid was removed via the filter compartment of the bag and centrifuged at 10,000xg for 20 min at 4°C to pellet residual food particles. A total of 1.4 ml chloroform (0.2 ml/ml TRI Reagent®) was added to the clarified supernatant and mixed for 15 sec. After incubation for 15 min at room temperature, samples were centrifuged at 10,000xg for 15 min at 4°C. The nucleic acids of a total of 1 ml of the aqueous phase containing the viral RNA were extracted using the NucliSENS® easyMAG system (bioMérieux, Geneva, Switzerland) according to the manufacturer's instructions and eluted in 60 µl of elution buffer. The extracts were either freeze-stored or immediately used for the viral RNA amplification. The quantitative real-time RT-PCR for HEV and Mengovirus were performed with commercial kits (ceeramTools®, Mengo Extraction Control an hepatitis@ceeramTools®, bioMérieux, Geneva, Switzerland) according to the manufacturer instructions, on a Rotor Gene Q system (Qiagen, Basel, Switzerland). Mengovirus recovery rates were >1%.

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Patient's fecal samples were diluted prior to RNA extraction as follow: one loop of sample was suspended in 1 ml of sterile water and centrifuged 1 min at 10,000 rpm. Total RNA from the supernatant was extracted with the QIAamp Viral RNA Mini Kit following the instructions provided by the manufacturer (Qiagen GmbH Germany).

RNA from patient's serum samples was extracted with the same kit without previous manipulations.

Sequencing and Phylogenetic Analysis

RNA from the fecal sample and the sausage were reverse-transcribed using the RevertAid H minus first strand cDNA synthesis kit (ThermoFisher, Reinach, Switzerland) following manufacturer's instructions. The cDNA was treated with RNase H (New England Biolabs, BioConept, Allschwil, Switzerland) for 20 min at 37°C to remove remaining RNA and a second strand was synthesised using Klenow fragment, exo- (ThermoFisher, Reinach, Switzerland) according to the instructions. During both, first and second strand cDNA synthesis, a tagged random primer was added to the reaction mixture (5'-GCTGGAGCTCTGCAGTCATC NNNNNN-3') to be incorporated in the growing cDNA strand. The double stranded cDNA was purified using the QIAquick PCR Purification kit (QIAGEN, Hombrechtikon, Switzerland). In the following sequence-independent single primer amplification (SISPA), the tag sequence (5'-GCTGGAGCTCTGCAGTCATC-3') was used to prime unspecific amplification in a PCR reaction using Hotstart Taq Polymerase (QIAGEN, Hombrechtikon, Switzerland) according to manufacturer's instruction in 18 cycles. The amplified DNA was purified using the PureLink DNA PCR Micro kit. The amplicons were subjected to NGS library preparation at the Functional Genomic Centre Zurich using the NebNext Ultra II library preparation kit and the NebNext barcoding kit for Illumina (both New England Biolabs, BioConept, Allschwil, Switzerland). A paired-end next generation sequencing run of 2 × 150 nt read-length was performed using the Illumina NextSeq500 machine and a mid-output flowcell. After quality-trimming, the reads were aligned to a manually prepared fasta database containing all available full-length Hepatitis E genomes of the genotypes 3 and 4 retrievable from GenBank using SeqMan NGen v14 software (DNASTAR Lasergene, Madison, USA). The highest number of reads matched to the recently published Swiss HEV-3 strain SW/16-0282 (KY780957.1). The consensus sequence of this alignment was near full-length with a 39 nt and 69 nt gap in the ORF2 region for the fecal sample and the sausage sample, respectively. This gap was bridged using specific primers binding up- and downstream of the gap (HEV_TI_gap-F 5'-CAC ATC ATG GCT ACT GAG-3' and HEV_TI_gap-R 5'-3'ACA CGG GTG TTA GTG TTC). The 442 nt long product covering nucleotides 5,690-6,132 relative to strain HEV-3_CH_16020189 (MF346772) was purified (QiaQuick PCR Purification kit, QIAGEN, Hombrechtikon, Switzerland) and sent to Microsynth GmbH (Balgach, Switzerland) for bidirectional Sanger sequencing. The two electropherograms from each sample were assembled using SeqMan Pro 13 software (DNASTAR Lasergene, Madison, USA) and aligned to the NGS consensus sequences using the Clone Manager Pro software (Sci-Ed software, Denver, USA). The 39 and 69 missing nucleotides were copied from the Sanger sequence and pasted into the NGS consensus sequence to obtain the full-length sequences. Both sequences were submitted to GenBank and received the accession numbers MF346772 (fecal sample, isolate HEV-3_CH_16020189) and MF346773 (sausage sample, HEV-3_CH_17LA00702).

For the phylogenetic analysis, the two sequences were aligned to 26 HEV genomes from GenBank representing the different subgroups of genotypes 1, 2, 3 and 4 using MUSCLE. Subgroup affiliation of the references sequences was based on the analysis of Vina-Rodrigues et al. [2]. The phylogenetic tree was computed in MEGA6 using the Kimura 2-parameter [3] and the Neighbor-Joining methods [4] with 1,000 bootstrap replicates. For this, only the coding region (7,128 nt length) of the 28 sequences was considered as not many of the reference strains available from GenBank include the non-coding ends of the genome.

Results and Discussion

Both sausages tested positive by quantitative real-time RT-PCR for HEV (2.45E+05 and 5.14E+05 copies/g sausage, respectively). The quantitative real-time PCR (RT-PCR) was positive in patient's fecal sample, but negative in serum. HEV IgM and IgG tested in serum were positive.

The extracted RNAs from the patient's fecal sample and one sausage sample were sequenced using next generation sequencing (NGS). Screening of the NGS reads to full-length HEV strains from GenBank showed best matches to the recently published Swiss HEV-3 isolate SW/16-0282 (KY780957.1, Wang et al. [5]), with 88,737 reads of the 34,266,144 total reads from the fecal sample and 6,955 reads of the 17,035,086 total reads from the sausage sample assembling to this reference sequence. The 7,244 nucleotide full-length sequences determined from the two samples were identical with exception of 21 positions. Nucleotide ambiguities were observed in one or the other isolate, suggesting quasispecies diversity. However, in each case of ambiguity, the other isolate showed one of the two possible nucleotides or even the same ambiguity. Hence, it is very likely that the two isolates belong to the same virus strain. Interestingly, while this strain clearly belongs to HEV-3 and is closely related to the strain SW/16-0282 (95% identity), it showed only 88% identity to other HEV full-length sequences. The phylogenetic tree reveals that the three Swiss HEV isolates form a distinct cluster within HEV-3. This is supported by high bootstrap values (Figure 1). While our two isolates are derived from the canton Ticino in Southern Switzerland, the isolate SW/16-0282 originates from Central Switzerland, north of

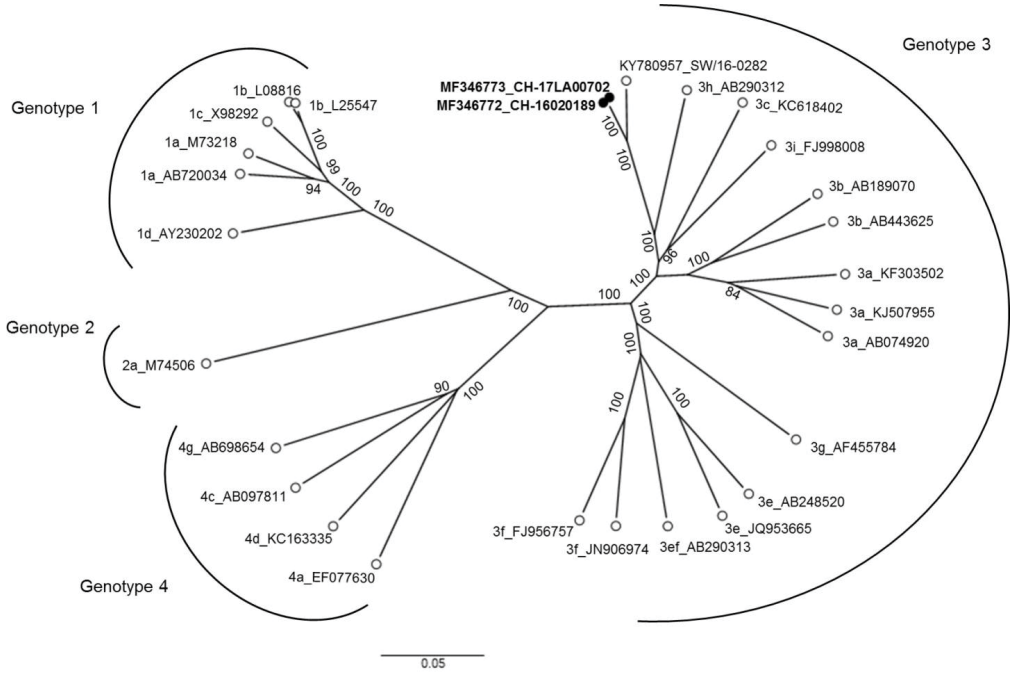

Figure 1: Phylogenetic analysis of HEV-1, 2, 3 and 4 was performed using Kimura-2 parameter and Neighbor-joining methods, based on complete sequences (excluding the non-coding genome ends). Numbers represent bootstrap values. The branch length is representative for the evolutionary distance (scale of substitution rate given). The new Swiss isolates (black circles) were compared to reference sequences from GenBank (open circles).

the Alps. Since these three isolates are the only full-length sequences of Swiss HEV strains, analysis of more viruses from different regions of Switzerland will be necessary to confirm the existence of a Swiss-specific HEV-3 subcluster.

In humans, clinical symptoms of hepatitis E are indistinguishable from other forms of acute hepatitis. The case fatality rate among patients is generally below 1% to 5%, with the exception of pregnancy where rates up to 25% have been reported [6]. In Switzerland, hepatitis E is not notifiable; therefore, the exact number of cases is unknown. Nevertheless, there are so far two published studies about the seroprevalence of HEV in the blood donor population. The seroprevalence depending on the assay which was used is 5% to 20% [7,8]. Recently, in Germany, a neighboring country, the number of notified hepatitis E cases has risen steeply. In 2017, 4,013 cases were reported (https://survstat.rki.de/) with is a significant increase compared to 607 cases in 2014 (Robert Koch Institute, 2015). Antibodies against HEV have been found in both the general population [8,9] and — with increased prevalence — in individuals with occupational exposure to swine and wild boars [10-12].

In Switzerland, a quantitative risk assessment following the Codex Alimentarius principles was recently performed in order to predict the exposure of consumers to hepatitis E virus through food consumption [13]. Pork products containing pork liver, in particular those sold raw were identified as posing the highest risk for the consumer.

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Authors' Contributions

- Claudia Bachofen, Jakub Kubacki, Cornel Fraefel: sequencing and phylogenetic analysis
- Marco Jermini, Petra Giannini: leading microbiological testing of food, information exchange between state authority and hospital
- Gladys Martinetti, Paolo Ripellino, Enos Bernasconi: clinical team
- Roger Stephan: scientific and coordination support; information exchange between state authorities and university, writing the manuscript

All authors have read and commented on the manuscript.

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