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# Complete genome sequencing of a genotype 3 hepatitis E virus strain identified in a swine farm in Italy

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ABSTRACT

In this study, we investigated hepatitis E virus (HEV) infection in piglets sampled in two farms in southern Italy. The virus was detected in 11 out of 15 animals tested. Based on sequence analysis, the 6 Italian strains examined belonged to two clusters containing both swine and human strains of either genotype 3 subtype e or f from Europe and Japan. The two Italian strain clusters shared nucleotide identity of 81.8% and 87.5% in the ORF2 (capsid protein) and ORF1 (RdRp) diagnostic fragments, respectively, confirming the heterogeneity of genotype 3 viruses circulating in pigs in Italy. The complete genome of one genotype 3 subtype e strain and the full ORF2 and ORF3 coding regions of one of the genotype 3f strains, obtained in this study, were compared to other HEV sequences available on line (NCBI database). The results of analysis showed that porcine strains clustered together with human and swine strains detected in Europe. Most changes in the coding region corresponded to synonymous mutations, and only the ORF3 showed a positive selection. Further, analyses are needed to understand the clinical significance of HEV genotypes and subtypes.



## 1. Introduction

Hepatitis E is an acute human disease caused by a small RNA virus, the hepatitis E virus (HEV). In developing countries, HEV has been associated with large waterborne outbreaks (Aggarwal and Naik, 2009). In industrialized countries, hepatitis E was firstly considered a travel-associated disease whilst in the last 10 years an increasing number of autochthonous cases and more recently also small outbreaks have been reported (Ruggeri et al., 2013). The disease is usually self-limiting with low mortality but it can be highly lethal among pregnant women, mostly in developing countries (Aggarwal and Jameel, 2011). Infection varies from asymptomatic to acute self-limited hepatitis, although it may lead to chronic liver disease in immunosuppressed patients. Except for this and the severe acute liver failure among pregnant women, complications are normally rare (Ahmed et al., 2015).

The hepatitis E viruses were recently classified in the genus *Orthohepevirus,* including 4 species: *Orthohepevirus* A includes strains infecting both humans and animals (pig, wild boar, deer, mongoose, rabbit and camel), whereas *Orthohepevirus* B (chicken), *Orthohepevirus* C (rat, greater bandicoot, Asian musk shrew, ferret and mink) and *Orthohepevirus* D (bat) were only identified in animals (Smith et al., 2014).

The genome of HEV is approximately 7.5 kb, and contains three open reading frames (ORFs) and short untranslated regions. The ORF1 encodes non-structural proteins including the methyltranferase (MTase) and the RNA-dependent RNA polymerase (RdRp), while the ORF2 and the ORF3 encode the glycoprotein that forms the viral capsid (Panda et al., 2007) and a small multifunctional protein involved in virus morphogenesis and release (Yamada et al., 2009), respectively. In addition to humans, HEV infects several animal species, apparently without evident clinical signs.

Although, only one HEV serotype is currently recognized, 4 mammalian HEV genotypes are distinguished based on the nucleotide sequence (Lu et al., 2006), which show different geographical distribution and epidemiology. Genotypes 1 and 2 are restricted to humans and include epidemic strains of developing countries that are often associated to waterborne outbreaks (Perez-Gracia et al., 2014). Genotypes 3 and 4 infect both humans and

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many animal species (e.g. pig, deer, wild boar, and rabbit), and circulate in developed countries usually causing sporadic cases. These two latter genotypes are considered zoonotic, and pigs and less frequently other animal species (wild boar, deer) are reservoir of viruses infecting humans (Van der Poel, 2014). Genotype 3 was the first HEV strain shown in animals, having been detected in pigs since the mid '90s (Meng et al., 1997). More recently, genotype 4 HEV, previously detected in Asia, has been detected in Europe in both pigs and humans (Bouamra et al., 2014; Hakze-van der Honing et al., 2011).

Food-borne transmission of HEV was reported in sporadic human cases and small outbreaks. These infections were associated to consumption of raw or under-cooked meat and sausages, and the genome sequences of the genotype 3 (g3) viruses identified in either clinical samples or animal-derived food (swine, deer, wild boar) were closely related to each other (Colson et al., 2010; Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003).

Based on intra-genotype sequence variability, HEV genotypes are further divided into subtypes: genotype 1, five subtypes (1a–1e); genotype 2, two subtypes (2a and 2b); genotype 3, ten subtypes (3a–3j); and genotype 4, seven subtypes (4a–4g) (Lu et al., 2006), the clinical relevance of different subtypes is unknown. Differential virulence between genotypes might at least partially explain the limited burden of human disease in contrast with the very high circulation of g3 HEV in pigs worldwide (Aggarwal and Naik, 2009; Ahmed et al., 2015).

Several studies conducted on pigs in Italy (Di Bartolo et al., 2008, 2011; Monne et al., 2015) indicate that swine HEV circulates largely within pig herds in this country, including detection of the virus in pig liver sausages that are frequently consumed raw (Di Bartolo et al., 2015). Genotype 3 HEV is widespread among Italian swine, and a single detection of genotype 4 HEV was reported recently in a swine farm in northern Italy (Monne et al., 2015).

In the present study, we investigated HEV infection in 15 piglets from two farms located in southern Italy. The complete genome of a g3 subtype e HEV strain and the complete ORF2 and ORF3 of a g3 subtype f strain were sequenced and compared to other HEV sequences available in the online NCBI database.

## 2. Materials and methods

### 2.1. Molecular detection of HEV

In July 2012, 15 fecal samples were collected from piglets (about 30 kg live body weight, two months old) in two farms of southern Italy. Pigs had been tested for norovirus, but not further test was performed. Total RNA was extracted from 10% fecal suspensions using the QIAamp Viral RNA mini kit (QIAGEN). The presence of HEV RNA was determined by three nested or semi-nested reverse transcription PCRs (nRT-PCR). The RT and the first-round PCR were carried out using the one-step RT-PCR kit (QIAGEN) and the second-round PCR was conducted with an amount of template corresponding to 10% of the final reaction volume, using the *Taq* DNA Polymerase (QIAGEN), according to manufacturer's instructions. A 467 bp region of the capsid protein gene (ORF2) was amplified by nRT-PCR (Mizuo et al., 2002) and a 329 bp fragment of the RdRp by a semi-nested RT-PCR (Zhai et al., 2006). The third reaction was a semi-nested RT-PCR, which amplifies a 748 bp fragment in the MTase. The latter was performed as described by Wang et al. (1999), with some modifications: the reverse primer used in both the first round RT-PCR and the second round of the semi-nested PCR, was designed in this study (named SwHEV853 Rw, 5′-GGGTAYGGGACATAAGGC-3′, annealing at position 840–858 referred to strain accession number JQ013795).

The PCR products were visualized in 1.5% agarose gel stained with GelRed® (Biotium). Bands of the expected size were excised and DNA was purified by Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions.

### 2.2. Amplification of HEV complete genome

Viral RNA was extracted from 400 μl of a 10% (w/v) fecal suspension from two samples collected in different farms. Reverse transcription was conducted with the SuperScript III reverse transcriptase (Lifetechnologies) with random hexamers, following the manufacturers' instructions. The full genome sequencing of swine HEV strain named E2 was performed, generating high-fidelity PCR fragments (Expand High fidelity PCR system, Roche) by primer walking (Table 1), amplifying nine overlapping regions. For the other HEV strain (named E14), only nRT-PCRs amplifying the entire 5′- portion of the ORF1, the entire ORF2 and ORF3 yielded DNA amplicons, which were sequenced. For the poly(A), the cDNA was obtained by an oligo-dT primer designed in this study, including a 10 residue-long T tail and a 3′ tag corresponding to the sequence of the universal primer M13 (5′-TAACAATTTCACACAAGG-3′). The cDNA was then subjected to PCR using the M13 primer and an upstream primer annealing in the ORF2. The amplicons obtained were cloned into the pGEM-T vector (Promega).

### 2.3. Sequencing and phylogenetic analysis

Sequencing was carried out by BioFab Research s.r.l. (Rome, Italy), using the BigDye Terminator Cycle Sequencing Ready reaction Kit v3.1 (Lifetechnologies), with the PCR amplification primers. For the entire ORF amplicons, sequencing reactions were performed on at least two clones (pGEM-T vector derivatives) using primers annealing in the plasmid, following the manufacturers' instructions.

Sequences were submitted to NCBI GenBank with accession numbers: KJ535112–116; KJ535119–123; KP698920; KP965762; KP698919; KP965762–64.

Sequence editing, assembly and multiple sequence alignments were performed using Bionumerics software packages v6.0 (Applied Maths, Kortrijk, Belgium). Phylogenetic analyses were carried out using the neighbor-joining (NJ) with the parameters suggested by the model test, using the Molecular Evolutionary Genetic Analysis (MEGA 6) software (Tamura et al., 2013). Confidence values at the nodes were obtained by performing 1000 bootstrap analyses.

Mean non-synonymous (*dN*) and synonymous (*dS*) substitutions per site (ratio, *dN*/*dS*) were calculated for each gene data set using the SLAC method within the DataMonkey web server (http://www.datamonkey.org) (Pond and Frost, 2005).

## 3. Results

HEV genome was detected in 11 of 15 swine fecal samples investigated. Eight samples were positive by all PCRs, 3 samples were positive by at least one PCR, and 4 samples were negative by all nested-RT-PCR performed. Six strains that yielded a sufficient amount of amplified DNA were subjected to sequencing and phylogenetic analysis.

The sequences obtained were compared with sequences from the NCBI database corresponding to HEV strains originating from humans, swine or other animals worldwide, belonging to the four known mammalian genotypes. Regardless of the genomic regions analyzed, the Italian swine HEV strains clustered tightly with several genotype 3 strains. Analyses of the 350 bp ORF2 fragments showed nucleotide identity of 85.2–100% between the swine Italian strains, which form two separate clusters corresponding to the