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SHORT REPORT
Hepatitis E virus genotype 4 in a pig farm, Italy, 2013

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SUMMARY

Zoonotic strains of hepatitis E virus (HEV) in Europe have been reported to belong to genotypes 3 and 4. In 2012 and 2013, 57 pig farms in Northern Italy that had previously resulted seropositive for HEV were surveyed for the presence of the virus, with positive samples subsequently genotyped. Hepatitis E RNA was identified in 17/57 (29·8%) seropositive farms. Phylogenetic analysis demonstrated that distinct subtypes of genotype 3 were circulating in the north-east of Italy; as well, for the first time in the Italian swine population, genotype 4 was identified and attributed to subtype d.

Key words: Genotype 4, hepatitis E, Italy, pig farm.

Hepatitis E virus (HEV) is an RNA virus of the genus *Hepevirus*, which includes four major genotypes [1]. Genotypes 1 and 2 are restricted to humans and associated with epidemics in developing countries, while genotypes 3 and 4 can cause acute liver diseases in humans and have their main reservoir in pigs and wild boars [1, 2]. In Europe, most infections in humans are attributable to genotype 3, as the geographical distribution of genotype 4 is considered limited to Asian countries [1]. However, European outbreaks of genotype 4 in people have been recorded in France [3], Germany [4] and Italy [5], none of which were attributable to travels to endemic regions. On the other hand, in the European swine population the only detection of genotype 4 had occurred on some pig farms in Belgium [6]. A sequence similarity of over 96% was observed between the HEV isolates from the human cases registered in France and the strain isolated from swine in Belgium, which was classified as subtype b [6]. Conversely, the human infection with HEV genotype 4 reported in Germany was attributed to subtype f [4]. The strain involved in the Italian outbreak showed a relatively low genetic similarity with any of the above mentioned strains, which suggests that different HEV genotype 4 strains may have recently been introduced in Europe [5].

In 2012 and 2013, 57/115 pig farms in northern Italy which had previously resulted seropositive for HEV were randomly recruited for laboratory investigations aimed at detecting the virus to provide updated information on the prevalence and genetic characteristic of HEV in Italian pig herds. Serological analyses had been performed by means of an ELISA commercial kit for HEV antibodies (ID Screen Hepatitis E indirect multi-species; ID-Vet, France) in 175 piggeries homogeneously distributed in northern Italy. Due to the increased likelihood of viral excretion in stools [7], 10 faecal pools from 10 different pens housing animals aged between 80 and 120 days were collected in each farm. After a pretreatment step, necessary to reduce the possible inhibition factors and to increase the

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sensitivity of the assay, the viral RNA was extracted from faecal samples using the High Pure Viral RNA kit (Roche, Germany). Briefly, faecal material thawed at 4 °C was diluted 1:5 in PBS (pH 7·4), shaken vigorously and incubated overnight at 4 °C. The next day, stool samples were centrifuged at 16000 ***g*** for 5 min to recover viral suspensions in the supernatants. Two hundred microlitres of supernatant were used for HEV RNA extraction using the High Pure Viral RNA kit (Roche), according to the manufacturer's recommendations in a final elution volume of 50 μl. The protocol was modified by adding 5 μl of *in vitro* transcribed internal control RNA (2×10^5 copies/μl) (In-type IC-RNA, Qiagen, USA) to each sample, to control the efficiency of RNA isolation and reverse transcription–polymerase chain reaction (RT–PCR), as described by Hoffmann *et al*. [8]. Recovered RNA was frozen at −70 °C until used.

Primers and a probe targeting a 70-bp-long ORF3 fragment of viral RNA have already been described by Jothikumar *et al.* [9], while primers and a probe for EGFP internal control RNA have been published by Hoffmann *et al.* [8]. In this study, a modified duplex rRT–PCR was performed on the Applied Biosystems 7900HT Fast Real-Time PCR instrument and was carried out and optimized using the AgPath-ID™ One-Step RT–PCR kit (Life Technologies, USA), in a total reaction volume of 25 μl.

The positive pooled samples were further analysed by a nested RT–PCR amplifying a 458 bp fragment of the ORF-2 encoding the constitutive protein of the capsid [10]. One positive sample was sequenced for each farm and sequences were generated using the BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, USA). The products of the sequencing reactions were cleaned-up using the PERFORMA DTR Ultra 96-well kit (Edge BioSystems, USA) and sequenced in a 16-capillary ABI PRISM 3130xl genetic analyzer (Applied Biosystems). Sequence data were assembled and edited with SeqScape software v. 2.5 (Applied Biosystems). ORF2 sequences were aligned and compared with the HEV sequences available in GenBank. Nucleotide sequence alignments were manually constructed for each gene fragment using the Se-Al program. To infer the evolutionary relationships, we employed the maximum likelihood (ML) method available in the PhyML program, incorporating a GTR model of nucleotide substitution with a gamma-distributed rate variation among sites procedure. A bootstrap resampling process was employed to assess the robustness of the individual phylogenetic nodes. Viral RNA was detected in at least one faecal pool in 17/57 (29·8%) farms. Sequence data obtained were deposited in GenBank (accession nos. KF939859–KF939868) and genetic typing of generated sequences indicated that 16/17 samples were phylogenetically grouped within genotype 3 [10], most commonly found in Italian pig farms [7]. According to the classification proposed by Lu *et al.* [11] 11 positive samples grouped in clusters 3e ($n=6$); 3f ($n=8$); 3 h ($n=1$) (Fig. 1). For 15/17 sequences, the highest values of similarity were found with viruses previously identified in the European swine and human population. In particular, sequences belonging to the subtype 3f presented a percentage of similarity $\leqslant$94% with European swine and human viruses available in GenBank. Within genotype 3e, a percentage of similarity of 99% was found between the Sw/ITA_2012_13rs366/1 sample and a virus identified in a human case in Italy (HM446628), while similarity values lower than 91% were shown between the remaining five sequences belonging to the 3e subtype and the European 3e genotype viruses available in GenBank. The Sw/ITA_2013_13rs985/5 virus clustered within the 3 h subtype and presented the highest similarity (91%) with a virus identified in pigs in France. One sample, Sw/ITA_2012_13rs366/25, obtained in September 2012 from a farrow-to-finish farm housing around 2000 animals in the province of Treviso (Veneto Region), fell into genotype 3 but on a separate branch of the phylogenetic tree. With less than 83% similarity to the ORF2 gene of previously described genotype 3 viruses and based on the high bootstrap value (100%), as suggested by Lu *et al.* [11], this virus could possibly represent a new genotype 3 subtype. However, since classification of HEV is currently in transition and questioned [12, 13], further sequence data will be essential to confirm subtyping of this variant. In this farm the pens had slatted floors, nipple drinkers and a trough for pelleted diet. The average mortality declared by the farmer was around 2–3%, within the expected range for this productive sector. Cleaning and disinfection procedures were performed with a high-pressure cold water jet and Virkon® (Dupont, USA). The manure was used as fertilizer on the soil surface after 6 months' storage.

Interestingly, an HEV isolate from another farm sampled in June 2013 was phylogenetically characterized as genotype 4 (subtype 4d) and showed 100% sequence similarity to the strain Hu/ITA_2011_E2104 (JX401928) obtained from a human HEV infection registered in central Italy. An additional RT–PCR

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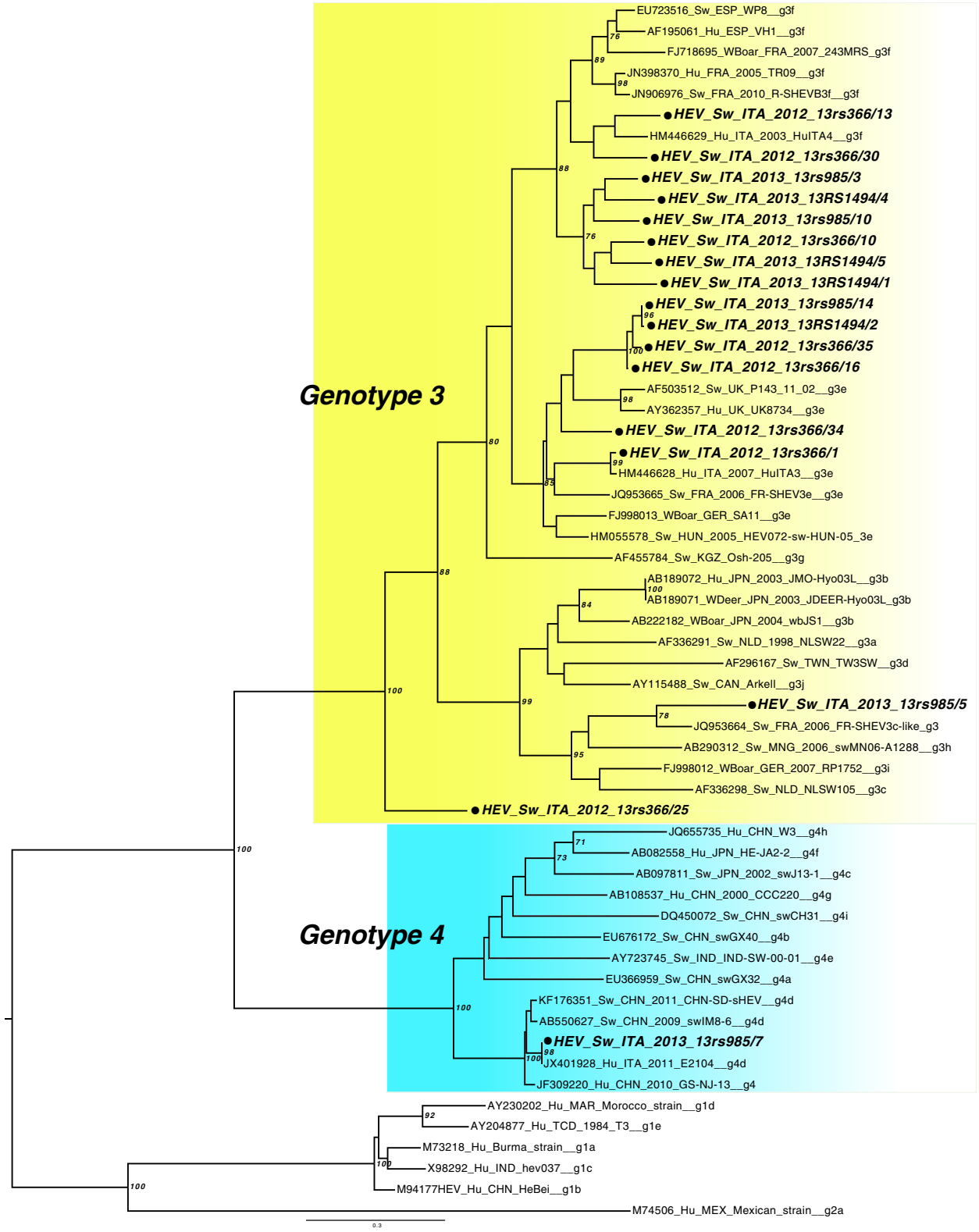

Fig. 1. Maximum-likelihood phylogenetic tree of the ORF2 region (450 bp) of HEV viruses identified in northern Italy. Viruses described in this study are indicated by a black dot. The numbers at nodes represent bootstrap values (>70%).

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and sequencing reaction targeting a 350 bp fragment of the ORF1, encoding the replication protein complex (primers available on request), was applied to this sample to confirm its classification as genotype 4. Phylogenetic analysis of the partial ORF1 sequence obtained (accession no. KF939857) indicates that this HEV variant is closely related to genotype 4d viruses (similarity between 96·5% and 98%), thus confirming the clustering obtained for the ORF2 region (Supplementary Fig. S1). This represents the first identification of HEV genotype 4 in the Italian swine population. The genotype 4-positive farm was a small-sized fattening farm (housing around 600 animals) with continuous-flow in the province of Vicenza (Veneto Region). The average mortality declared by the farmer was around 3%. The pens had solid floors, nipple drinkers and a trough for pelleted diet. The manure was daily removed by means of cold water and drainpipes. Cleaning and disinfection procedures were performed with a high-pressure cold water jet and Delegol NF® (Bayer, Germany). The manure was used as fertilizer on the soil surface after 6 months' storage. All the animals were supplied at age 11 weeks by a single farm situated in the same region (in the province of Verona). The farmer declared he was the only worker involved in animal management and that no foreign personnel had ever been employed. Moreover, he reported not to have been abroad in recent times, nor to have ever travelled to Asia. The other people with access to the farm were veterinarians, truck drivers, and private customers who had directly purchased one or a few pigs for backyard production.

The epidemiological investigation did not allow for the virus introduced into the swine population to be traced back to its origins. The supplier piggery resulted seronegative (i.e. the sampling had been carried out assuming an intra-herd prevalence higher than 30% with a 95% confidence interval). Oro-faecal transmission can be hypothesized by means of epidemiological contacts between animals, in case batches from different farms had been transported together, or vehicles not cleaned and disinfected properly. However, contamination from humans entering the farm cannot be excluded. In either case the continuous flow of the farm and the inefficacy of the disinfection procedures may have contributed to the maintenance of the infection within herds of different fattening cycles. Transmission from wild animals is considered very unlikely, as the premises were well fenced and functioned as a fully indoor operation farm. The detection of a genotype 4 virus with a close phylogenetic relation to a recent human strain with no links to imported food or people travelling from endemic areas [5], indicates that HEV4 is likely circulating in Italian pig herds and poses possible risks of transmission to humans. HEV4 infections have been increasing in frequency in Europe over the last decade and have been reported to cause a more severe clinical course in humans [6]. Combining these facts with the increasing genetic diversity of swine HEV isolates reported herein, the implementation of appropriate surveillance programmes aimed at HEV characterization, as well as epidemiological studies to outline the main risk factors for virus spread in swine and human populations will be of great importance in the coming years. Moreover, populations of wild animals (wild boars and deer) should also be monitored in view of their possible role as reservoir [2]. Collectively, these actions should enhance preparedness for emergent genotypes in the animal reservoirs and contribute to reduce the risks of interspecies transmission of HEV.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268814001150.

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