



Short communication

Reduced prevalence of genotype 3 HEV in Shanghai pig farms and hypothetical homeostasis of porcine HEV reservoir

Zhen Li^{a,c,1,*}, Shuisheng Yu^{a,c,1}, Shijuan Dong^{a,c}, Yumin Zhu^{a,c}, Fusheng Si^{a,b}, Shiyuan Shen^{a,c}, Zhongqi Jiang^d, Ruisong Yu^{a,c}, Sixiang Zou^b

^a Institute of Animal Science and Veterinary Medicine, Shanghai Academy of Agricultural Sciences, 2901 Beidi Road, Shanghai 201106, China

^b College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

^c Shanghai Key Laboratory of Agricultural Genetics and Breeding, Shanghai 201106, China

^d College of Animal Sciences, Zhejiang University, Hangzhou 310029, China

1. Introduction

Hepatitis E virus (HEV) is the principal cause of non-A, non-B hepatitis infections transmitted through the oral-fecal route. Hepatitis E outbreaks can be sporadic or epidemic and are normally cause for serious public health concerns in developing countries where poor sanitary conditions often lead to contamination of drinking water supplies (Krawczynski, 1993). Recent studies have documented the occurrence of HEV-associated hepatitis among

individuals in developed countries with no travel history to HEV-endemic areas (Ijaz et al., 2005). Currently, four major phylogenetically related HEV groups or genotypes are

regarded as a zoonotic pathogen since it has been isolated from pigs and several other animals including chickens, deer and wild boar (Meng et al., 1997; Haqshenas et al., 2001; Tei et al., 2003; Tamada et al., 2004). In fact, the zoonotic distribution of the four HEV genotypes is quite different. Genotype 1 is found associated mainly with human infections although a strain of HEV belonging to this genotype was detected recently on a farm in Cambodia (Caron et al., 2006). So far, there have been no reports of HEV genotype 2 involvement in animal infections, and most animal HEV isolates belong to genotypes 3 and 4 (Meng et al., 1997; Lu et al., 2006). Swine are believed to be the principal reservoir of these latter two genotypes because of their wide distribution, high infection rates and close association with humans (Meng, 2000; Zheng et al., 2006).

In our previous study involving Shanghai pig farms, 48.6% (18/37) of the farms investigated and 16.2% (69/426) of the fecal samples tested were contaminated with HEV genotype 3 (Ning et al., 2008). This is surprising considering that HEV genotype 3 was most probably only recently imported to the area. During 2006/2007, the Chinese pig farming industry was severely affected by a febrile disease epidemic ('pig high fever' disease), the suspected cause of which was a mutated form of porcine reproductive and respiratory syndrome virus (PRRSV) (Li et al., 2007). Thereafter, stricter sanitary measures were introduced in the pig farms. Since such procedures usually bring about changes in the local microecology, we initiated this investigation in mid-2007 with the aim of determining if the prevalent states of genotype 3 and the native HEV genotype 4 had been affected.

2. Materials and methods

2.1. Sampling and RNA extraction

A total of 493 fresh fecal samples were collected from 39 middle- or large-scale pig farms (200–2000 sows each) located in ten Shanghai suburban districts and tested for the presence of HEV RNA. The farms included 37 facilities that were included in our previous investigation (Ning et al., 2008). Fecal samples were collected from pens housing pigs aged 2–5 months, and one or two samples were collected from each pen or building. The pigs showed no obvious symptoms of ill-health at the time of sampling.

2.2. RT-PCR

2.2.1. Viral RNA extraction and first cDNA strand synthesis

Viral RNA was extracted from 100 µl of fecal suspension using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized using the SuperScript™ III First-strand Synthesis System (Cat. No.: 18080-051, Invitrogen).

2.2.2. HEV RNA detection

HEV RNA was detected by nested RT-PCR using the first strand cDNA (see Section 2.2.1) as the template and the degenerate primers described in Ning et al. (2007). As

expected, the size of the amplified HEV ORF2 fragment was 150 bp.

The positive PCR product of 150 bp was sequenced for the genotype analysis. The corresponding genotype 4 positive sample was then checked with Section 2.2.3 method to detect genotype 3 HEV co-infection. The corresponding genotype 3 positive sample was checked with Section 2.2.4 method to detect genotype 4 HEV co-infection.

2.2.3. Detection of HEV genotype 3 RNA

Detection of genotype 3 HEV was carried out using the first strand cDNA synthesized in Section 2.2.1 as a template and PCR primers specifically targeted to 164 bp ORF2 segment of the genome of genotype 3 HEV. Primers HE1 (5'-CAAATTGAAGGTGATTACCGC-3') and HE2 (5'-CCTAGGGCGCATGGTGTTC-3') were used for the first round of PCR, and primers HE3 (5'-CACTGCCCGATGTGGTGCCT-3') and HE4 (5'-GGCTAACTCCATAGACACGG-3') were used for the second round of PCR. Amplification was carried out in 12.5 µl reaction volumes containing 2.5 µl template, 0.5 µl (each) of primer, 6.25 µl 2× Taq PCR MasterMix (Tiangen Biotech Co., Ltd.) and 2.75 µl sterile water. Amplification conditions were: 36 cycles of 94 °C for 40 s, 53 °C for 40 s and 72 °C for 60 s (first round PCR), and 30 cycles of 94 °C for 40 s, 53 °C for 40 s and 72 °C for 40 s (second round PCR).

2.2.4. Detection of HEV genotype 4 RNA

Detection of genotype 4 HEV was conducted using the first strand cDNA synthesized in Section 2.2.1 as a template and PCR primers specifically targeted to 440 bp ORF1-ORF2 segment of the genome of genotype 4 HEV. Primers HE5 (5'-ACTGATGTCCGSATYCTTGT-3') and HE6 (5'-CCTGCTGAGCATTCTCGACTG-3') were used for the first round of PCR, and primers HE7 (5'-GTGATYCTAGY-GAGCGCTG-3') and HE8 (5'-GTCGGCTCGCCATTGGCTG-3') (Y = C or T/U) were used for the second round of PCR. Amplification was carried out in 12.5 µl reaction volumes as described in Section 2.2.3. Amplification conditions were: 36 cycles of 94 °C for 50 s, 53 °C for 50 s and 72 °C for 80 s (first round PCR), and 32 cycles of 94 °C for 50 s, 57 °C for 40 s and 72 °C for 60 s (second round PCR).

2.2.5. Negative and positive controls

Negative (water) and positive (HEV RNA) controls were included to exclude the possibility of contamination. Positives were scored when no false positives were obtained with the negative controls and when HEV RNA was detected in the positive controls. The HEV RNA sample for the positive control was obtained and confirmed as described in Ning et al. (2008).

2.3. Nucleotide sequencing

Amplified fragments were separated by 1% (w/v) agarose gel electrophoresis, excised and purified with a gel extraction kit (Invitrogen). Both strands of the positive PCR products were sequenced for phylogenetic analysis using a 3730 DNA Analyzer (Applied Biosystems).

Table 1
Prevalence and genotypic analysis of HEV RNA in pig fecal samples.

| Farm | No. of fecal samples | No. of positive HEV RNA samples | Genotype | District | Isolated strain |
|------|----------------------|---------------------------------|--------------|------------|--|
| 1 | 12 | 2 | 4 | Min Hang | SAAS-MHZX1 |
| 2 | 12 | 8 | 4 | Min Hang | SAAS-MHZF2 |
| 3 | 12 | 4 | 4 | Min Hang | SAAS-MHZN5 |
| 4 | 12 | 0 | – | Min Hang | – |
| 5 | 18 | 0 | – | Nan Hui | – |
| 6 | 12 | 3 | 4 | Nan Hui | SAAS-XX2 |
| 7 | 18 | 3 | 4 | Nan Hui | SAAS-XC31 |
| 8 | 18 | 5 | 4 | Nan Hui | SAAS-WX27 |
| 9 | 12 | 4 | 4 | Jin Shan | SAAS-JSSL5 |
| 10 | 12 | 5 | 4 | Jin Shan | SAAS-JSTL4 |
| 11 | 12 | 3 | 4 | Jin Shan | SAAS-JSZH4 |
| 12 | 12 | 2 | 4 | Jin Shan | SAAS-JSQY2 |
| 13 | 12 | 3 | 4 | Song Jiang | SAAS-SJZS1 |
| 14 | 12 | 4 | 3(1) 4(3) | Song Jiang | SAAS-SJR3 SAAS-SJR9 SAAS3-3 ^a SAAS3-4 ^a |
| 15 | 12 | 2 | 4 | Song Jiang | SAAS-SJWG1 |
| 16 | 12 | 0 | – | Song Jiang | – |
| 17 | 12 | 0 | – | Song Jiang | – |
| 18 | 12 | 2 | 4 | Song Jiang | – |
| 19 | 6 | 4 | 4 | Bao Shan | SAAS-BS4 |
| 20 | 12 | 2 | 4 | Bao Shan | SAAS-BSLZ1 |
| 21 | 12 | 6 | 4 | Bao Shan | SAAS-BSSY3 |
| 22 | 12 | 0 | – | Bao Shan | – |
| 23 | 12 | 1 | 4 | Qing Pu | SAAS-QPM3 |
| 24 | 12 | 2 | 4 | Qing Pu | SAAS-QPT1 |
| 25 | 12 | 5 | 4 | Qing Pu | SAAS-QPFX1 |
| 26 | 12 | 3 | 3 | Qing Pu | SAAS-QPXP1 |
| 27 | 12 | 2 | 4 | Chong Ming | – |
| 28 | 12 | 0 | – | Chong Ming | – |
| 29 | 12 | 3 | 4 | Chong Ming | SAAS-CMYZ3 |
| 30 | 12 | 2 | 4 | Chong Ming | – |
| 31 | 12 | 0 | – | Chong Ming | – |
| 32 | 12 | 2 | 4 | Jia Ding | SAAS-XQ1 |
| 33 | 12 | 5 | 4 | Jia Ding | SAAS-MS3 |
| 34 | 12 | 3 | 3 | Jia Ding | SAAS-JDY5 |
| 35 | 19 | 4 | 4 | Feng Xian | SAAS-FX3 |
| 36 | 18 | 3 | 3(1) 4(2) | Feng Xian | SAAS-FX8 SAAS-FX6 |
| 37 | 12 | 5 | 4 | Pu Dong | SAAS-PDRL1 |
| 38 | 12 | 6 | 4 | Pu Dong | SAAS-PDQM2 |
| 39 | 12 | 2 | 4 | Pu Dong | SAAS-PDDF2 |
| | 493 | 110 (22.2) | | | |

^a Fecal sample in which both HEV genotypes 3 and 4 were identified. The samples were included when the overall prevalence of HEV RNA was calculated, but omitted when the incidence of specific genotypes was determined. Figures in parentheses in the fourth column represent the numbers of each sample.

2.4. Phylogenetic analysis of HEV genotypes

Sequence alignments were generated by CLUSTAL-W (version 1.8). Genetic distances between pairs of viral isolates were calculated with the MEGA software (Version 4.0) using the Kimura 2-parameter method. Percent identity was calculated with Lasergene (version 5.03; DNASTar), and phylogenetic trees were constructed by the neighbor-joining method. Bootstrap values were determined on 1000 re-samplings of the data sets. Geographic origins of the HEV strains used in the phylogenetic and sequence analysis were as follows. Genotype 1: B1 (Burma); B2 (Burma); pSK-HEV-2 (Pakistan); Yam-67 (India); P2 (Pakistan); Ind1 (India);

C3 (Nepal); I2 (India); I3 (India); C4 (Nepal); MOR (Morocco); CH-HB (China); TS (Chad); TK15/92 (Nepal). Genotype 2: M1 (Mexico). Genotype 3: Arkell (Canada); US-1 (USA); US-2 (USA); US-SW (USA); JRA1 (Japan); SWJ12-4 (Japan); SWJ8-5 (Japan); wbJYG1 (Japan); JSO-Hyo03L (Japan); wbJSG1 (Japan); JBOAR1-Hyo04 (Japan); JMNG-Okio2C (Japan); HEVN1 (Japan); Kyrg (Kyrgyzstan); Genotype 4: SwJ13-1 (Japan); JAK-Sai (Japan); CCC220 (China); swCH25 (China); HE-JK4 (Japan); JSN-Sap-FH (Japan); JKK-Sap (Japan); HE-JI4 (Japan); JYI-ChiSai01C (Japan); T1 (China); IND-SW-00-01 (India); SH-SW-zs1 (China); swCH31 (China); T21 (China). Sub-type analysis was undertaken using the method of Lu et al. (2006).

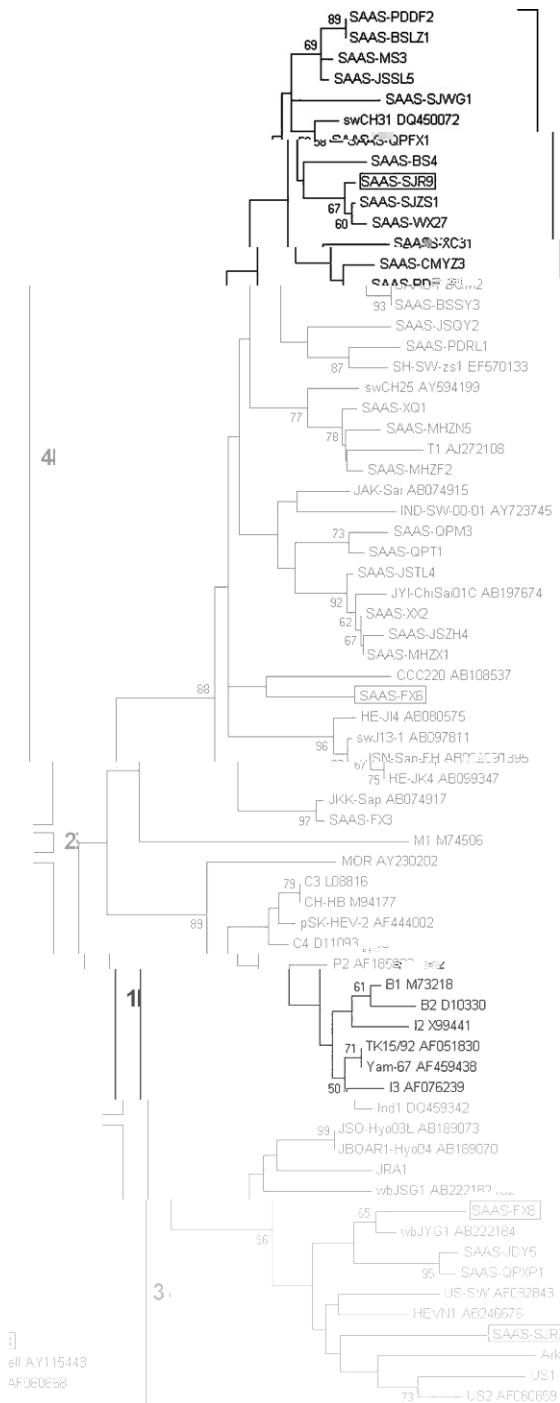


Fig. 1. Phylogenetic tree based on 150 bp ORF2 fragments. The tree was constructed using the neighbor-joining method and the bootstrap values (expressed as percentages) were determined on 1000 re-samplings of the data sets (only bootstrap values >50 are shown). The tree includes 37 animal and human HEV reference isolates and the 31 isolates from the present investigation. Boxed strains are the isolates from farms 14 and 36 that were contaminated by HEV genotypes 3 and 4.

3. Results

3.1. Prevalence of HEV in pig farms located in Shanghai suburbs and genotype analysis

One hundred and eleven (22.3%) of the 493 fecal samples and 32 (82.0%) of the 39 farms investigated were HEV RNA positive (Table 1). Phylogenetic alignment of the amplified sequences with 37 reference sequences showed that all the isolates belonged to either HEV genotype 3 or genotype 4 (Fig. 1). Two farms were genotype 3 positive, 28 farms were genotype 4 positive, while farms 14 and 36 were found to be contaminated with both HEV genotypes. The incidence of HEV genotypes 3 and 4 relative to the total number of fecal samples tested was 1.6% and 20.6%, respectively (Table 1).

The nucleotide sequences of the four HEV genotype 3 strains isolated from mono-infected (i.e. only one genotype present) fecal samples were 94.0–95.7% homologous with the corresponding sequence of the Japanese isolate WbJYG1 belonging to sub-type 3b. The HEV genotype 4 isolates were assignable to three sub-groups: 7 were 93.3–98.7% homologous with the Japanese isolate JYI-ChiSai01C belonging to sub-type 4c, 19 shared the same phylogenetic branch with Chinese isolates swCH31, T1 and SH-SW-zs1 belonging to sub-type 4d, and strain SAAS-FX6 was 93.7% homologous with Chinese isolate CCC220 belonging to sub-type 4g.

3.2. Identification of HEV genotypes 3 and 4 in the same (co-infected) fecal sample

One fecal sample, which amplification with universal PCR primers and sequencing had initially scored positive for HEV genotype 4, was also found to be positive for HEV genotype 3 when amplification was carried out using primers specific for this genotype. The nucleotide sequence of the HEV genotype 4 strain in the sample (designated SAAS3-4) was 94.5% homologous with that of sub-type 4a strain T21 of Chinese origin (Fig. 2). The nucleotide sequence of the HEV genotype 3 in the sample (designated SAAS3-3) was 95.7% homologous with that of sub-type 3a strain US2 of United States origin (Fig. 3).

4. Discussion

Comparison of the data from this present study with results from our previous investigation (Ning et al., 2008) reveal that, while the overall occurrence of HEV RNA positives decreased by only 3.7%, the incidence of HEV genotype 3 decreased from 16.2% to 1.6% whereas the incidence of HEV genotype 4 increased from 9.8% to 20.6%. Therefore, although there were considerable fluctuations in the frequency of the two individual genotypes, the overall incidence of HEV remained relatively stable. Since essentially the same procedures were adopted in both studies, it is unlikely that the observed fluctuations in the relative frequencies of genotypes 3 and 4 were due to differences in the sensitivity of the methods used to detect the respective genotypes. One possible explanation for the lower incidence of HEV genotype 3 is the introduction of

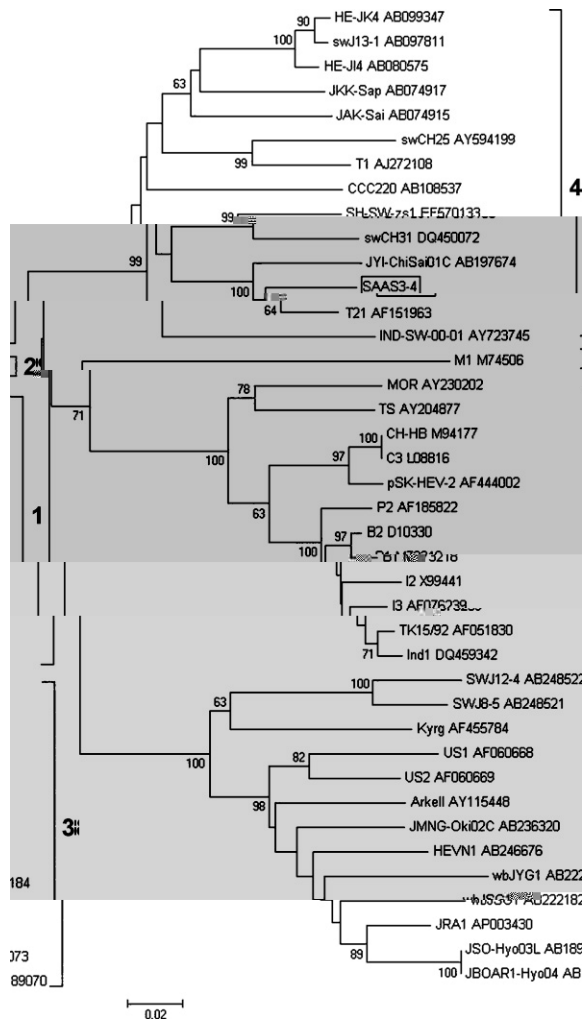


Fig. 2. Phylogenetic tree based on a 440 bp partial nucleotide sequence of the ORF2 region. The tree was constructed by the neighbor-joining method and the bootstrap values (expressed as percentages) were determined on 1000 re-samplings of the data sets (only bootstrap values >50 are shown). The tree includes 39 animal and human HEV reference isolates and the SAAS3-4 strain isolated from the mixed-genotype sample collected from farm 14.

stricter sanitation measures following the completion of the earlier study. These included more frequent pen and environment sterilization, the use of antibiotics, stricter vaccination regimes, improved pig-raising conditions and adopting an all in/all out management system. While the origin of the HEV genotype 3 strains first reported in fecal samples from pig farms in the Shanghai suburbs in 2007 remains unclear, the ease of present-day travel and transportation has created conditions for the transmission of infectious diseases over huge distances. Therefore, these strains possibly represented new ‘immigrants’ to China that were less able to survive the unfavorable conditions resulting from the introduction of improved sanitation regimes compared with the better adapted ‘native’ HEV genotype 4 strains. Lower sensitivity to, and/or ability to recover more quickly from, environmental perturbations

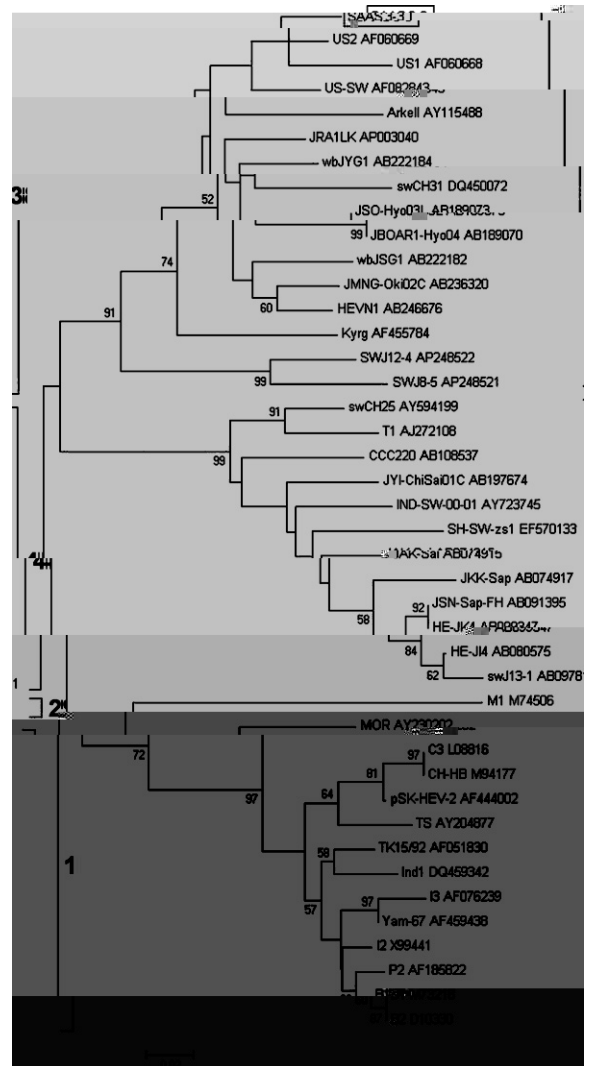


Fig. 3. Phylogenetic tree based on a 164 bp partial nucleotide sequence of the ORF1 region. The tree was constructed by the neighbor-joining method and the bootstrap values (expressed as percentages) were determined on 1000 re-samplings of the data sets (only bootstrap values >50 are shown). The tree includes 41 animal and human HEV reference isolates and the SAAS3-3 strain isolated from the mixed-genotype sample collected from farm 14.

would enable the latter to colonize those ecological niches previously occupied by the HEV genotype 3 strains. The similar overall HEV positive rates recorded in the two investigations indicated that there is a mechanism for maintaining homeostasis within the HEV reservoir. A similar explanation for viral stability in individuals, the underlying mechanism of which correlates with quasispecies expansion and immune escape, has been proposed for hepatitis C, hepatitis B and human immune deficiency viruses (Sallie, 2004, 2005).

In our previous report (Ning et al., 2008), both genotypes 3 and 4 were detected in fecal samples taken from farm 36 located in Feng Xian District. This was confirmed in the present study, and both genotypes were also detected in fecal samples taken from farm 14 located

in Song Jiang District. In an earlier Japanese study, 127 strains of HEV genotype 3 and 10 strains of HEV genotype 4 were isolated from 25 pig farms, but no co-contamination of the same pig farm with the two different genotypes was reported (Takahashi et al., 2003). Where a single farm is co-contaminated with two HEV genotypes, the possibility exists of animals on that farm becoming infected with both genotypes at the same time. This proved to be the case when a fecal sample taken from one of the co-contaminated farms tested positive for both HEV genotypes 3 and 4. Although genotypes 3 and 4 co-infections have been reported in humans (Takahashi et al., 2002), to our knowledge this is the first report of such an occurrence in pigs.

The nucleotide sequences of HEV genotype 3 strains identified in the present study exhibited highest homology with either strain US2 (sub-type 3a) or strain WbjYG1 (sub-type 3b), first reported in the US and Japan, respectively (Erker et al., 1999; Nishizawa et al., 2005). The possibility that genotype 3 strains identified in Shanghai pig farms may have originated from two different geographical regions would explain why the SAAS-SJR3 and SAAS3-3 strains isolated from the same farm belonged to different sub-types. However, it should be noted that, in the investigation, different segments of the two genomes were amplified for alignment with reference strain sequences; i.e. a 164 bp segment in the ORF1 region of the SAAS3-3 genome and a 150 bp segment in the ORF2 region of the SAAS-SJR3 genome. Since intra-genotype recombination may occur among HEV strains (van Cuyck et al., 2005), there is a possibility that a single HEV strain might be assigned to different sub-types when different segments of the genome are used for alignment.

Shanghai HEV genotype 4 strains were relatively diverse. Most genotype 4 strains isolated in this study exhibited highest homology with Chinese isolates belonging to sub-types 4c, 4d and 4g. However, 7 strains belonging to sub-type 4c displayed highest homology with strain JYI-ChiSaio1c that was originally isolated from Japanese patients who had travelled to China.

It is clear that HEV genotypes 1, 3 and 4 are all prevalent in China (Wang et al., 1999; Wei et al., 2006; Ning et al., 2007). Prior to the discovery of genotype 4, HEV genotype 1 was assumed to be dominant (Wei et al., 2006). However, at present, most cases of non-A, non-B hepatitis are caused by HEV genotype 4, suggesting this has now become the most important HEV genotype (Wang, 2003; Zheng et al., 2006). The coexistence of different HEV genotypes within the same fecal sample implies the possibility of complex infection scenarios in Shanghai pig farms and the potential for more virulent HEV strains to develop as a result of genetic recombination and species evolution.

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