



RESEARCH ARTICLE



Cross-sectional Seroprevalence and Genotype of Hepatitis E Virus in Humans and Swine in a High-density Pig-farming Area in Central China

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Abstract

Hepatitis E virus (HEV) infection is a common public health problem in developing countries. However, the current prevalence of HEV and the relationship of HEV genotype between swine and human within high-density pig-farming areas in central China are still inadequately understood. Here, cross-sectional serological and genotypic surveys of HEV among the 1232 general population, 273 workers occupationally exposed to swine, and 276 pigs in a high-density pig-breeding area, were undertaken by ELISA and nested RT-PCR methods. Anti-HEV IgG was detected in 26.22% of general population and 48.35% of occupational workers. The prevalence of swine serum HEV-Ag was 6.52%. The prevalence of anti-HEV IgG was significantly higher among the workers occupationally exposed to swine than among the general population. An increased HEV seropositivity risk among the general population was associated with either being a peasant or male and was very strongly associated with the increase of age. Among the occupationally exposed group, the prevalence of anti-HEV IgG antibodies increased with age and working years. Among the 30 HEV-IgM-positive people, the infection rates of clerks in the public, peasants, pork retailers, and pig farmers were higher than those of others. A phylogenetic analysis revealed that all the isolates belonged to subgenotype 4_a, and four people and four pigs shared 97.04%–100% sequence homology. This study revealed a high HEV seroprevalence among the general population and workers occupationally exposed to swine in the Anlu City, and supports the notion that swine are a source of human HEV infection.

Keywords Hepatitis E virus (HEV) · Seroepidemiological study · Zoonosis · Cross-sectional study · Genotype

Yilin Shu, Yameng Chen and Sheng Zhou authors contributed equally to this work.

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Introduction

Hepatitis E virus (HEV) is one of the commonest global causes of hepatitis (Aggarwal and Naik 2009). Seven closely related HEV genotypes (HEV-1–7) have been identified, but only four major HEV genotypes cause human

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infection, each of which has different epidemiological features (Woo *et al.* 2016). HEV genotype 1 had been isolated in Asia and Africa and genotype 2 was originally identified in Mexican and some African isolates (Kamar *et al.* 2012). Genotype 3 was initially isolated from human patients in the United States and has since been detected on most continents, including Europe (Vina-Rodriguez *et al.* 2015; Adlhoch *et al.* 2016; Mesquita *et al.* 2016; Rosa *et al.* 2016; Matos *et al.* 2018). Genotype 4 includes strains from sporadic human cases in Asia and more recently in Europe (Meng 2010; Hakze-van der Honing *et al.* 2011; Colson *et al.* 2016; Jeong *et al.* 2017; Wu *et al.* 2017a, b). Recently, a novel HEV genotype 7 was isolated from a liver transplant recipient with chronic HEV infection in the Middle East (Lee *et al.* 2016). Hepatitis A and hepatitis E viruses are the two main hepatitis etiological factors causing enterically transmitted infections in developing countries and among indigenous people in developed countries (Caruso *et al.* 2016). The mortality rate of HEV, which is usually low (1%–2%) in the general population, can be as high as 10%–25% in pregnant women, and over 75% of liver disease patients lose their livers as a result of HEV infection (Marano *et al.* 2015; Bazerbachi *et al.* 2016). A World Health Organization (WHO) report estimated that 20 million people are infected with HEV worldwide, 3.3 million of whom are symptomatic, and that ~ 56,600 people die each year from HEV-related liver failure (WHO 2016). The possibility that it is a zoonotic disease is supported by a large body of evidences, including prevalence reports of HEV RNA detected in pigs, wild boars, rodents, and deer (Kamar *et al.* 2012; Song *et al.* 2014). From the human perspective, current evidence supports a zoonotic origin of HEV infections, with a statistically significant association identified between occupational exposure to pigs and anti-HEV immunoglobulin G (IgG) levels (Wilhelm *et al.* 2014). Moreover, genotyping a section of open reading frame 2 (ORF2) has shown both homology and sequence identities ranging from 70% to 100% between human- and animal-derived isolates (Wilhelm *et al.* 2011). With the exception of sporadic cases of HEV infection, which are predominantly caused by HEV genotype 4, large outbreaks rarely occur in China. However, the prevalence of HEV infection is high in China and it is suspected to be zoonotically transmitted from wild boars, rabbits, and domestic pigs, and pigs are thought to be a major repository of genotypes 3 and 4 (Liu *et al.* 2012; Dai *et al.* 2013; Wang and Wang 2016; Wu *et al.* 2017a, b). A serological survey showed that HEV infection in pigs is very common in different regions of China, with positivity rates ranging from 30% to 100% (Ding *et al.* 2018; Gong *et al.* 2018; Shuai *et al.* 2009; Huang *et al.* 2012; Zhang *et al.* 2010). In fact, a high seroprevalence of HEV among swine farmers and swine veterinarians has been reported in

previous studies (Kang *et al.* 2016; Bansal *et al.* 2017; Lange *et al.* 2017; Mughini-Gras *et al.* 2017; Teixeira *et al.* 2017; Ukuli and Mugimba 2017). Residence in areas with high rates of swine contact and sharing drinking-water resources with swine have been significantly associated with HEV infection, and zoonotic spread is a major mode of HEV genotypes 4 transmission from pigs to humans in eastern China (Khuroo and Khuroo 2016).

In townships and rural areas of central China, the major pork production and the local meat economy depend on a swine breed, which is defined by the local Food and Drug Administration as an autochthonous small-sized breed of Landrace and Large White pigs. In the previous study, we investigated the acute HEV infections in commercial pigs in Anlu city of Hubei Province of China and found a high anti-HEV IgM prevalence of 46.7% in towns and an average anti-HEV prevalence of 37.9% (Zhang *et al.* 2016, 2017). Following this, we used seroprevalence and HEV RNA detection to assess the presence of HEV and its genotypes in the general population, swine workers, and swine in Anlu city of Hubei Province of China.

Materials and Methods

Study Design for Human and Swine Sampling

Anlu city of Hubei province (31°0.250'N, 113°0.690'E) is a high-density pig-farming area in central China. Sera were randomly collected from 1505 human blood samples (1232 from the general population and 273 from occupationally swine-exposed workers) during February to August in 2016. The specific sampling locations are shown in Supplementary Figure S1. The 273 swine workers included 137 pig farmers, 104 pork retailers, 24 slaughterers, and 8 veterinarians. At the time of the study, all the participants were healthy and showed no signs or symptoms of hepatitis within the 6 months preceding the survey. To assess the human exposure risk, we collected data on the demographic characteristics, employment status, and liver disease history of all subjects using a questionnaire that we designed, after conducting advanced reliability and validity analyses. Informed consent was obtained from all participants.

Forty-seven pig-fattening farms from 15 villages and towns were sampled over a 7-months period (from February to August) in 2016 (Supplementary Figure S1). Blood samples were taken from the jugular veins of 276 pigs. The two blood samples were collected at the same time from each pig and used for HEV-Ag or HEV RNA detection. The samples were centrifuged at 1000 ×g for 20 min to separate the sera, which were then stored at – 80 °C before analysis.

Serological Analyses

Acute HEV was determined by the presence of anti-HEV IgM, and previous viral exposure was confirmed by anti-HEV IgG reactivity without anti-HEV IgM. The human serum samples were screened for HEV IgM and HEV IgG using anti-HEV IgM/IgG ELISA kit (Wantai Biopharmaceutical Co., Ltd, Beijing, China), according to the manufacturer's instructions. The swine serum samples were screened for HEV ORF2 antigen (HEV-Ag) using an HEV-Ag ELISA kit (Shanghai Meilian Biological Scientific Co. Ltd, Shanghai, China), according to the manufacturer's instructions.

HEV RNA Detection and Isolation of the Partial ORF2 Region

Total RNA was extracted from 140 μ L of serum collected from the human and swine, using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions, and then resuspended in 30 μ L of DNase/RNase-free water. The total RNA was used as the template for reverse transcription (RT)-PCR with the PrimeScript First-Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan). The 5' region of ORF2 provides a phylogenetic signal comparable to a full-genome analysis and was therefore used for a phylogenetic analysis (Lu *et al.* 2006). After nested RT-PCR was performed, the amplicons were sequenced. In brief, the HEV ORF2 domain sequences were amplified using RT products as the templates. The two pairs of primer sets were: 5'-CCCTTATCCTGCTGAGCATTCTC-3' (F) and 5'-AAATATGCMCAGTACCGGGTTG-3' (R) (first primer set), and 5'-GTYATGYTYTGCATACATGGCT-3' (F) and 5'-AGCCGACGAAATYAATTCTGTC-3' (R) (second primer set), with product lengths 731 bp and 438 bp, respectively. The PCRs were run on an EppendorfTM MastercyclerTM pro PCR System (Thermo Fisher Scientific, Inc., Hercules, CA, USA) under the following three-step cycling conditions: one initial template denaturation step at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 50 s; and a final elongation step at 72 °C for 7 min. The second primer set was used during the second round of PCR, using the same procedure. The amplification products were analyzed on a 1% agarose gel, and then purified with a PCR clean-up system (QIAquick PCR Purification Kit, cat no./ID: 28104, Qiagen). After purification, the amplicons were sequenced at Sangon Biotech Company (Shanghai, China).

Phylogenetic Analysis

Four-three HEV strains with different genotypes were selected as reference sequences for constructing phylogenetic trees. A phylogenetic analysis was performed with the MEGA version 6 (Tamura *et al.* 2013) software. We constructed phylogenetic trees with the neighbor-joining method and evaluated them with the interior branch test method. The Kimura model was then used to calculate the genetic distances. The statistical robustness and reliability of the branching order were confirmed with a bootstrap analysis of 1000 resampled data points. The similarity and divergence ratios of the strains were calculated with the Lasergene software package MegAlign (DNASTAR, Madison, WI, USA).

Statistical Analyses

We descriptively estimated the seroprevalence of HEV in humans after grouping them by age, sex, and work experience. The rates were compared with either the χ^2 or Fisher's exact test. We estimated the odds ratios (ORs) and Wald's 95% confidence intervals (CIs) for the conditional logistic regression models to identify significant risk factors in the occupationally swine-exposed group. We used Wald's χ^2 to test the significance of each item in the model. Statistical significance was set at $P < 0.05$.

Results

Demographic Characteristics of HEV Seroprevalence in Individuals

Among the 1505 human serum samples tested, 455 subjects (30.23%) from the study cohort were positive for anti-HEV IgG. Out of these, 30 (1.99%) were also positive for anti-HEV IgM and 7 (0.47%) were positive for HEV RNA. Further analysis showed that the prevalence of anti-HEV IgG in the 273 workers with occupational swine contact was significantly higher than in the 1232 representatives from the general population (χ^2 characteristic of independence [χ^2] for this distribution = 51.908, with degrees of freedom [df] = 5, $P < 0.001$; Table 1). No participants had any history of travel abroad. Among the 276 swine serum samples, we found a prevalence of 6.52% (18/276) for HEV-Ag and 2.54% (7/276) for HEV RNA.

Prevalence of Anti-HEV IgG among the General Population according to Sex, Age, and Current Occupation

We divided the general population into 11 age subgroups, ranging from 0 to 90 years old. Table 1 shows that 323 (26.22%) out of the 1232 people from the general population had detectable anti-HEV IgG levels. Surprisingly, the prevalence of anti-HEV IgG ranged from its lowest value (1.82%) in the 0 to 5-years age group, up to its highest value (44.44%) in the 61 to 70-years age group, and the age-specific differences in prevalence were statistically significant ($\chi^2 = 160.925$, $P < 0.001$), with a linear distribution. The male:female ratio for anti-HEV IgG was 1.22:1, which indicated a significant difference by sex

($\chi^2 = 10.874$, $P = 0.01$), insofar as the prevalence was higher in men (30.58%, 178/582) than in women (22.31%, 145/650). Among the general population, we expected occupation to be a key differentiating factor for the anti-HEV IgG positivity. In fact, we found a significantly higher occurrence of HEV infection in peasants (46.51%) than in other groups, whereas the prevalence was lowest in preschool children (2.76%). Our result showed high prevalence of HEV among blue-collar workers (37.50%), managerial staff (38.24%), and clerks in the public (37.19%) compared with the other occupational groups. Retirees and other people not belonging to specific occupational groups were classified into the “others” group, and the prevalence in this group was high, at 34.86% (Table 1).

Table 1 The positive rates of anti-HEV IgG among recruited cases.

Variables	No. detected	Positive numbers	Positive rate (%)	Chi square test
<i>Crowd</i>				$\chi^2 = 51.908$
General population (GP)	1232	323	26.22	$P = 0.00$
Workers occupationally contact with swine	273	132	48.35	
Total	1505	455	30.23	
<i>Gender in GP</i>				$\chi^2 = 10.874$
Male	582	178	30.58	$P = 0.01$
Female	650	145	22.31	
Total	1232	323	26.22	
<i>Age grouping in GP (years)</i>				$\chi^2 = 160.925$
0–5	165	3	1.82	$P = 0.00$
6–10	89	5	5.62	
11–15	61	4	6.56	
16–20	96	9	9.38	
21–30	104	24	23.08	
31–40	131	43	32.82	
41–50	133	49	36.84	
51–60	113	48	42.48	
61–70	99	44	44.44	
71–80	181	69	38.12	
81–90	60	25	41.67	
Total	1232	323	26.22	
<i>Occupational grouping in GPs</i>				$\chi^2 = 155.871$
Preschool children	181	5	2.76	$P = 0.00$
Students	219	15	6.85	
Peasants	129	60	46.51	
Blue-collar workers	112	42	37.5	
Managerial staffs	102	39	38.24	
Medical staffs	84	18	21.43	
Clerks in public place	121	45	37.19	
Others	284	99	34.86	
Total	1232	323	26.22	

Multivariable Conditional Logistic Regression Analysis of Risk Factors for HEV Infection among Occupationally Swine-Exposed Workers

Anti-HEV IgG is a standard marker of an immune response to previous HEV infection. We used a multivariable model to identify the factors that predicted anti-HEV IgG positivity among the 273 workers with occupational swine contact. We included age, working years, sex, education level, occupational pattern, and working hours per day in the model. Overall, younger workers were significantly less likely to show evidence of previous HEV infection (OR = 2.067, $\chi^2 = 14.649$, $P < 0.001$; Table 2), and people > 40 years old had a higher HEV-infection risk and were more likely to test positive for anti-HEV IgG than people < 30 years old. A similar trend was also detected in working years (OR = 6.0, $\chi^2 = 11.903$, $P = 0.018$), and more working years was associated with an increased risk of HEV. The statistical analysis showed no significant differences in the sex ($P = 0.491$), education level ($P = 0.321$), occupation ($P = 0.163$), or working hours per day ($P = 0.331$) among workers who had swine contact.

Analysis of Epidemic and Clinical Characteristics of 30 Anti-HEV-IgM-Positive Patients

There was no significant difference in anti-HEV IgM prevalence between males and females (1.83% [14/767] and 2.17% [16/738], respectively). There was also no any significant age-related ($\chi^2 = 0.268$, $P = 8.793$) or occupation-related differences in anti-HEV IgM prevalence detected (Table 3). The epidemiological questionnaire showed that two out of the 30 patients with anti-HEV IgM were also diagnosed with liver dysfunction; one was female with no history of pig exposure and the other was a male swine worker. The female patient was coinfecting with HBV and HCV, and presented with chronic liver disease without jaundice. Her alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and direct bilirubin (DBIL) levels were 92.9 U/L, 108.9 U/L, 13.63 $\mu\text{mol/L}$, and 4.6 $\mu\text{mol/L}$ respectively. The ALT, AST, TBIL and DBIL levels of the male patient were 462.5 U/L, 138.4 U/L, 155.37 $\mu\text{mol/L}$ and 115.73 $\mu\text{mol/L}$, respectively, and were accompanied by evident clinical jaundice. Only two of the 30 people with anti-HEV-antibody-positive sera had abnormal liver function, which suggests that HEV-infected people often have asymptomatic liver disease.

Phylogenetic Analysis of HEV ORF2 RNA Sequence

A sequence of 100% homology in the same species is considered to be the same sequence. There were 4 different sequences after comparing and analyzing the homology of 7 swine HEV ORF2 sequences. After the homology analysis of 7 human HEV ORF2 sequences, 4 different sequences were found. Homology and phylogenetic analyses were performed on the partial ORF2 sequences from pig samples (GenBank nos KX463414–KX463417) and human samples (GenBank nos KX463410–KX463413). The human and swine HEV isolates shared 97.04%–100% sequence homology, and ORF2 from a human sample (KX463411) shared 100% sequence identity with ORF2 from a swine sample (KX463417). The four human HEV isolates shared 97.04%–99.34% sequence homology and the swine isolates shared 98.68%–99.67% sequence homology. The eight HEV isolates also shared 75.33%–79.28%, 77.96%–78.95%, 75.33%–78.62%, and 82.89%–94.08% identity with GenBank reference sequences of HEV genotypes 1–4, respectively. Homology and phylogenetic analyses confirmed that the eight novel HEV sequences detected in this study were subtype 4_d, and shared 96.05%–97.37% nucleotide sequence identity with the most closely matched strain (GenBank no. FJ461765), which was isolated from a person in Hubei Province (Fig. 1), and 89.14%–90.46% identity with another strain (GenBank no. FJ461762) that was isolated from a swine in Hubei Province. Further analysis of the ORF2 amplicons revealed higher sequence homologies (96.05%–97.04% and 95.72%–96.38% identity) with genotype 4_d from Shandong (KF176370) and Beijing (KP196807) HEV swine strains, respectively.

Discussion

In Asia and Africa, HEV is generally considered an endemic disease that causes acute self-limiting hepatitis in young people, with the exception of pregnant women, in whom fatality rates are usually 10%–25% or greater (Kamar *et al.* 2012). In Spain, the HEV rate has remained stable at 12%–17%. In European Union and European Economic Area countries, HEV infections are predominantly indigenous and are caused by HEV genotype 3, the commonest viral genotype in the human and animal reservoirs in Europe (Mesquita *et al.* 2016; Adlhoch *et al.* 2016; Rosa *et al.* 2016; Matos *et al.* 2018). Several cross-sectional studies in Norway, Canada, and other countries have also shown an increased HEV-infection risk in people with direct exposure to pigs or pork products

Table 2 Analysis for influent factors of the positive rates of anti-HEV IgG among workers occupationally contacted with swine using logistic regression model.

Factors	Variables	β	Wald χ^2	P value	OR (95% CI)	Chi square test
Gender	1 = male, 0 = female	0.15	0.474	0.491	1.162 (0.759–1.779)	
Age group (years)			24.699	0	2.067	$\chi^2 = 14.649$
	1 \leq 30 years	– 1.371	12.969	0	0.254 (0.120–0.535)	$P = 0.002$
	2 = 31–40 years	– 0.824	6.551	0.01	0.439 (0.234–0.825)	
Years of Working	3 = 41–50 years	– 1.044	19.379	0	0.352 (0.221–0.560)	
			23.243	0	6	$\chi^2 = 11.903$
	1 = 0–5 years,	– 3.007	13.445	0	0.049 (0.010–0.247)	$P = 0.018$
	2 = 6–10 years,	– 2.772	11.946	0.001	0.063 (0.013–0.301)	
Educational background	3 = 11–15 years,	– 2.986	13.313	0	0.050 (0.010–0.251)	
	4 = 16–20 years,	– 1.576	2.881	0.09	0.207 (0.033–1.276)	
			3.5	0.321	0.923	$\chi^2 = 1.749$
Job pattern	1 = Primary school	– 0.811	1.987	0.159	0.444 (0.144–1.373)	$P = 0.417$
	2 = Junior school	– 0.483	0.769	0.381	0.617 (0.210–1.815)	
	3 = Senior school	– 0.826	2.067	0.151	0.438 (0.142–1.350)	
Working hours per day			1.95	0.163	1.2	$\chi^2 = 5.637$
	1 = pig farmers	1.713	7.134	0.068	5.545 (1.011–30.424)	$P = 0.060$
	2 = Slaughters	2.396	3.89	0.049	10.984 (1.650–73.130)	
Working hours per day	3 = Pork retailers	2.303	6.139	0.013	10.009 (1.533–65.349)	
			2.214	0.331	1.506	$\chi^2 = 8.077$
	1 \leq 6 h	– 0.008	0	0.985	1.008 (0.452–2.247)	$P = 0.018$
	2 = 7–9 h	– 0.398	1.012	0.315	0.670 (0.307–1.462)	

(Walachowski *et al.* 2014; Kang *et al.* 2016; Bansal *et al.* 2017; Lange *et al.* 2017; Mykytczuk *et al.* 2017; Mughini-Gras *et al.* 2017; Teixeira *et al.* 2017; Ukuli and Mugimba 2017). In Europe, anti-HEV antibody prevalence among the general population was 1.3%–52%, whereas a high seroprevalence of HEV IgG (13%–51.1%) was detected among swine farmers (Lapa *et al.* 2015). Surprisingly, anti-HEV IgG prevalence was very low (5.6%) among swine farmers in Croatia (Vilibic-Cavlek *et al.* 2016). In a previous study, we investigated acute HEV infection in commercial pigs in Anlu city of Hubei Province, and found a high anti-HEV IgM prevalence of 46.7% in towns and an average anti-HEV-antibody-positive prevalence of 37.9% (Zhang *et al.* 2016, 2017). Our results indicate that HEV is prevalent in pigs in Anlu city of Hubei Province and that the seroprevalence among workers who are occupationally exposed to pigs (48.35%) is almost twice that of the general population not exposed to pigs (26.22%). This indicates an association between direct swine contact and a high risk of HEV infection.

In addition to global variations in the epidemiology of HEV, the seroprevalence of HEV within a single country can vary widely. Consistent with previous findings, we found that anti-HEV IgG prevalence increased with age,

with people > 50 years old more likely to be infected with HEV than people < 50 years old (Kamar *et al.* 2014; Taherkhani and Farshadpour 2016; Lange *et al.* 2017). We also reported that the overall prevalence of anti-HEV IgG in people < 20 years old was only 5.11% (21/411), which is below the 14.93% reported in northern China or even the 10% estimated in the Netherlands (Meng *et al.* 2015; van *et al.* 2017). As expected, the mean prevalence of anti-HEV IgG among people aged \geq 50 years exceeded 40% in the general population of Anlu city, which is higher than the 33% reported in the Netherlands among similarly aged people. This further supports the conclusion that viral exposure has a cumulative age-dependent lifetime pattern that correlates with decreasing immunity (van *et al.* 2017). This result also indicates that previous infections reflect an age-cohort effect. In the majority of studies, HEV seroprevalence has been higher in males than in females and HEV antibody positivity has also been higher in male blood donors and males in the general population (Mohebbi *et al.* 2012; Fearon *et al.* 2017; van Gageldonk-Lafeber *et al.* 2017). We also found a significant sex difference in exposure rate ($\chi^2 = 10.874$, $P = 0.01$), which may be attributable to the higher frequency with which men in China participate in business entertainment,

Table 3 The positive rates of anti-HEV IgM using Chi square test among general population and workers occupationally contacted with swine.

Variables	No. detected	Positive numbers	Positive rate (%)	Chi square test
<i>Gender</i>				$\chi^2 = 0.634$
Male	767	14	1.83	$P = 0.226$
Female	738	16	2.17	
<i>Age grouping (years)</i>				$\chi^2 = 0.268$
0–10	254	0	0	$P = 8.793$
11–20	161	1	0.62	
21–30	132	0	0	
31–40	174	7	4.02	
41–50	246	7	2.85	
51–60	189	4	2.12	
61–70	107	4	3.74	
71–80	182	5	2.75	
81–90	60	2	3.33	
<i>Occupation grouping</i>				$\chi^2 = 76.158$
Preschool children	181	0	0	$P = 0.000$
Students	219	1	0.46	
Peasants	129	10	7.75	
Blue-collar workers	112	0	0	
Managerial staffs	102	0	0	
Medical staffs	84	0	0	
Clerks in public place	121	11	9.09	
Others	284	0	0	
Pig farmers	137	3	2.19	
Pork retailers	104	5	4.81	
Slaughterers	24	0	0	
Veterinarians	8	0	0	

including eating dinner outside the home, compared with women. We also found that HEV seroprevalence (anti-HEV IgG) differed significantly by occupation among the general population, insofar as only 2.76% of preschool children were positive for anti-HEV IgG, whereas 46.51% of peasants in the general population were positive. This finding may indirectly support the prediction that poor sanitation increases the risk of HEV infection. Age and working years were the main factors positively associated with HEV seropositivity among workers with occupational swine contact ($\chi^2 = 14.649$, $P = 0.002$ and $\chi^2 = 11.903$, $P = 0.018$, respectively), which is consistent with a previous study (Song *et al.* 2014).

Several studies have found that genotype 4 is not only prevalent in developed countries, but also distributed across China, Vietnam, and India, where subtypes 4_{a-d} have been identified (Tian *et al.* 2015; Bansal *et al.* 2017; Jeong *et al.* 2017; Mykytczuk *et al.* 2017). Subtype 4_d is mainly distributed in China and Italy, among both humans and swine (Garbuglia *et al.* 2013; Monne *et al.* 2015). In this study, we also found that HEV genotype 4 was the main genotype, and a phylogenetic analysis confirmed that

this subtype was 4_d. Using nested RT-PCR, followed by amplicon sequencing, we amplified 14 HEV sequences, seven sequences from human populations and another seven from swine herds. A homology analysis identified four novel human HEV sequences and four novel swine HEV sequences. We detected sequence identity of 100% between one swine HEV sequence (KX463417) and one human HEV sequence (KX463411); in total, the four human HEV sequences and four swine HEV sequences shared 97.04%–100% homology. Therefore, we speculate that HEV genotype 4d is epidemic in this specific local region, and that swine may be one of the sources of the HEV genotype 4d infections in the local human population. In fact, one older HEV-infected man was also a pig farmer. Interestingly, the 304 bp HEV ORF2 sequences that we sequenced shared only 89.14%–90.46% homology with another previously identified sequence (FJ461762) from Hubei, which further supports the argument that epidemics of different HEV subtypes have occurred in the local region, rather than a widespread epidemic of a single genotype. By contrast, genotype 4_d identified in this study shares greatest sequence homology with Shandong

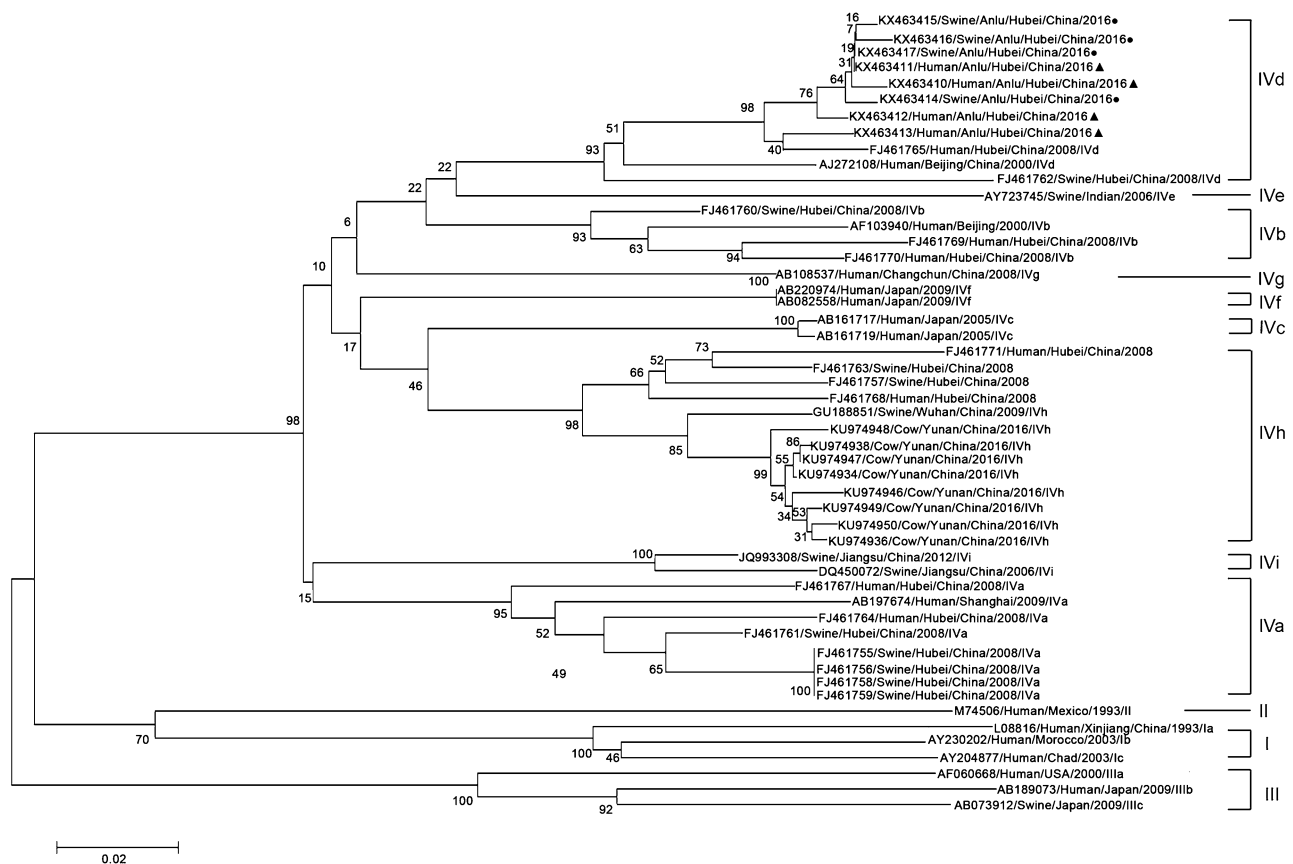


Fig. 1 Phylogenetic analysis of HEV isolates from human populations and swine herds. The phylogenetic tree was constructed by the neighbor-joining method and evaluated by interior branch test, based on partial nucleotide sequence of the ORF2 region. Forty-three known

(KF176370) and Beijing (KP196807) strains, which suggests that this genotype 4_d strain was imported to the city of Anlu from other provinces or cities.

This study had several limitations. First, we only recruited individuals from Anlu city of Hubei province, so our estimates of HEV seroprevalence cannot be generalized across central China. Further studies should be performed in other cities with pig-breeding activities similar to those in Anlu. Second, pig-farm workers who were exposed to pigs in the swine age group with the highest viral shedding rate also had the highest risk of HEV infection (Wilhelm *et al.* 2016). However, we did not subdivide this group from the 273 members of the occupationally swine-exposed group, so did not control for this effect. Third, we did not collect data on other potential HEV-infection-related activities, such as alcohol use, diet, or pork consumption.

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representative strains published in GenBank were used as references. The human or swine's isolates were signed with filled triangle or filled circle, respectively.

Author Contributions YS, JZ, LZ and HW designed the study. YS, YC, SZ and QW performed the experiments. YS, SZ, CZ and ZZ analyzed the data. HC, YS and LZ drafted the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement All institutional and national guidelines for the care and use of animals were followed. Additional informed consent was obtained from all patients for which identifying information is included in this article.

References

- Adlhoch C, Avellon A, Baylis SA, Ciccaglione AR, Couturier E, de Sousa R, Epstein J, Ethelberg S, Faber M, Feher A, Ijaz S, Lange H, Mandakova Z, Mellou K, Mozalevskis A, Rimhanen-Finne R, Rizzi V, Said B, Sundqvist L, Thornton L, Tosti ME, van Pelt W, Aspinall E, Domanovic D, Severi E, Takkinen J, Dalton HR (2016) Hepatitis E virus: assessment of the epidemiological situation in humans in Europe, 2014/15. *J Clin Virol* 82:9–16
- Aggarwal R, Naik S (2009) Epidemiology of hepatitis E: current status. *J Gastroenterol Hepatol* 24:1484–1493
- Bansal M, Kaur S, Deka D, Singh R, Gill JPS (2017) Seroepidemiology and molecular characterization of hepatitis E virus infection in swine and occupationally exposed workers in Punjab, India. *Zoonoses Public Health* 00:1–11
- Bazerbachi F, Haffar S, Garg SK, Lake JR (2016) Extra-hepatic manifestations associated with hepatitis E virus infection: a comprehensive review of the literature. *Gastroenterol Rep* 4:1–15
- Caruso C, Peletto S, Rosamilia A, Modesto P, Chiavacci L, Sona B, Balsamelli F, Ghisetti V, Acutis PL, Pezzoni G, Brocchi E, Vitale N, Masoero L (2016) Hepatitis E virus: a cross-sectional serological and virological study in pigs and humans at zoonotic risk within a high-density pig farming area. *Transbound Emerg Dis* 64:1443–1453
- Colson P, Brunet P, Lano G, Moal V (2016) Hepatitis E virus genotype 4 in Southeastern France: still around. *Liver Int* 36:765–767
- Dai X, Dong C, Zhou Z, Liang J, Dong M, Yang Y, Fu J, Tian H, Wang S, Fan J, Meng J, Purdy MA (2013) Hepatitis E virus genotype 4, Nanjing, China, 2001–2011. *Emerg Infect Dis* 19:1528–1530
- Ding J, Zhang XJ, Yu KR, Zhou TM, Li HX, Sheng YM, Chen YY, Liu BY, Sun YN, Zhou EM, Zhou Q (2018) Development and application of an indirect ELISA for detecting antibodies against swine hepatitis E virus. *Acta Vet Zootech Sin* 49:2240–2248
- Fearon MA, O'Brien SF, Delage G, Scalia V, Bernier F, Bigham M, Weger S, Prabhu S, Andonov A (2017) Hepatitis E in Canadian blood donors. *Transfusion* 57:1420–1425
- Garbuglia AR, Scognamiglio P, Petrosillo N, Mastroianni CM, Sordillo P, Gentile D, La SP, Girardi E, Capobianchi MR (2013) Hepatitis E virus genotype 4 outbreak, Italy, 2011. *Emerg Infect Dis* 19:110–114
- Gong G, Wang YF, Yi XC, Suolang SZ (2018) Serological epidemiological investigation on hepatitis E virus in Tibetan pigs in some areas of Tibet. *China Anim Health Insp* 35:14–16
- Hakze-van der Honing RW, van Coillie E, Antonis AF, van der Poel WH (2011) First isolation of hepatitis E virus genotype 4 in Europe through swine surveillance in the Netherlands and Belgium. *PLoS ONE* 6:e22673
- Huang F, Yu WH, Ma TW, Jing SR, Zeng WK (2012) Molecular epidemiology of swine HEV in a village of Yunan. *Chin J Zoonoses* 28:274–277
- Jeong SH, Park BJ, Kim YH, Choi YS, Ahn HSc, Han SH, Choi IS (2017) Isolation of hepatitis E virus genotype 4 from patients with acute cryptogenic hepatitis in Korea. *J Clin Virol* 89:10–13
- Kamar N, Bendall R, Legrand-Abravanel F, Xia NS, Ijaz S, Izopet J, Dalton HR (2012) Hepatitis E. *Lancet* 379:2477–2488
- Kamar N, Dalton HR, Abravanel F, Izopet J (2014) Hepatitis E virus infection. *Clin Microbiol Rev* 27:116–138
- Kang YH, Cong W, Zhang XY, Wang CF, Shan XF, Qian AD (2016) Hepatitis E virus seroprevalence among farmers, veterinarians and control subjects in Jilin province, Shandong province and Inner Mongolia Autonomous Region, China. *J Med Virol* 89:872–877
- Khuroo MS, Khuroo NS (2016) Transmission of hepatitis E virus in developing countries. *Viruses* 8:E253
- Lange H, Overbo J, Borgen K, Dudman S, Hoddevik G, Urdahl AM, Vold L, Sjurseth SK (2017) Hepatitis E in Norway: seroprevalence in humans and swine. *Epidemiol Infect* 145:181–186
- Lee GH, Tan BH, Teo EC, Lim SG, Dan YY, Wee A, Aw PP, Zhu Y, Hibberd ML, Tan CK, Purdy MA, Teo CG (2016) Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk. *Gastroenterology* 150:355–357
- Liu P, Li L, Wang L, Bu Q, Fu H, Han J, Zhu Y, Lu F, Zhuang (2012) Phylogenetic analysis of 626 hepatitis E virus (HEV) isolates from humans and animals in China (1986–2011) showing genotype diversity and zoonotic transmission. *Infect Genet Evol* 12:428–434
- Lu L, Li C, Hagedorn CH (2006) Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol* 16:5–36
- Marano G, Vaglio S, Pupella S, Facco G, Bianchi M, Calizzani G, Candura F, Catalano L, Farina B, Lanzoni M, Piccinini V, Liumbro GM, Grazzini G (2015) Hepatitis E: an old infection with new implications. *Blood Transfus* 13:6–17
- Matos A, Mesquita JR, Gonçalves D, Joana Abreu-Silva, Luxo C, Nascimento MSJ (2018) First detection and molecular characterization of hepatitis E virus in water from wastewater treatment plants in Portugal. *Ann Agric Environ Med* 25:364–367
- Meng XJ (2010) Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol* 140:256–265
- Meng QF, You HL, Wang WL, Zhou N, Dong W, Cong W (2015) Seroprevalence and risk factors of hepatitis E virus infection among children in China. *J Med Virol* 87:1573–1577
- Mesquita JR, Oliveira D, Rivadulla E, Joana Abreu-Silva, Varela MF, Romalde JL, Nascimento MSJ (2016) Hepatitis E virus genotype 3 in mussels (*Mytilus galloprovincialis*). *Food Microbiol* 58:13–15
- Mohebbi SR, Rostami Nejad M, Tahaei SM, Pourhoseingholi MA, Habibi M, Azimzadeh P, Naghoosi H, Karayiannis P, Zali MR (2012) Seroepidemiology of hepatitis A and E virus infections in Tehran, Iran: a population based study. *T Roy Soc Trop Med H* 106:528–531
- Monne I, Ceglie L, DI Martino G, Natale A, Zamprogna S, Morreale A, Rampazzo E, Cattoli G, Bonfanti L (2015) Hepatitis E virus genotype 4 in a pig farm, Italy, 2013. *Epidemiol Infect* 143:529–533
- Mughini-Gras L, Angeloni G, Salata C, Vonesch N, D'Amico W, Campagna G, Natale A, Zuliani F, Ceglie L, Monne I, Vascellari M, Capello K, DI Martino G, Inglese N, Palù G, Tomao P, Bonfanti L (2017) Hepatitis E virus infection in North Italy: high seroprevalence in swine herds and increased risk for swine workers. *Epidemiol Infect* 145:3375–3384
- Mykytczuk O, Harlow J, Bidawid S, Corneau N, Naseri N (2017) Prevalence and molecular characterization of the hepatitis E virus in retail pork products marketed in Canada. *Food Environ Virol* 9:208–218
- Rosa GL, Libera SD, Brambilla M, Bisaglia C, Pisani G, Ciccaglione AR, Bruni R, Taffon S, Equestre M, Iaconelli M (2016) Hepatitis E virus (Genotype 3) in slurry samples from swine farming activities in Italy. *Food Environ Virol* 9:219–229
- Shuai JB, Zhang XF, Xu JJ, Chen N, Fang WH (2009) Seroprevalence of hepatitis E virus infections in pigs from Zhejiang Province during 2005–2008. *Acta Vet Zootech Sin* 40:1037–1042
- Song YJ, Park WJ, Park BJ, Lee JB, Park SY, Song CS, Lee NH, Seo KH, Kang YS, Choi IS (2014) Hepatitis E virus infections in humans and animals. *Clin Exp Vaccine Res* 3:29–36
- Taherkhani R, Farshadpour F (2016) Epidemiology of hepatitis C virus in Iran. *World J Gastroenterol* 22:5143–5153

- Tamura K, Stecher G, Peterson D, Filipiński A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729
- Teixeira J, Mesquita J, Pereira SS, Oliveira RM, Abreu-Silva J, Rodrigues A, Myrnel M, Stene-Johansen K, Øverbø J, Gonçalves G, Nascimento MS (2017) Prevalence of hepatitis E virus antibodies in workers occupationally exposed to swine in Portugal. *Med Microbiol* 206:77–81
- Tian H, Fu X, Li W, Huang Y, Sun J, Zhou G, Zhou C, Shen Q, Yang S, Zhang W (2015) Genotype 4 Hepatitis E virus prevalent in eastern China shows diverse subtypes. *Hepat Mon* 15:e25367
- Ukuli AQ, Mugimba KK (2017) Seroprevalence of hepatitis E in swine abattoir workers. *Afr Health Sci* 17:1022–1028
- van Gageldonk-Lafeber AB, van der Hoek W, Borlee F, Heederik DJ, Mooi SH, Maassen CB, Yzermans CJ, Rockx B, Smit LA, Reimerink JH (2017) Hepatitis E virus seroprevalence among the general population in a livestock-dense area in the Netherlands: a cross-sectional population-based serological survey. *BMC Infect Dis* 17:21
- Vina-Rodriguez A, Schlosser J, Becher D, Kaden V, Groschup MH, Eiden M (2015) Hepatitis E virus genotype 3 diversity: phylogenetic analysis and presence of subtype 3b in wild boar in Europe. *Viruses* 7:2704–2726
- Walachowski S, Dorenlor V, Lefevre J, Lunazzi A, Eono F, Merbah T, Eveno E, Pavio N, Rose N (2014) Risk factors associated with the presence of hepatitis E virus in livers and seroprevalence in slaughter-age pigs: a retrospective study of 90 swine farms in France. *Epidemiol Infect* 142:1934–1944
- Wang L, Wang L (2016) Animal Models for Hepatitis E Virus. *Adv Exp Med Biol* 948:161–173
- WHO (2016) Hepatitis E. <http://www.who.int/mediacentre/factsheets/fs280/en/>. Accessed 4 July 2016
- Wilhelm BJ, Rajic A, Greig J, Waddell L, Trottier G, Houde A, Harris J, Borden LN, Price C (2011) A systematic review/meta-analysis of primary research investigating swine, pork or pork products as a source of zoonotic hepatitis E virus. *Epidemiol Infect* 139:1127–1144
- Wilhelm B, Leblanc D, Houde A, Brassard J, Gagne MJ, Plante D, Bellon-Gagnon P, Jones TH, Muehlhauser V, Janecko N, Avery B, Rajic A, McEwen SA (2014) Survey of Canadian retail pork chops and pork livers for detection of hepatitis E virus, norovirus, and rotavirus using real time RT-PCR. *Int J Food Microbiol* 185:33–40
- Wilhelm B, Fazil A, Rajic A, Houde A, McEwen SA (2016) Risk profile of hepatitis E virus from pigs or pork in Canada. *Transbound Emerg Dis* 64:1694–1708
- Woo PC, Lau SK, Teng JL, Cao KY, Wernery U, Schountz T, Chiu TH, Tsang AK, Wong PC, Wong EY, Yuen KY (2016) New hepatitis E virus genotype in bactrian camels, Xinjiang, China, 2013. *Emerg Infect Dis* 22:2219–2221
- Wu CH, Ho CM, Tsai JH, Sun HY, Hu RH, Lee PH (2017a) First case genotype 4 hepatitis E infection after a liver transplant. *Exp Clin Transpl* 2:228–230
- Wu Q, An J, She R, Shi R, Hao W, Soomro M, Yuan X, Yang J, Wang J (2017b) Detection of genotype 4 swine hepatitis E virus in systemic tissues in cross-species infected rabbits. *PLoS ONE* 12:e0171277
- Zhang HY, Chen DS, Zhang Y, Wu YQ (2010) Epidemiological survey of hepatitis E virus in industrialized swine herds. *Acta Vet Zootech Sin* 41:1268–1273
- Zhang SD, Shu YL, Wan Q, Zhou S, Zhang XF, Wei YQ, Zhang L (2016) Acute hepatitis E virus infection situation of commercial pigs in Xiaogan Area of Hubei Province. *China Trop Med* 16:945–946
- Zhang SD, Shu YL, Peng J, Zhou S, Xie J, Zhang L (2017) The seroprevalence study on hepatitis E in swine at Anlu City, Hubei province. *Prev Med* 24:88–89