Research paper

Potential effects of hepatitis E virus infection in swine on public health in China

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A B S T R A C T

Hepatitis E virus (HEV), a zoonotic pathogen, is the main cause of acute hepatitis worldwide. Swine serves as the main reservoir, and its infection is mainly transmitted via fecal-oral route. Due to huge consumption of pork in China, close human-swine interactions at pig farms likely contribute to high risk in zoonotic transmission of HEV. Thus, we aim to investigate the HEV prevalence in pig farm in seven provinces across the east to west China and estimate the potential effects of swine HEV on public health in China. In this study, serum samples of pig were collected for detection of anti-HEV antibodies from the seven provinces. A high seroprevalence of 67.1% was found, and no clear difference was observed among these regions. However, the age and the breeding purpose (for meat supplier or breeding offspring) play significant roles in the risk of swine HEV infection. In addition, sequence comparison of various HEV genomes isolated in China displayed that swine HEV posed obvious threats to ruminant breeding and public health. The high level of seroprevalence of swine HEV strongly plays an important role in cross-species of HEV infection. Therefore, effective measures should be performed to prevent HEV infection from infected pigs to human and other ruminants.

1. Introduction

Hepatitis E virus (HEV) is an etiological agent of hepatitis E, mainly transmitted via fecal-oral route. As an emerging global health issue, epidemics of hepatitis E occurs periodically in developing countries and sporadic cases are frequently reported in developed countries (Meng, 2016). It is a zoonotic virus with expanding host range and cross-species infection. Ample studies have indicated zoonotic infection of genotypes 3 and 4 HEV (Kamar et al., 2013). Swine is regarded as the primary reservoir with high risk of transmission to human (Huang et al., 2002b; Meng et al., 2002a). Contact exposure to HEV-infected swine results in an increased risk of zoonotic HEV infection in public health (Drobeniuc et al., 2001).

Domestic swine is the important breed of livestock in China. It is widely distributed across the country, and generally used for meat production. Although several studies have investigated the prevalence of HEV in pig farms in some provinces (i.e. Beijing, Tibet, Shandong and Guangdong) of China (Li et al., 2009; Liang et al., 2014; Wang et al., 2014; Zhang et al., 2017), the epidemiological information of vaster

area of China remains segmented. In this study, we aim to investigate the prevalence of HEV infection in pig farms in seven provinces across the east to west China and estimate the potential effects of HEV derived from swine on public health in China by sequence comparison of HEV genomes isolated in China.

2. Materials and methods

2.1. Blood sample collection

This study was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval No. LVRIAEC 2013-010). The animals were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China. From the seven provinces in China (Supplementary Fig. S1), the pigs were selected in random, after obtaining the verbal consent from the owners of pig farm. Finally, a total of 1768 blood samples were collected from swine in pig farms. As for extracting serum from these

samples, blood samples were transported to the laboratory and left at room temperature for 2 to 3 h, then centrifuged at 3000*g* for 10 min to separate serum. The serum samples were kept at −20 °C until further analysis.

2.2. Serological detection for HEV infection

To estimate HEV antibody status in sampled pigs, all serum samples for total antibodies against HEV were tested by using a double-antigen sandwich ELISA kit (Wantai Biological, Beijing, China). As for this ELISA kit, the recombinant peptide of open reading frame 2 (AA 394-606) is used for the viral antigen (Wang et al., 2002).

2.3. Risk factors of HEV infection

The independent factors (pig age stages, breeding pig purposes and geographical origins) were considered in the examination, respectively. The age of sampled pigs was divided into four categories: < 30 birth days, 60 birth days, 90 birth days and adult. According to breeding pig purposes, the serum samples were divided into two groups, namely hog used for food supplier and boar used for breeding. The geographical origin of sampled pigs was classified by different provinces. Variables related to HEV infection in pigs of different age groups were analyzed by Chi-sequre test method using SPSS software for Windows. Probability (*p* value < 0.05) was considered as statistically significant between the independent factor (age, breeding purpose or geographical origin) and seroprevalence of HEV infection. To estimate the degrees of risk factors of HEV infection, odds-ratios (OR) with 95% confidence intervals based on likelihood ratio statistics was calculated for quantification of the strength of the association between HEV antibody status (dependent variable) and the available independent variables including age, breeding purpose and geographical origin by SPSS software.

2.4. Detection of HEV RNA sequence by nested RT-PCR

The positive serum samples which had been identified by ELISA kit were diluted 1:1 in phosphate-buffered saline (PBS; pH 7.4), and then total RNA was extracted from 500 μl of the above sample mixture using TRlzol reagent according to the manufacture's instructions (Invitrogen, USA). Total RNAs were resuspended in DNase- and RNase-free water. 10 μl of each RNA suspension was used for complementary DNA (cDNA) synthesis using the external anti-sense primer E04 (reverse, 5′-TTRTC-CTGCTGWGCRTTCTC-3′) with a reaction mix containing M-MLV Reverse Transcriptase (Promega, USA) at 42 °C for 1 h followed by incubation at 72 °C for 15 min. Afterwards, the nested PCR was performed for detecting partial HEV ORF2 sequence, based on the established method (Huang et al., 2002a; Zhou et al., 2018b) with the following modifications. In brief, the same primers to include external primers E01 (forward, 5′-TGGCGTTCDGTTGAGA-3′), E04 (reverse, 5′-TTRTC-CTGCTGWGCRTTCTC-3′), and internal primers E02 (forward, 5′-GGB-GTBGCKGAGGAGGAG-3′), E03 (reverse, 5′-CCGACGAAATCAATTCT GTC-3′). 5 μl of each synthesized cDNA sample was used for template for the first round of PCR using amplification parameters as follows: 40 cycles of 94 °C for 30s, 48 °C for 45 s and 72 °C for 45 s. Next, 5 μl of the first round PCR product was used as template for second round PCR as follows: 35 cycles of 94 °C for 30s, 50 °C for 45 s and 72 °C for 30s. Premix rTaq kit (TAKARA, Dalian, China) was used in the two rounds of PCR. PCR products predicted from the HEV ORF2 were observed, with sizes of amplification products of first-round and second-round PCRs of 497 bp and 397 bp, respectively. Due to the small quantity of available serum for each sample, only partial sequences of HEV open reading frame 2 (ORF2) were amplified. Positive PCR products were purified using a Gel Extraction Mini Kit (TaKaRa) and inserted into the pGEM-T Easy Vector (Promega, USA). The recombinant plasmid was transformed into DH5-α competent *Escherichia coli* cells according to the manufacturer's instructions. Positive plasmids containing the inserted

fragment were identified by PCR. The positive clones were sequenced by the Sangon Biotech (shanghai) Co., Ltd.

2.5. Phylogenetic and sequence analyses

To better estimate potential effects of HEV in swine on public health in China, HEV genomes isolated from China with the size > 7000 nt were downloaded from Genbank at the National Center for Biotechnology (NCBI) and listed in Supplementary Table S1. The total of 61 complete genomes were isolated from several susceptible hosts (human, swine, rabbit, camel, cow, goat and avian) in China (Supplementary Table S1). These HEV genomes were analyzed by producing UPGMA trees, based on the distribution of nucleotide *p*-distances using MEGA 5.

3. Results

3.1. Seroprevalence of HEV infection in pigs

To estimate the seroprevalence of HEV infection in pigs, each serum sample was tested for the anti-HEV antibodies. The overall serological prevalence is 67.14% in pigs in the seven provinces of China (Table 1). It ranges from 62.50% to 73.73% across the provinces from east to west of China (Supplementary Fig. S1). Among the different age groups, swine of 30 birth days has the highest seroprevalence rate (74.58%); whereas swine with 60 birth days has the lowest rate (56.67%) (Table 2). In addition, the seroprevalence in boar (74.58%, 483 of 687 samples) appears higher than that (65.12%, 704 of 1081 samples) in the common swine which is used for food supplier (Chi-sequre test, p < 0.05).

3.2. Relations of age and breeding purpose to HEV infection in swine

To further investigate the association between HEV seroprevalence in swine and pig age stage, breeding pig purpose or geographical region, respectively, odds ratios for the independent factors were calculated. Four categories of age were estimated and the age group 'adult' served as reference. No significant difference of seroprevalence is found between the group with 30 birth days and adult, although swine with 30 birth days is a high risk factor for HEV infection (OR > 1.0). Significant difference is found between the groups with 60 or 90 birth days and the adult group (Table 3). In comparison to hog used for food supplier, boar used for breeding is at high risk of HEV infection (OR > 1.0) (Table 3). In addition, no significant difference is observed among the different provinces (Table 3).

3.3. Amplification of the partial ORF2 of HEV from pig serum samples

To further identify HEV RNA in the positive pig serum samples, the partial ORF2 gene segments were amplified from these positive serum samples by nested RT-PCR method, and the resulting PCR products were amplified from three serum samples derived from Gansu province.

Table 1
Serological prevalence for antibodies against HEV in pigs from different provinces.

Province	No. samples	No. positive	Prevalence (%)
Sichuan	596	374	62.75
Fujian	91	61	67.03
Hubei	120	75	62.50
Anhui	121	76	62.81
Jiangxi	99	73	73.73
Ningxia	145	106	73.10
Gansu	596	422	70.81
Total	1768	1187	67.14

Table 2
Serological prevalence for antibodies against HEV in different ages of pig.

Age group	No. samples	No. positive	Prevalence (%)
30 days	118	88	74.58
60 days	120	68	56.67
90 days	104	60	57.69
Adult	1426	971	68.09

Table 3
Odds ratios for different risk factors in HEV infection.

Category	No. negative	No. positive	OR (95% CI)	p value
Adult	455	971	Reference	–
30 days	30	88	1.38 (0.69–1.22)	$p > 0.05$
60 days	52	68	0.61 (0.42–0.89)	$p < 0.05$
90 days	44	60	0.64 (0.43–0.96)	$p < 0.05$
Hog	377	704	Reference	–
Boar	204	483	1.27 (1.03–1.56)	$p < 0.05$
Western region	435	902	Reference	–
Eastern region	146	285	0.94 (0.75–1.19)	$p > 0.05$

The three positive clones were selected for sequencing, and sequences were analyzed by the NCBI BLAST program. It was shown that the sequences shared 100% identification with corresponding sequences from HEV genome (swCH189, FJ610232) available in GenBank. As for HEV strain swCH189, we have obtained the genome information which was classified into genotype 4 and isolated from swine in Gansu province, China (Zhou et al., 2018b). However, no HEV RNA sequence was amplified from the rest positive serum samples in this study.

3.4. Genetic diversity in HEV strains in China

To characterize the genetic diversity of HEV strain circulating in China, we constructed a phylogenetic tree using UAPMG method based on the 61 complete HEV genomes. Currently, genotype 4 dominated in HEV infection of swine in China, according to the information about HEV genomes in China. The phylogenetic tree displays the four major evolutional groups in HEV genotype 4 derived from swine (Fig.1), namely Group 1 (GU119961, GU188851, KM253769, FJ610232, HM152568, GU206559, GU361892, KC163335, KF176351, DQ450072), Group II (JQ655736, KC692453, EU366959, GU119910), Group III (DQ279091, EU676172, JX855794) and Group IV (EF570133, JF915746, JQ993308). Of note, the four distinct branches in genotype 4 HEV isolated from swine do not reflect the specific-geographic features. Based on the genetic diversity of HEV isolated from swine mentioned above, we further analyzed the potential effects of HEV in swine on public health. Without surprise, the HEV genome (GaHEV, GU954430) derived from avian which belongs to *Orthohepevirus B* is grouped into an independent evolutionary branch. As shown in Fig.1, HEV strains classified into either genotype 1 derived from human or genotype 3 derived from rabbit have distinct evolutionary branches, compared with evolutionary branches of HEV genotype 4. As for the four major evolutionary branches in HEV genotype 4, the groups I and II of swine HEV had stronger threats to public health than the groups III and IV (Fig.1). As for the group I in HEV genotype 4, HEV strain (swCH31, DQ450072) derived from swine and strain (T1, AJ272108) are obviously divergent from the rest strains in group I. HEV strain (KM01, KJ155502) derived from swine and strains (KU356188, KU356189, KU356187 isolated from cow; KU356183, KU356184, KU356185 isolated from goat; KR872417, KR872415 isolated from human) are grouped into one branch (Fig.1). As for the Group II in HEV genotype 4, the distribution of nucleotide *p*-distances among HEV strains either from human or swine also displays a complex pattern with multiple hierarchies of relatedeness (Fig.1). As for the Group III in HEV genotype

4, these HEV strains from swine have the relatively independent evolutionary pathway. As for the group IV in HEV genotype 4, HEV strain (JF915746) derived from swine have a similar genetic feature with strain (JQ993308) derived from goat. In addition, one main branch, which includes the Group IV, displays an evolutional relatedness among HEV strains (KT727028 and FJ527832) of genotype 3 and ones (KT633715, EF570133, JF915746, JQ993308) of genotype 4 (Fig.1). These results indicate that swine HEV is able to pose threats to the local livestock breeding and public health. Interestingly, HEV strain (HM439284) isolated from human is grouped into a distance branch with the three genotype 8 HEV strains (KX387865, KX387866, KX387867) derived from camel.

4. Discussion

HEV is an emerging and zoonotic pathogen which is expanding its host ranges. Swine is regarded as a major reservoir, and meat-consumption is a major factor for HEV infection (Slot et al., 2017). Although swine is the main reservoir of HEV infection which can infect a variety of animals, HEV infection is often ignored due to non-obvious clinical symptom in healthy persons (Meng et al., 1997; Meng et al., 2002b). HEV is an extremely understudied but important human pathogen (Dimeglio et al., 2018; Sooryanarain and Meng, 2018; Zhou et al., 2018a). To satisfy with a huge meat-consumption, the swine industry has made great progress and pigs are frequently transferred to the farms in different provinces in China. Thus, the nation-wide seroprevalence of HEV infection can benefit for estimating the risk of HEV infection in the swine industry to public health. Currently, there are two main detection methods (RT-PCR and ELISA) for HEV exposure/infection. However, ELISA is simple, safe and reliable in assess the prevalence of anti-HEV antibodies in a specific population. According to the threat of transmission from swine to human, the epidemiological information on seroprevalence of HEV infection in swine is largely concerned globally (Aniţă et al., 2014; Bansal et al., 2017; Caruso et al., 2017; Ivanova et al., 2015; Lange et al., 2016; Liu et al., 2015; Merino-Ramos et al., 2016). Here, we have shown a high prevalence (67.1%) of HEV-specific antibodies in domesticated pigs in the seven provinces of China. Having a huge market for swine meat consumption in China, there are several studies investigated HEV prevalence in swine farms in particular geographical regions of China (Geng et al., 2009; Li et al., 2011; Liang et al., 2014; Ma et al., 2010; Malikin et al., 1989). These previous studies were focused in particular geographical regions in China. Food-borne cases of hepatitis E in humans are increasingly reported and likely underestimated in the medical community due to the lack of information about the nation-wide HEV epidemiology. Here, we undertook a nation-wide approach to estimate the HEV seroprevalence in swine, showing that no significant differences of seroprevalence of HEV infection in pig farms but the significant effects of pig ages and breeding pig purposes on HEV prevalence. The reasons for the high seroprevalence of pig farms in China could be associated with common practices in pig farms (i.e. high population density) and pig transportation between provinces, and shorter production cycles that significantly increased the risk for HEV infection (Walachowski et al., 2014). This lower than the seroprevalence in pigs in Italy (80%), Brazil (82%) and Norway (90%) (Lange et al., 2016; Oliveira-Filho et al., 2017; Pérez-Rosas and Hazen, 1989), but is higher than that in Scotland (29%), Mexican (31%), Vietnam (36%) and Philippines (50%) (Crossan et al., 2015; Huang et al., 2002b; Liu et al., 2015; Merino-Ramos et al., 2016) and similar to that in Germany, Netherlands and India (Baechlein et al., 2010; Bansal et al., 2017; Rutjes et al., 2010). Among the provinces that we have investigated, we did not observe the impact of geographic factor in HEV prevalence. This phenomenon could be explained that water and food supplier contaminated by HEV would directly threat pig population (Kozak and Saelinger, 1988). At different age stages, the seroprevalence in swine with 30 birth days was highest, while the relatively low rate was found in swine with 60 or 90 birth
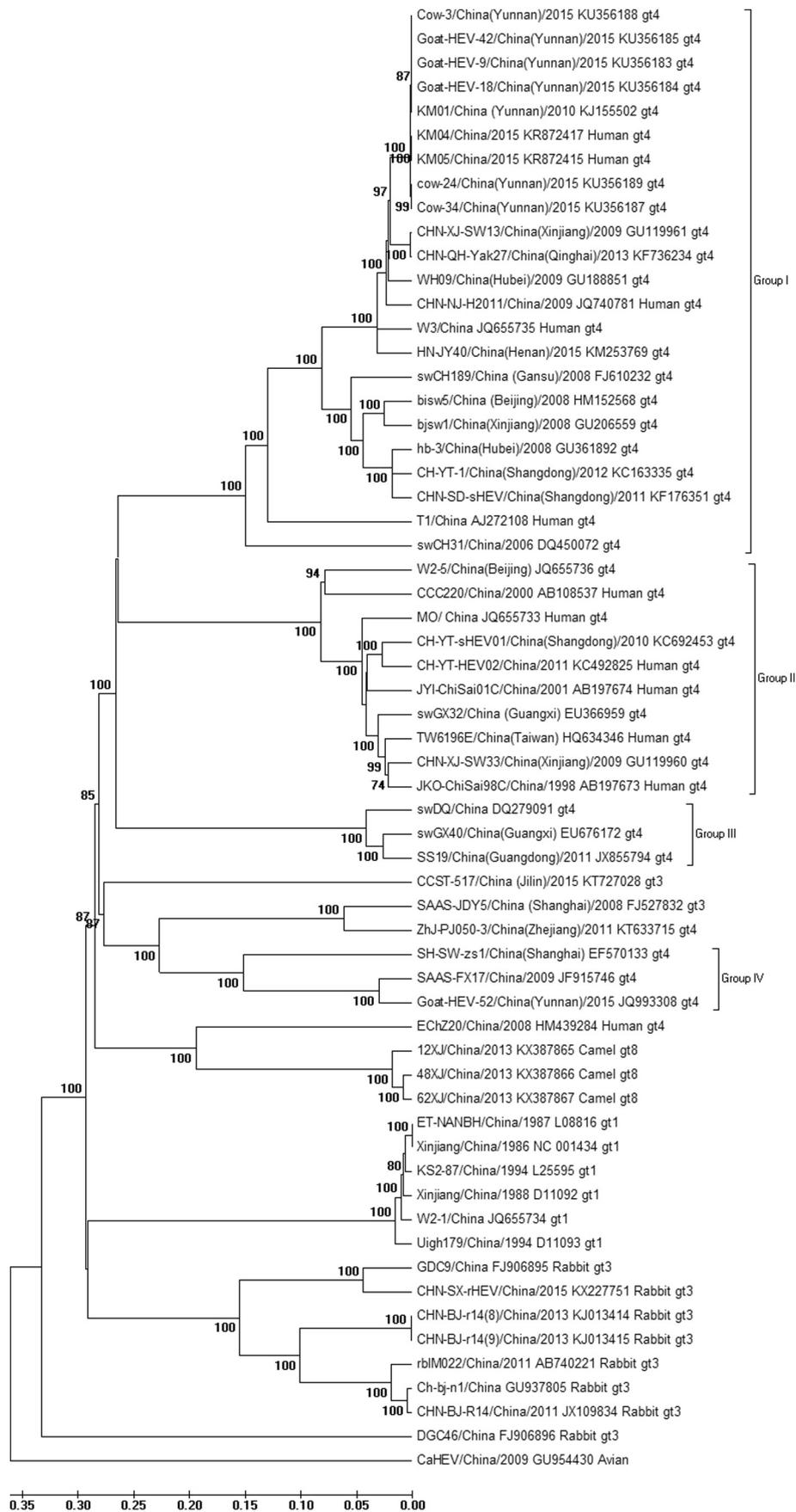
Fig. 1. Phylogenetic analyses of HEV complete genome sequences. A UPGMA tree of *p*-distances is displayed. Branches supported by > 70% of bootstrap replicates are shown. The two HEV genomes have not been assigned to a subtype, namely (i) the strain 'CaHEV/China/2009' (GU954430, belonging to *Orthohepevirus* B) isolated from avian and (ii) the strain 'DGC46/China' (FJ906896, classified into genotype 3) isolated from rabbit.

days. This is probably related to the swine breeding separation carried out according to the specific age stage. Our finding indicated that the seroprevalence in boar used for breeding increased the risk in HEV infection in swine. This result further supports the previous report which indicated that pig semen contaminated by HEV could enhance the risk of HEV infection in pigs by artificial insemination (Li et al., 2018). This study has extended the previous investigation and performed estimation of HEV seroprevalence in pig farms in seven provinces across the east to west China.

HEV RNA detection is the gold standard to identify viremia (Vollmer et al., 2014). In this study, just three resulting PCR products were amplified from these positive serum samples which have been identified by ELISA, implying that the development of viremia is not compatible with antibody response to HEV infection. Generally, viremia and fecal shedding begin 1–2 weeks before and last 2–4 weeks after the onset of symptoms, namely the peak of viremia prior to that of IgM response (Aggarwal and Jameel, 2011). The immune response is marked by an initial increase in anti-HEV immunoglobulin (IgM, followed closely by an IgG response; whereas IgM titers wane off in 4–6 months, IgG persists for longer periods (Khuroo et al., 1993). Anti-HEV IgM might not reach detectable levels at early stages of infection (window period), where HEV RNA usually peaks in blood (Pawlotsky, 2014). Although detection rate of HEV RNA from serum is lower than that of viral RNA from liver or feces, HEV seroprevalence can be rapidly and easily analyzed by ELISA method from swine serum. According to the development of pig transportation between provinces or shorter production cycles in pig farms, rapidly obtaining information about HEV infection can benefit for estimating the risk of HEV-infected pigs to public health.

Meeting with the relatively high levels of seroprevalence in swine in China, investigating the genetic diversity of HEV strains existing in China is important for understanding its present distribution and epidemic / pandemic potential. The 61 HEV genomes derived from different animals during 1986 and 2015 in China display unique evolutionary process of HEV genotype 1 and considerable sequence variety of HEV strains derived from livestock, swine and human. Although rabbit HEV is able to infect swine and human HEV also reportedly infected rabbits (Meng, 2010), rabbit HEV seems to pose a little threats to public health in China. Of note, genotypes 3 and 4 HEV have a much broader host range and can infect across species barriers (Meng, 2016). In China, genotype 4 HEV derived from swine has complex interplay with HEV derived from human and other livestock. The relatively high levels of seroprevalence in pig farms need to appeal to effective control measures to prevent swine HEV to infect human.

In summary, we revealed a high seroprevalence of HEV in swine farms across different provinces in China. In addition, we presented the first intra-epidemic analysis of HEV genome by studying available HEV trains isolated from different susceptible hosts in China and demonstrated the potential of transmission of HEV from swine to other livestock and human. These results call for effective measures that should be carried out to improve sanitation and breeding management in swine production, and to prevent transmission of HEV from infected swine to human.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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