**Hepatitis E vaccine immunization for rabbits to prevent animal HEV infection
and zoonotic transmission**

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Abstract

Hepatitis E virus (HEV) infection has become a significant global public health concern as increasing cases of acute and chronic hepatitis E are reported. HEV of animal origin was proved to be a possible source of human infection and a previous study showed that the recent licensed HEV 239 vaccine can serve as a candidate vaccine to manage animal sources of HEV infection. However, previous immunization strategy for rabbits was the same as that for human, which is too costly to conduct large-scale animal vaccination. In an effort to reduce the costs, three vaccination schemes were assessed in the present study. Forty specific-pathogen-free (SPF) rabbits were divided randomly into five groups with eight animals for each and inoculated intramuscularly with different doses of HEV 239 and placebo, respectively. All animals were challenged intravenously with swine HEV-4 and rabbit HEV of different titers seven weeks after the initial immunization and then fecal virus excretion was monitored for 10 weeks. The results indicated that immunizing rabbits with two 10 µg doses of the vaccine is superior to vaccination with two 20 µg doses or a single 30 µg dose, which can protect rabbits against homologous and heterologous HEV infection. These findings could enable implementation of large-scale animal vaccination to prevent rabbit HEV infection and zoonotic transmission.

Keywords: Hepatitis E virus; Zoonotic transmission; Rabbits; HEV 239 vaccine; Immunization strategy.

39 **Abbreviations:** rHEV: HEV strain isolated from rabbit; sHEV-4: HEV-4 strain
40 isolated from swine.

1. Introduction

Hepatitis E virus (HEV) infection is a major cause of acute viral hepatitis in areas with poor sanitation, and recently became a significant global public health concern as increasing cases of hepatitis E have been reported in developed countries [1]. HEV is a non-enveloped positive sense single-stranded RNA virus that is transmitted mainly through the fecal-oral route. Most cases of hepatitis E are self-limited and the mortality rate is about 1% in the general population [2]. However, HEV infection can lead to poor outcomes in the context of pre-existing liver diseases or pregnancy [3]. The mortality rate rises substantially to approximately 20% in pregnant women infected with HEV [4]. Moreover, chronic hepatitis E has been identified in immunocompromised individuals, including organ transplant recipients [5], patients receiving chemotherapy [6], and HIV-infected individuals [7]. Additionally, non-hepatic complications associated with HEV infection, such as neurologic disorders and pancreatitis, have been reported [8-11].

HEV belongs to the genus *Hepevirus* in the *Hepeviridae* family, which includes at least four major genotypes that can infect humans. Genotypes 1 and 2 infections are restricted to human and are the major cause of sporadic cases and waterborne epidemic acute viral hepatitis in resource-limited regions. Genotypes 3 and 4 are responsible for sporadic cases of hepatitis E in both developing and developed areas and are thought to be zoonotically transmitted from animal reservoirs to humans via the consumption of uncooked or undercooked infected meat or by direct contact with infected animals

[12-18]. In the last decade, the sporadic cases of autochthonous HEV infection caused by genotype 3 and 4 reported in industrialized countries have increased, which are mainly zoonotic and transmitted by ingesting uncooked or poorly cooked pork or game meat [1,12,19-27]. Moreover, in recent years, considerable cases of fulminant hepatitis E and chronic hepatitis E have been increasingly reported after eating undercooked meats and entrails of pigs, wild boars and deer [18,28-31]. Therefore, it is essential to manage HEV zoonotic transmission from animal reservoirs to humans. However, prevention of human infection remains challenging as the target population is currently uncertain and possible transmission routes are not fully understood. Therefore, preventing the spread of HEV from animal hosts seems to be a practical measure. Strategies might include avoiding uncooked or undercooked meat products, ensuring the proper disposal of animal feces and vaccinating animal reservoirs. However, the first two ways are too complicated to control, especially for people who in general likes eating something uncooked or undercooked. In addition, soft fruits or vegetables irrigated with contaminated surface water may also constitute potential sources of zoonotic transmission [32-33], which are not easy to control. Hence, vaccination of primary hosts could be the optimal option, but this would be a costly undertaking with the same vaccine program as for humans. Thus, it is of interest to explore an immunization strategy for animal reservoirs of HEV to prevent animal HEV infection and zoonotic transmission as well.

HEV was isolated from rabbits in China, USA and France recently [34-36].

Phylogenetic analysis showed that the rabbit HEV isolates are most closely related to

genotype 3 HEV, but sequences of the full-length genome have < 80% identity to the genotype 1-4 HEV. Besides, rabbit HEV is characterized by an insertion of 31-aa in the X domain of ORF1, which does not exist in genotype 1-4 HEV, but is present in all rabbit strains isolated worldwide. Thus, some researchers have suggested that it represents a new genotype [34,37], while others considered it as a distant member of the zoonotic genotype 3 [35,38].

Accumulative lines of evidence have indicated that rabbits are sensitive to HEV infection and seem to constitute an animal reservoir of HEV [34,39]. The fact that rabbits can be experimentally infected by sHEV-4 [40] or human HEV-4 [41] and that rHEV is transmissible from rabbits to cynomolgus macaques [42] both suggest that rabbits may be a new source of human HEV infection. Interestingly, a HEV strain in rabbits closely related to human HEV strain has been identified in France [36], and recently, a HEV strain closely related to a human HEV sequence was identified for the first time in the pet house rabbit [43]. These findings might shed new light on the potential of zoonotic HEV transmission from rabbits to humans. Furthermore, HEV infection in rabbits could lead to adverse outcomes in pregnancy and vertical transmission [44]. In order to explore the immunization strategy for rabbits to prevent animal HEV infection and zoonotic transmission, we tested in the present the protective efficacy of HEV vaccine with various dosages and immunization times on rabbits challenged by HEV strains with different titers and genotypes.

2. Materials and Methods

2.1. Ethics statement

The animal experiments were approved by the Committee of Laboratory Animal Welfare and Ethics, Peking University Health Science Center. This study was performed in strict accordance with the protocol for the review on Laboratory Animal Welfare and Ethics, Peking University Health Science Center.

2.2. Vaccines

The HEV 239 vaccine (Hecolin; Xiamen Innovax Biotech, Xiamen, China) is a bacterially expressed recombinant peptide corresponding to amino acid residues 368-606 of ORF2 of HEV-1, which contains 30 µg of the purified antigen in 0.5 mL buffered saline adsorbed to 0.8 µg aluminum hydroxide [45]. The licensed hepatitis B vaccine (10 µg of Engerix-B, GlaxoSmithKline, Belgium) was used as the placebo.

2.3. Specific-pathogen-free rabbits

Forty 7-week-old, specific-pathogen-free (SPF) Japanese white rabbits (1-1.5kg) were obtained from the Department of Laboratory Animal Science of Peking University Health Science Center. Prior to vaccination, all rabbits were confirmed

negative for anti-HEV antibodies by an enzyme-linked immunosorbent assay (ELISA) and negative for HEV RNA in fecal/serum by RT-nPCR [40]. Each rabbit was placed in a separate cage and fed twice a day.

2.4. Challenge Viruses

A rabbit HEV strain of CHN-BJ-R14 (rabbit feces, JX109834, rHEV) and a swine HEV genotype 4 strain of SD-sw2 (pig feces, KP284140, sHEV-4) isolated from feces, which were prepared as suspension in phosphate-buffered saline (PBS, pH 7.4), were used as the inocula in this study. The viral suspension was centrifuged at 5000 rpm at 4 °C for 30 min and filtered sequentially through 0.45-μm and 0.22-μm filters before inoculation. The viral load of each HEV strain was adjusted to both 6×10^4 copies/mL and 6×10^6 copies/mL, according to their original titers determined by TaqMan RT-qPCR as described previously [46].

2.5. Immunization and challenge

All rabbits were divided randomly into five groups (A-E) with eight rabbits per group. The rabbits in group A, B and C were inoculated intramuscularly in the thigh on week 0 with a 10 μg, 10 μg and 20 μg dose of the vaccine in 0.5 mL, respectively, and exactly the same vaccination procedure was repeated on week 4. The rabbits in group D were only vaccinated intramuscularly in the thigh on week 0 with a 30 μg dose

in 0.5 mL. Similarly, 10 µg dose of the hepatitis B vaccine were administered twice to rabbits in group E on week 0 and 4. On week 7, eight vaccinated rabbits in each group were further divided into two subgroups of four rabbits, which were then challenged intravenously with 1 mL of inocula (the rabbits in subgroup A1 and A2 were challenged with 6×10^4 copies/mL of rHEV or sHEV-4, and those in other subgroups were challenged with 6×10^6 copies/mL of rHEV or sHEV-4, Fig. 1). At the same time, eight placebo-vaccinated rabbits were divided into four subgroups with two rabbits each, which were also challenged intravenously with 1 mL of inocula (the rabbits in subgroup E1 and E2 were challenged with 6×10^6 copies/mL of rHEV or sHEV-4, and those in subgroup E3 and E4 were challenged with 6×10^4 copies/mL of rHEV or sHEV-4, Fig. 1).

2.6. Sample Collection and Processing

Samples of serum and feces were collected prior to inoculation and weekly for 10 weeks post-challenge. The amount of HEV RNA in feces was determined by TaqMan RT-qPCR [46]. Anti-HEV antibody was detected using standard methods [40]. Antibody level was expressed in titer, which was defined as the maximum dilution of the test sample that yields an average OD value that exceeded the cut-off value. The anti-HEV levels were also calibrated against a World Health Organization (WHO) reference standard for anti-HEV (National Institute for Biological Standards and Control (UK), catalogue no. 95/584), which was from Wantai, Biopharmaceutical,

Beijing, China and had been assigned an arbitrary value of 100 U/mL [47].

2.7. Statistical methods

All data was processed by SAS 9.1. The mean antibody levels of different groups were compared using two a sample independent t-test with the assumption that the mean antibody levels of each group follow a normal distribution. Vaccine efficacy against infection was calculated as $100(1 - R)\%$, where R is the ratio of the proportion of infection among vaccinated rabbits to the proportion of infection among placebo recipients. Vaccine efficacy was evaluated based on the assumption that the number of HEV infection in each group is independent variable following a Poisson distribution. Proportions were compared using a two-sided Fisher's exact test. A P -value ≤ 0.05 was considered statistically significant; while a P -value > 0.05 indicates no significant difference [45].

3. Results

3.1. Antibody responses of rabbits to vaccinations

All vaccinated rabbits mounted anti-HEV responses while each of the control animals remained negative for antibodies to HEV during the 7-week pre-challenge period. The first dose of 10 μ g, 20 μ g and 30 μ g was found to have induced 93.8%,

87.5% and 100% seroconversion in animals on week 4, respectively (Table 1). Meanwhile, we also observed a direct correlation between the level of anti-HEV titer and vaccine dose at this time, with the geometric mean antibody titers (GMT) of 13, 20, and 32 for 10- μ g, 20- μ g and 30- μ g dose, respectively. When the booster dose was administered, more dramatic rise GMT values was, however, observed in groups receiving two 10 μ g and two 20 μ g doses compared with that receiving a single 30 μ g dose. The former rose up to approximately 690, whereas the latter only rose slightly to a level less than 100 (Fig. 2). Anti-HEV levels in every subgroup at the time of challenge are showed in Fig. 3. Two 10 μ g and two 20 μ g doses of vaccine triggered greater antibody responses, with the maximum mean antibody levels reaching up to (571.98 $\pm$ 431.31) U/mL, while that induced by a single 30 μ g dose merely rose to less than 200 U/mL (Fig. 3 and Table 2). Statistical analysis indicated that there was no significant difference among group A, B and C ($P = 0.6493$), whereas the mean antibody level of group D was significantly lower than other three groups ($P < 0.0001$). Also, the mean antibody levels of two subgroups in each group were comparable at the time of challenge ($P > 0.05$).

3.2. Protective efficacy against HEV infection post-challenge

The course of HEV infection after challenge was monitored for 10 weeks by measuring serum antibody responses and fecal shedding (Table 2). As expected, all of the control rabbits excreted virus in stool concurrent with anti-HEV seroconversion.

Unlike a single 30 µg dose of the vaccine, two 10 µg and two 20 µg doses both achieved a more robust and reliable protection against HEV infection, as confirmed by the statistical analysis that the number of rabbits shedding virus in group A, B or C was significantly less than that in group D and E ($P < 0.05$), while there was no statistical difference between group A, B and C ($P > 0.05$), or between group D and E ($P > 0.05$). Accordingly, the post-challenge mean antibody levels in group D and E, rather than those in group A, B and C, were four times higher than the corresponding base line levels prior to challenge, which was considered to indicate the occurrence of infection [48]. Additionally, the protective efficacy for various vaccine dosages and immunization times was also evaluated in this study. Compared with the placebo, two 10 µg and two 20 µg doses were effective (100% and 86% efficacy, 95% confidence interval of 63-100% and 42-100%, respectively, $P < 0.05$) in preventing HEV infection, while a single 30 µg dose was only 43% effective (95% confidence interval of 63-82%), which had no statistical difference compared with the placebo ($P > 0.05$). The two 20 µg doses of the vaccine were unsuccessful for protection of rabbits from HEV infection since 25% rabbits (1/4) in group C1 were infected. Furthermore, as in the control groups E1 and E2, four rabbits receiving a single 30 µg dose developed sustained fecal shedding, though with lower levels and shorter duration of viral excretion as compared to those in group E (Fig. 4). It is also of note that the anti-HEV levels of these five rabbits rose by more than four-fold (Table 3). Therefore, it was most likely that HEV infection occurred in these animals.

Interestingly, among 25 uninfected rabbits in this experiment, 23 developed

anti-HEV levels higher than 54 U/mL pre-challenge, while the antibody levels for those 5 infected rabbits were consistently lower than 51 U/mL. Based on this observation, it is reasonable to speculate that anti-HEV levels with ≥ 54 U/mL might be sufficient to protect rabbits against rHEV or sHEV-4 infection.

4. Discussion

Hepatitis E has been traditionally supposed to be endemic in developing countries, where it is a major health issue. Notably, very recently, compelling evidence has suggested that most cases of human HEV infection are autochthonous in industrialized areas, which are mainly caused by HEV-3 or HEV-4 and transmitted zoonotically [12]. Growing bodies of evidence indicate that animal reservoirs of HEV might be a possible source of human infection. The demonstration that hepatitis E occurred in groups of people who had consumed raw or undercooked meat from infected animals provided direct evidence of HEV cross-species transmission to humans [13,49-50]. Furthermore, it has been demonstrated in experimental settings that swine and rabbit HEVs are able to infect non-human primates [42,51]. In addition, the findings that HEV strains circulating in animals are genetically related to strains identified in indigenous human cases also support the speculation that animal reservoirs might be a common source of zoonotic HEV [52-53]. The zoonotic nature of HEV dictates that zoonotic transmission can possibly be prevented through vaccination of animal reservoirs such as pigs and rabbits. HEV vaccine is the world's first commercial hepatitis E

vaccine with a protective efficacy of 100% (95% CI 72.1-100.0) and shows no unexpected side effects in the general population in Phase III trials [54] and pregnant women [55]. Moreover, our group has revealed that HEV 239 vaccine could completely protect rabbits against rHEV and sHEV-4 infections using the same immunization strategy as for humans, which, however, is expensive and not feasible for large-scale animal vaccinations [56]. Hence, in the present study, we explored the hepatitis E immunization strategy for rabbits by testing the protective efficacy of HEV 239 vaccine with different dosages and immunization times on rabbits against the challenge by different titers and genotypes of HEV.

In this study, two 10 µg doses of the vaccine showed more excellent immunogenicity in rabbits than two 20 µg doses and a single 30 µg dose. Besides, it was also shown that the GMT values rose mildly in response to the first vaccination, as is characteristics of primary antibody response and rose sharply in response to the second immunization, as is characteristics of booster antibody response. Nevertheless, the anti-HEV levels induced by two 20 µg doses varied across different rabbits (<1000U/mL) in this study, similar as what has been observed for rhesus monkeys (>1000U/mL) in a previous research [45], which was likely due to the variation in immune status between different animals. In regard to the protective efficacy, two 10 µg and two 20 µg doses of the vaccine, rather than a single 30 µg dose, could protect most rabbits against rHEV and sHEV-4 infections. Thus, vaccinating rabbits with the vaccine could contribute to decrease the risk of HEV zoonotic transmission.

The rabbit HEV strains we used here were isolated and identified by our group and

may cause severe hepatitis in SPF rabbits and are transmissible from rabbits to cynomolgus macaques [40,42,44]. Genotype 4 HEV was dominant in China [52] and sHEV-4 used in this study could infect rabbit and cause severe hepatitis in our pre-experiment, thus this study selected these two particular strains of HEV. In addition, the range of HEV RNA titers in samples of rabbits from natural environment and in our laboratory settings were 10^2 - 10^5 copies/mL [57] and 10^3 - 10^6 copies/mL [our data unpublished], respectively. The viral loads of HEV reported for human shedding were 10^2 - 10^6 copies/mL in general [58-59]. Also, the dosages of 6×10^4 copies/mL and 6×10^6 copies/mL were proved to be infectious for rabbit in our pilot experiment. Therefore, we selected 6×10^4 copies/mL as the low viral load and 6×10^6 copies/mL as the high viral load. Moreover, an intravenous challenge was performed in this study, which is more efficient than fecal-oral route [60-61] in natural environment and could ensure all rabbits infected. Thus, if the vaccine could protect rabbits against intravenous challenge with the high viral load of 6×10^6 copies/mL, it could also protect rabbits against HEV infection by fecal-oral route in field conditions.

Furthermore, the relationship of anti-HEV level and infection was analyzed on the basis of the large-scale animal vaccinations in the current study. Antibody levels with ≥ 54 U/mL might be sufficient to protect against infection, which would be likely to provide laboratory evidence for developing the immunization strategy to prevent animal HEV infection and zoonotic transmission.

According to literatures, the criterion to judge an effective immunizing dose or vaccine strategy is that the vaccine could confer complete protection against HEV

infection on the vaccinated subjects [45,62], that is, to the current setting, none of the vaccinated rabbits showed fecal shedding accompanied by more than four-fold rise in anti-HEV levels. Based on this criterion, we concluded that two 10 µg doses of the HEV 239 vaccine were superior to two 20 µg doses and a single 30 µg dose in preventing rabbits against the challenge with rHEV or sHEV-4. Whether two 5 µg doses or less have a better effect needs further study. The results of this research demonstrated that HEV 239 vaccine could be capable of cross protection and could be suitable for protecting animal reservoirs (rabbits) from HEV infection, and thus could also contribute to the implementation of large-scale animal vaccination to prevent HEV zoonotic transmission worldwide. In addition, a similar and interesting study on pigs should be performed in the future considering that pigs have been recognized as a major animal reservoir of zoonotic HEV and could potentially pose additional impact on human health.

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References

- [1] Kamar N, Bendall R, Legrand-Abravanel F, Xia NS, Ijaz S, Izopet J, et al. Hepatitis E. *Lancet* 2012;379(9835):2477-88.
- [2] Mast EE, Krawczynski K. Hepatitis E: an overview. *Annu Rev Med* 1996;47:257-66.
- [3] Kamar N, Dalton HR, Abravanel F, Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev* 2014;27(1):116-38.
- [4] Navaneethan U, Al MM, Shata MT. Hepatitis E and pregnancy: understanding the pathogenesis. *Liver Int* 2008;28(9):1190-9.
- [5] Kamar N, Selves J, Mansuy JM, Ouezzani L, Peron JM, Guitard J, et al. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* 2008;358(8):811-7.
- [6] Tavitian S, Peron JM, Huynh A, Mansuy JM, Ysebaert L, Huguet F, et al. Hepatitis E virus excretion can be prolonged in patients with hematological malignancies. *J Clin Virol* 2010;49(2):141-4.
- [7] Dalton HR, Bendall RP, Keane FE, Tedder RS, Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. *N Engl J Med* 2009;361(10):1025-7.
- [8] Deniel C, Coton T, Brardjanian S, Guisset M, Nicand E, Simon F. Acute pancreatitis: a rare complication of acute hepatitis E. *J Clin Virol* 2011;51(3):202-4.
- [9] Kamar N, Weclawiak H, Guilbeau-Frugier C, Legrand-Abravanel F, Cointault O, Ribes D, et al. Hepatitis E virus and the kidney in solid-organ transplant patients. *Transplantation* 2012;93(6):617-23.
- [10] Cheung MC, Maguire J, Carey I, Wendon J, Agarwal K. Review of the neurological manifestations of hepatitis E infection. *Ann Hepatol* 2012;11(5):618-22.
- [11] Colson P, Payraudeau E, Leonnet C, De Montigny S, Villeneuve L, Motte A, et al. Severe thrombocytopenia associated with acute hepatitis E virus infection. *J Clin Microbiol* 2008;46(7):2450-2.
- [12] Dalton HR, Kamar N, Izopet J. Hepatitis E in developed countries: current status and future perspectives. *Future Microbiol* 2014;9(12):1361-72.
- [13] Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, et al. Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis* 2005;11(12):1958-60.

356 [14] Perez-Gracia MT, Suay B, Mateos-Lindemann ML. Hepatitis E: an emerging disease. *Infect Genet*
357 *Evol* 2014;22:40-59.

358 [15] Renou C, Cadranet JF, Bourliere M, Halfon P, Ouzan D, Rifflet H, et al. Possible zoonotic
359 transmission of hepatitis E from pet pig to its owner. *Emerg Infect Dis* 2007;13(7):1094-6.

360 [16] Meng XJ, Wiseman B, Elvinger F, Guenette DK, Toth TE, Engle RE, et al. Prevalence of
361 antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in
362 the United States and other countries. *J Clin Microbiol* 2002;40(1):117-22.

363 [17] Kim YM, Jeong SH, Kim JY, Song JC, Lee JH, Kim JW, et al. The first case of genotype 4
364 hepatitis E related to wild boar in South Korea. *J Clin Virol* 2011;50(3):253-6.

365 [18] Miyashita K, Kang JH, Saga A, Takahashi K, Shimamura T, Yasumoto A, et al. Three cases of
366 acute or fulminant hepatitis E caused by ingestion of pork meat and entrails in Hokkaido, Japan:
367 Zoonotic food-borne transmission of hepatitis E virus and public health concerns. *Hepatol Res*
368 2012;42(9):870-8.

369 [19] Dalton HR, Stableforth W, Thuraiajah P, Hazeldine S, Remnarace R, Usama W, et al.
370 Autochthonous hepatitis E in Southwest England: natural history, complications and seasonal
371 variation, and hepatitis E virus IgG seroprevalence in blood donors, the elderly and patients with
372 chronic liver disease. *Eur J Gastroenterol Hepatol* 2008;20(8):784-90.

373 [20] Mitsui T, Tsukamoto Y, Hirose A, Suzuki S, Yamazaki C, Masuko K, et al. Distinct changing
374 profiles of hepatitis A and E virus infection among patients with acute hepatitis, patients on
375 maintenance hemodialysis and healthy individuals in Japan. *J Med Virol* 2006;78(8):1015-24.

376 [21] Dalton HR, Fellows HJ, Gane EJ, Wong P, Gerred S, Schroeder B, et al. Hepatitis E in new
377 zealand. *J Gastroenterol Hepatol* 2007;22(8):1236-40.

378 [22] Mansuy JM, Peron JM, Abravanel F, Poirson H, Dubois M, Miedouge M, et al. Hepatitis E in the
379 south west of France in individuals who have never visited an endemic area. *J Med Virol*
380 2004;74(3):419-24.

381 [23] Drobeniuc J, Greene-Montfort T, Le NT, Mixson-Hayden TR, Ganova-Raeva L, Dong C, et al.
382 Laboratory-based surveillance for hepatitis E virus infection, United States, 2005-2012. *Emerg*
383 *Infect Dis* 2013;19(2):218-22; quiz 353.

384 [24] Colson P, Borentain P, Queyriaux B, Kaba M, Moal V, Gallian P, et al. Pig liver sausage as a
385 source of hepatitis E virus transmission to humans. *J Infect Dis* 2010;202(6):825-34.

- [25] Mirazo S, Ramos N, Mainardi V, Gerona S, Arbiza J. Transmission, diagnosis, and management of hepatitis E: an update. *Hepat Med* 2014;6:45-59.
- [26] Dalton HR, Bendall R, Ijaz S, Banks M. Hepatitis E: an emerging infection in developed countries. *Lancet Infect Dis* 2008;8(11):698-709.
- [27] Hoofnagle JH, Nelson KE, Purcell RH. Hepatitis E. *N Engl J Med* 2012;367(13):1237-44.
- [28] Masuda J, Yano K, Tamada Y, Takii Y, Ito M, Omagari K, et al. Acute hepatitis E of a man who consumed wild boar meat prior to the onset of illness in Nagasaki, Japan. *Hepatol Res* 2005;31(3):178-83.
- [29] Legrand-Abravanel F, Kamar N, Sandres-Saune K, Garrouste C, Dubois M, Mansuy JM, et al. Characteristics of autochthonous hepatitis E virus infection in solid-organ transplant recipients in France. *J Infect Dis* 2010;202(6):835-44.
- [30] Tei S, Kitajima N, Ohara S, Inoue Y, Miki M, Yamatani T, et al. Consumption of uncooked deer meat as a risk factor for hepatitis E virus infection: an age- and sex-matched case-control study. *J Med Virol* 2004;74(1):67-70.
- [31] Dalton HR, Bendall RP, Pritchard C, Henley W, Melzer D. National mortality rates from chronic liver disease and consumption of alcohol and pig meat. *Epidemiol Infect* 2010;138(2):174-82.
- [32] Brassard J, Gagne MJ, Genereux M, Cote C. Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. *Appl Environ Microbiol* 2012;78(10):3763-6.
- [33] Kokkinos P, Kozyra I, Lazic S, Bouwknegt M, Rutjes S, Willems K, et al. Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. *Food Environ Virol* 2012;4(4):179-91.
- [34] Zhao C, Ma Z, Harrison TJ, Feng R, Zhang C, Qiao Z, et al. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. *J Med Virol* 2009;81(8):1371-9.
- [35] Cossaboom CM, Cordoba L, Dryman BA, Meng XJ. Hepatitis E virus in rabbits, Virginia, USA. *Emerg Infect Dis* 2011;17(11):2047-9.
- [36] Izopet J, Dubois M, Bertagnoli S, Lhomme S, Marchandeau S, Boucher S, et al. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. *Emerg Infect Dis* 2012;18(8):1274-81.
- [37] Geng J, Wang L, Wang X, Fu H, Bu Q, Zhu Y, et al. Study on prevalence and genotype of hepatitis E virus isolated from Rex Rabbits in Beijing, China. *J Viral Hepat* 2011;18(9):661-7.

416 [38] Purdy MA, Khudyakov YE. Evolutionary history and population dynamics of hepatitis E virus.
417 PLoS One 2010;5(12):e14376.

418 [39] Pavio N, Meng XJ, Doceul V. Zoonotic origin of hepatitis E. *Curr Opin Virol* 2015;10:34-41.

419 [40] Han J, Lei Y, Liu L, Liu P, Xia J, Zhang Y, et al. SPF rabbits infected with rabbit hepatitis E virus
420 isolate experimentally showing the chronicity of hepatitis. *PLoS One* 2014;9(6):e99861.

421 [41] Ma H, Zheng L, Liu Y, Zhao C, Harrison TJ, Ma Y, et al. Experimental infection of rabbits with
422 rabbit and genotypes 1 and 4 hepatitis E viruses. *PLoS One* 2010;5(2):e9160.

423 [42] Liu P, Bu QN, Wang L, Han J, Du RJ, Lei YX, et al. Transmission of hepatitis E virus from
424 rabbits to cynomolgus macaques. *Emerg Infect Dis* 2013;19(4):559-65.

425 [43] Caruso C, Modesto P, Prato R, Scaglione FE, De Marco L, Bollo E, et al. Hepatitis E Virus: First
426 Description in a Pet House Rabbit. A New Transmission Route for Human? *Transbound Emerg*
427 *Dis* 2015;

428 [44] Xia J, Liu L, Wang L, Zhang Y, Zeng H, Liu P, et al. Experimental infection of pregnant rabbits
429 with hepatitis E virus demonstrating high mortality and vertical transmission. *J Viral Hepat* 2015;

430 [45] Li SW, Zhang J, Li YM, Ou SH, Huang GY, He ZQ, et al. A bacterially expressed particulate
431 hepatitis E vaccine: antigenicity, immunogenicity and protectivity on primates. *Vaccine*
432 2005;23(22):2893-901.

433 [46] Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR. A broadly reactive one-step
434 real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J Virol Methods*
435 2006;131(1):65-71.

436 [47] Ferguson M, Walker D, Mast E, Fields H. Report of a collaborative study to assess the suitability
437 of a reference reagent for antibodies to hepatitis E virus. *Biologicals* 2002;30(1):43-8.

438 [48] Zhang J, Zhang XF, Zhou C, Wang ZZ, Huang SJ, Yao X, et al. Protection against hepatitis E
439 virus infection by naturally acquired and vaccine-induced immunity. *Clin Microbiol Infect*
440 2014;20(6):O397-405.

441 [49] Matsuda H, Okada K, Takahashi K, Mishiro S. Severe hepatitis E virus infection after ingestion of
442 uncooked liver from a wild boar. *J Infect Dis* 2003;188(6):944.

443 [50] Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to
444 human beings. *Lancet* 2003;362(9381):371-3.

445 [51] Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, et al. Genetic and

experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol* 1998;72(12):9714-21.

[52] Liu P, Li L, Wang L, Bu Q, Fu H, Han J, et al. 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* 2012;12(2):428-34.

[53] Purdy MA, Khudyakov YE. The molecular epidemiology of hepatitis E virus infection. *Virus Res* 2011;161(1):31-9.

[54] Zhu FC, Zhang J, Zhang XF, Zhou C, Wang ZZ, Huang SJ, et al. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. *Lancet* 2010;376(9744):895-902.

[55] Wu T, Zhu FC, Huang SJ, Zhang XF, Wang ZZ, Zhang J, et al. Safety of the hepatitis E vaccine for pregnant women: a preliminary analysis. *Hepatology* 2012;55(6):2038.

[56] Liu P, Du Rj, Wang L, Han J, Liu L, Zhang Y, et al. Management of hepatitis E virus (HEV) zoonotic transmission: protection of rabbits against HEV challenge following immunization with HEV 239 vaccine. *PLoS One* 2014;9(1):e87600.

[57] Jirintai S, Jinshan, Tanggis, Manglai D, Mulyanto, Takahashi M, et al. Molecular analysis of hepatitis E virus from farm rabbits in Inner Mongolia, China and its successful propagation in A549 and PLC/PRF/5 cells. *Virus Res* 2012;170(1-2):126-37.

[58] Abravanel F, Sandres-Saune K, Lhomme S, Dubois M, Mansuy JM, Izopet J. Genotype 3 diversity and quantification of hepatitis E virus RNA. *J Clin Microbiol* 2012;50(3):897-902.

[59] Tabatabai J, Wenzel JJ, Soboletzki M, Flux C, Navid MH, Schnitzler P. First case report of an acute hepatitis E subgenotype 3c infection during pregnancy in Germany. *J Clin Virol* 2014;61(1):170-2.

[60] Purcell RH, Emerson SU. Animal models of hepatitis A and E. *ILAR J* 2001;42(2):161-77.

[61] Cheng X, Wang S, Dai X, Shi C, Wen Y, Zhu M, et al. Rabbit as a novel animal model for hepatitis E virus infection and vaccine evaluation. *PLoS One* 2012;7(12):e51616.

[62] Purcell RH, Nguyen H, Shapiro M, Engle RE, Govindarajan S, Blackwelder WC, et al. Pre-clinical immunogenicity and efficacy trial of a recombinant hepatitis E vaccine. *Vaccine* 2003;21(19-20):2607-15.

Figure captions

Fig. 1. Experimental design.

There was one rabbit in group C2 and D1 each suffered from an accidental death before challenge.

Fig. 2. Dynamics of GMT induced by various types of vaccination pre-challenge.

Y axis represents Geometric mean titers of anti-HEV; X axis represents the time after vaccination in weeks; The rabbits in group A, B and C were inoculated intramuscularly on week 0 with a 10 µg, 10 µg and 20 µg dose of the vaccine, respectively, and were vaccinated again with the same dose in the same way like before on week 4. The rabbits in group D were only vaccinated intramuscularly on week 0 with a 30 µg dose. Similarly, 10 µg dose of the Hepatitis B vaccine were administered twice to the rabbits in group E on week 0 and 4; all animals were challenged on week 7.

Fig. 3. The comparison of anti-HEV levels in each group pre-challenge.

Y axis represents anti-HEV levels on week 7 at the time of the challenge expressed in WHO unit/mL; Statistical analysis indicated that there was no significant difference among group A, B and C ($P = 0.6493$), whereas the mean antibody level of group D was significantly lower than other three groups ($P < 0.0001$).

Fig. 4. Dynamics of viral load in feces of infected rabbits in group D and group E.

Y axis represents $\text{Log}_{10}[\text{copies/g}]$ of HEV RNA in feces; X axis represents the time after challenge in weeks; The upper figure: 16[#], 38[#] (group D1) and 18[#] (group E1)

496 which were challenged with 10^6 copies/mL of rHEV; The under figure: 29[#], 39[#]
497 (group D2) and 21[#] (group E2) which were challenged with 10^6 copies/mL of sHEV-4.

Table 1

The seroconversion rates of anti-HEV antibody after vaccination.

Groups (No.)	HEV 239 (μg)	The seroconversion rates of anti-HEV (%)		
		1w ^a	4w ^b	5w ^c
A+B (16)	2×10	10/16 (62.5)	15/16 (93.8)	16/16 (100)
C (8)	2×20	5/8 (62.5)	7/8 (87.5)	8/8 (100)
D (8)	2×30	4/8 (50)	8/8 (100)	8/8 (100)
E (8)	2×0	0	0	0

^a A week after the first immunization.

^b Before the second immunization.

^c A week after the second immunization.

Table 2

Protection of rabbits against challenge with rHEV/sHEV-4

Subgroup ^a (No.)	HEV 239 (μg)	Pre-challenge	Post-challenge		
		Mean anti-HEV level $\pm$ SD (U/mL) ^b	Number of rabbits shedding virus in feces ^c	Ab resp. ^d	Vaccine efficacy and 95% CI (%) <i>P</i> values
A1 (4)	2×10	192.90 ± 135.30	0/4	-	100 (63-100) A vs. E
A2 (4)	2×10	442.15 ± 235.91	0/4	-	<i>P</i> < 0.0001
B1 (4)	2×10	313.63 ± 173.47	0/4	-	100 (63-100) B vs. E
B2 (4)	2×10	394.47 ± 436.43	0/4	-	<i>P</i> = 0.0014
C1 (4)	2×20	571.98 ± 431.31	1/4	-	86 (42-100) C vs. E
C2 (4)	2×20	280.42 ± 189.01	0/3	-	<i>P</i> = 0.0014
D1 (4)	1×30	145.17 ± 80.00	2/3 ^e	+ (>30 fold)	43 (10-82) D vs. E
D2 (4)	1×30	190.98 ± 98.20	2/4 ^e	+(>4 fold)	<i>P</i> = 0.0769
E1 (2)	2×0	< 1	2/2	+(>4 fold)	0
E2 (2)	2×0	< 1	2/2	+(>4 fold)	0
E3 (2)	2×0	< 1	2/2	+(>4 fold)	0
E4 (2)	2×0	< 1	2/2	+(>4 fold)	0

^a 1: rHEV, 2: sHEV-4.

^b The mean antibody level $\pm$ standard deviation (SD) in every subgroup pre-challenge.

^c Number of rabbits shedding virus in feces/total number of rabbits in the group.

^d Ab resp. (+) represents the mean anti-HEV level in every subgroup post-challenge is four times higher than that pre-challenge.

^e Number of rabbits shedding virus in group D was more than that in group A, B and C (*P* < 0.05).

There was one rabbit in group C2 and D1 each suffered from an accidental death before challenge.

Table 3

Virus shedding and anti-HEV responses of infected rabbits after vaccination

Sub-groups	Rabbit ID	Vaccine (µg)	Challenge viruses		Virus shedding		Anti-HEV level (U/mL)	
			Virus genotype	Viral load (copies/mL)	Duration (w) ^a	Log ₁₀ [peak] ^b	pre-challenge	post-challenge ^c
C1	17 [#]	2×20	rHEV	6×10 ⁶	1	4.33	34.66	488.42
D1	16 [#]	1×30	rHEV	6×10 ⁶	3	5.81	6.89	9486.02
D1	38 [#]	1×30	rHEV	6×10 ⁶	6	6.27	7.88	6026.38
D2	29 [#]	1×30	sHEV-4	6×10 ⁶	6	7.1	50.06	451.23
D2	39 [#]	1×30	sHEV-4	6×10 ⁶	5	5.69	34.06	1855.97

^a Duration (w) is the duration of virus shedding in feces.^b Log₁₀[peak] is the log₁₀[peak of fecal HEV RNA].^c Anti-HEV level on week 5 post-challenge.

Fig. 1

Fig. 2

Fig. 3

Fig. 4