Egg whites from eggs of chickens infected experimentally with avian hepatitis E virus contain infectious virus, but evidence of complete vertical transmission is lacking

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Avian hepatitis E virus (HEV) is genetically and antigenically related to human HEV. Vertical transmission of HEV has been reported in humans, but not in other animals. In this study, we showed that avian HEV could be detected in chicken egg-white samples. Subsequently, avian HEV in egg white was found to be infectious, as evidenced by the appearance of viraemia, faecal virus shedding and seroconversion in chickens inoculated with avian HEV-positive egg white, but not in chickens inoculated with HEV-negative egg white. To further assess the possibility of vertical transmission of avian HEV, batches of embryonated eggs from infected hens were hatched, and hatched chicks were monitored for evidence of avian HEV infection. However, no virus was detected in samples collected from the hatched chicks throughout this study, suggesting that avian HEV could not complete the vertical transmission cycle. The possible implications of our findings are also discussed.

Hepatitis E virus (HEV), which causes epidemic and sporadic self-limiting acute hepatitis, is an important public health disease in many developing countries where sanitation conditions are poor (Purcell & Emerson, 2001). Although only sporadic cases of domestically acquired hepatitis E have recently been reported in industrialized countries, including the USA, the UK, France and Japan (Amon et al., 2006; Sadler et al., 2006; Mansuy et al., 2004; Mizuo et al., 2005), anti-HEV antibodies are detected in 1–20% of the general population in these countries (Purcell & Emerson, 2004). The mortality associated with HEV infection is generally as low as less than 1% in the general population; however, in pregnant women, the mortality rate can reach up to 20%, the reason for this high rate is unknown (Purcell & Emerson, 2001). HEV is also reportedly transmitted from infected mothers to their babies, causing significant perinatal morbidity and mortality (Kumar et al., 2001, 2004; Khuroo et al., 1995).

Hepatitis E is a zoonosis (Meng, 2005). Anti-HEV antibodies have been detected in a number of animal species including pigs, chickens and rodents (Favorov et al., 2000; Tien et al., 1997). Several animal strains of HEV, including swine HEV from pigs and avian HEV from chickens (Meng et al.,1997; Haqshenas et al., 2001; Takahashi et al., 2004; Nishizawa et al., 2005), have been definitively isolated. Cross-species infections have been demonstrated, as a US-2 strain of human HEV infected specific-pathogen-free (SPF) pigs and a swine HEV strain infected non-human primates (Halbur et al., 2001; Meng et al., 1998a). Swine were considered an important source of infection for swine veterinary workers and swine farmers (Zheng et al., 2006; Meng et al., 2002). However, the detection of a high level of anti-HEV prevalence in human populations who have no history of exposure to pigs suggests that multiple sources of exposure may exist (Meng et al., 2002). Rodents could serve as a reservoir for HEV, since they are widely distributed in urban as well as rural environments; however, HEV has not yet been isolated from rodents (Emerson & Purcell, 2003). HEV transmission via the consumption of undercooked or

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The GenBank/EMBL/DDBJ accession numbers for the avian HEV sequences of virus isolated from egg white are DQ660974, DQ660975, DQ660976, DQ660977 and DQ660978.
raw pig liver or deer meats has been reported, and could be a source of the high anti-HEV prevalence observed (Yazaki et al., 2003).

Rhesus monkeys are frequently used as the animal model for HEV study; however, infected pregnant monkeys failed to transmit the virus to offspring (Tsarev et al., 1995). An attempt to demonstrate vertical transmission of HEV from pregnant gilts to offspring was unsuccessful (Kasorndorkhua et al., 2003). Avian HEV was first isolated from chickens with hepatitis–splenomegaly (HS) syndrome in the USA, and was shown to be genetically and antigenically related to human HEV (Hqaqshenas et al., 2001, 2002; Huang et al., 2002; Sun et al., 2004; Billam et al., 2005). The question of whether HEV can be vertically transmitted under controlled experimental conditions remains unknown.

We previously produced an avian HEV infectious faecal stock with a titre of $10^8$ genome equivalents (GE) ml$^{-1}$ (Guo et al., 2006a), by inoculating specific-pathogen-free (SPF) chickens intravenously with 200 μl avian HEV. During the process of preparing the virus stock, some chickens started to lay eggs. Twenty intact eggs were collected at 3 weeks post-infection (p.i.). It is of interest to know whether avian HEV can be detected in these eggs. The detection of avian HEV in eggs was performed with a modified protocol that is routinely used for avian influenza virus detection in the Veterinary Diagnostic Laboratory of Iowa State University (Ames, USA). All egg shells were washed with 70% ethanol before the separation of white and yolk. White sample was diluted 1 : 3 with 0.01 M PBS buffer (pH 7.2) and centrifuged for 5 min at 5000 g. The supernatants (100 μl) were used for RNA extraction with an RNeasy Mini kit (Qiagen). RNA extraction and nested RT-PCR were performed as described previously (Guo et al., 2006a). Five out of 20 eggs collected (nos 4, 13, 14, 16 and 17) had detectable avian HEV RNA in egg white. Positive egg-white samples were further titrated by a semi-quantitative nested RT-PCR method as described previously (Meng et al., 1998b). Briefly, positive egg-white samples were diluted 10-fold serially with 0.01 M PBS buffer (pH 7.2), and each diluent was used for RNA extraction and nested RT-PCR. One GE was defined as the number of viral genomes present in the highest 10-fold dilution that is positive by nested RT-PCR (Meng et al., 1998b). The results showed that egg-white samples no. 4 and 14 contained $10^5$ GE avian HEV RNA ml$^{-1}$, sample no. 13 had $10^4$ GE avian HEV RNA ml$^{-1}$, and samples no. 16 and 17 both had $10^3$ GE avian HEV RNA ml$^{-1}$. The final PCR products amplified from these egg-white samples were sequenced, and sequence analysis confirmed that the viruses recovered from egg white originated from the inocula, as more than 98% nucleotide sequence similarity was found between viruses in the samples and the inocula. The avian HEV sequences from egg-white samples no. 4, 13, 14, 16 and 17 were deposited in GenBank with the accession numbers DQ660974, DQ660975, DQ660976, DQ660977 and DQ660978, respectively. Thus, our finding suggested that avian HEV can be passed into eggs, in addition to its distribution in liver, bile, serum and faeces in infected chickens (Hqaqshenas et al., 2001; Huang, et al., 2002; Sun et al., 2004; Billam et al., 2005).

To evaluate the infectivity of avian HEV in egg whites, 38 SPF hens and two SPF roosters (all 22-week-old), negative for antibodies to avian HEV, were tagged and divided into 3 groups. Groups 1 and 2 had 18 hens each (nos 1–18 in Group 1, and nos 19–36 in Group 2), and chickens in each group were housed in six cages with three hens per cage. Group 3 contained two hens (no. 37 and 38) and two roosters (no. 39 and 40) that were housed together in a room. All chickens in Group 1 were inoculated intravenously with 400 μl each of egg-white sample no. 13 (10$^4$ GE ml$^{-1}$). All chickens in Group 2 were similarly inoculated with 400 μl normal egg-white sample (negative for avian HEV RNA by RT-PCR). The two hens in Group 3 were each inoculated intravenously with 400 μl avian HEV infectious stock with a 10$^4$ GE ml$^{-1}$ titre. Serum and faeces were collected from the three groups of chickens weekly for 5 weeks. Three chickens from each of Groups 1 and 2 were necropsied weekly and bile samples were collected. All samples were tested for the presence of avian HEV RNA by the nested RT-PCR method. In Group 1, chickens inoculated with RT-PCR-positive egg-white sample no. 13, viraemia and faecal virus shedding were detected from 3 weeks p.i. (Table 1). Virus was also detected variably in bile samples collected during necropsies in Group 1 chickens. However, viraemia and virus shedding in faeces or bile were not detected from Group 2 chickens, which were inoculated with RT-PCR-negative egg-white samples. As expected, both hens in Group 3 that received an infectious stock of avian HEV had detectable viraemia and faecal virus shedding starting at 1 week p.i.

Anti-avian HEV antibody response was measured by an indirect ELISA as previously described (Guo et al., 2006b). Prior to inoculation, all chickens in the three groups were seronegative for avian HEV. Anti-avian HEV IgG was detected at 2 weeks p.i. in both hens in Group 3, which were inoculated with an avian HEV infectious stock, and remained seropositive throughout the study (Fig. 1). For the six chickens from Group 1 that were continuously monitored for 5 weeks, anti-avian HEV IgG was detected at 4 and 5 weeks p.i. As expected, anti-avian HEV antibody was not detected in any of the chickens from Group 2, which were inoculated with RT-PCR-negative egg-white samples. The results of the infectivity study demonstrated that the virus in the egg white is infectious, as evidenced by the detection of viraemia and faecal virus shedding in chickens inoculated with the RT-PCR-positive egg-white samples (Table 1). Seroconversion and slight elevation of serum levels of lactate dehydrogenase (data not shown) were also detected in the chickens inoculated with RT-PCR-positive egg-white sample, although these occurred later than in
chickens inoculated with an avian HEV infectious stock (Fig. 1), which is possibly due to avian HEV in faecal stock being distributed to the liver (the avian HEV primary replication site) more quickly than that contained in egg whites.

To know whether avian HEV can be further transmitted into newborn chicks, embryonated eggs were collected daily from Group 3 chickens, from 1 week before virus inoculation to 5 weeks p.i. Eggs collected during the same period were hatched as one batch in an RX1 incubator with automatic egg turning (Lyon Electric Company). Temperature and humidity were adjusted according to the manufacturer’s instructions. A total of six batches of eggs were hatched. Each batch of chicks was housed separately and monitored daily for faecal virus shedding and viraemia for 1 week before necropsy. As shown in Table 2, 11–13 eggs were laid weekly by the two hens in Group 3, and the fertility of each batch of eggs reached from 91 to 100%. The hatchability of eggs collected from 1 week before virus inoculation to 4 weeks p.i. ranged from 82 to 85%. Eggs collected at 5 weeks p.i. had a relatively low hatchability (75%). Each batch of chicks was monitored daily for viraemia and faecal shedding by nested RT-PCR. Bile and liver samples collected from all chicks necropsied at 7 days after hatching were also subjected to nested RT-PCR. However, all the hatched chicks were negative for avian HEV infection.

The finding of infectious avian HEV in egg white raised a question as to whether infected chicken eggs could be a potential source for the high seroprevalence of IgG antibodies in chickens experimentally inoculated with RT-PCR-positive egg white and with avian HEV infectious stock. Sera from 14 chickens continuously monitored throughout the 5-week study were diluted at 1:100 and tested for the presence of anti-avian HEV ORF2 antibodies by ELISA. Recombinant avian HEV ORF2 antigen was coated at 100 ng per well on the ELISA plates. Each point represents the mean values (±SEM) of the A490 obtained from duplicate ELISA results. Chickens no. 13–18 were inoculated with egg-white sample no. 13 that is RT-PCR-positive for avian HEV RNA. Chickens no. 31–36 were inoculated with an RT-PCR-negative egg-white sample. Chickens no. 37 and 38 were inoculated with avian HEV infectious stock as positive controls. Chicken numbers and Groups are indicated on the right.

Fig. 1. Seroconversion to avian HEV IgG antibodies in chickens experimentally inoculated with RT-PCR-positive egg white and with avian HEV infectious stock. Sera from 14 chickens continuously monitored throughout the 5-week study were diluted at 1:100 and tested for the presence of anti-avian HEV ORF2 antibodies by ELISA. Recombinant avian HEV ORF2 antigen was coated at 100 ng per well on the ELISA plates. Each point represents the mean values (±SEM) of the A490 obtained from duplicate ELISA results. Chickens no. 13–18 were inoculated with egg-white sample no. 13 that is RT-PCR-positive for avian HEV RNA. Chickens no. 31–36 were inoculated with an RT-PCR-negative egg-white sample. Chickens no. 37 and 38 were inoculated with avian HEV infectious stock as positive controls. Chicken numbers and Groups are indicated on the right.

### Table 1. Detection of avian HEV RNA in sera, faecal swabs and bile samples from inoculated chickens

<table>
<thead>
<tr>
<th>Chicken group</th>
<th>Weeks p.i.</th>
<th>No. of positive sera (faecal swabs)/total tested</th>
<th>No. of positive bile samples/total tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td>0</td>
<td>0(0)/18</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0(0)/18</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0(0)/15</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10(8)/12</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9(9)/9</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6(5)/6</td>
<td>2/6</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td>0</td>
<td>0(0)/18</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0(0)/18</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0(0)/16</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0(0)/12</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0(0)/12</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0(0)/6</td>
<td>0/6</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td>0</td>
<td>0(0)/2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2(2)/2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2(2)/2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2(2)/2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2(2)/2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2(1)/2</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Chickens were inoculated with an egg-white sample positive for avian HEV RNA by RT-PCR.
†Chickens were inoculated with normal egg white negative for avian HEV.
§Only the two hens were inoculated with an avian HEV infectious stock.
§ND, Not done.
anti-HEV observed in the general population of industrialized countries, including the USA (Purcell & Emerson, 2004), where avian HEV is highly prevalent, as anti-avian HEV antibodies have been found in approximately 71% of the chicken flocks in five states (Huang et al., 2002). Although it was previously shown that avian HEV failed to infect two rhesus monkeys (Huang et al., 2004), avian HEV does have the ability to cross species barriers and infect turkeys (Sun et al., 2004). Until now, there is no direct evidence as to whether avian HEV can infect humans.

Chicken eggs are a popular and highly nutritious food, and eating raw eggs is common in many parts of the world (Doorduyn et al., 2006; Lievonen et al., 2004; Shiferaw et al., 2000; Sun et al., 2000). A multistate surveillance conducted in the USA between 1995 and 1996 found that as many as 50% of the responders ate undercooked or raw eggs, and more than 60% did not wash hands after cracking raw eggs, and this habit has remained unchanged for years (Yang et al., 1998; Fein et al., 2002). In addition, several kinds of food contain raw eggs, such as meringue, hollandaise sauce, cookie dough, homemade mayonnaise and Caesar salad dressing (Mazurek et al., 2005; McNally, 2006). The significant percentage of individuals eating raw and undercooked eggs suggests that even if avian HEV does not infect humans, repeated exposures to the same foreign micro-organism could cause the production of anti-HEV IgG antibody, especially since avian HEV could survive the acidic stomach environment after its oral inoculation into chickens (Billam et al., 2005).

We have previously identified common antigenic epitopes in the ORF2 protein of avian and human HEV (Guo et al., 2006b). Western blot and ELISA results indicated that the avian HEV ORF2 capsid protein reacted with anti-human HEV antiserum. Convalescent serum from SPF chickens experimentally infected with avian HEV also reacted with the recombinant ORF2 capsid proteins of human HEV (Haqshenas et al., 2002). Therefore, it is possible that the IgG anti-HEV antibodies detected from healthy individuals in the USA and other industrialized countries could be the result of repeated exposures to avian HEV through the consumption of raw chicken eggs. Further studies are warranted to determine definitively if there is a correlation between IgG anti-HEV seroprevalence and egg-eating habits in humans.

Vertical transmission of human HEV was first reported in India, where six out of eight infants from infected mothers had HEV infection (Khuroo et al., 1995). In another study, approximately two-thirds of the infected pregnant women had preterm deliveries (Kumar et al., 2004), and approximately 8% of HEV RNA-positive infants born to the infected mothers developed acute clinical disease that could cause early neonatal deaths (Kumar et al., 2001). The mortality rate among HEV-infected pregnant women is as high as 20% (Purcell & Emerson, 2001); however, attempts to reproduce severe hepatitis experimentally in pregnant rhesus monkeys and pregnant sows were unsuccessful, and the infected pregnant animals failed to transmit the virus to newborns (Tsarev et al., 1995; Kasorndorkbua et al., 2003).

Although avian HEV could be transmitted to egg white, it was not able to establish vertical transmission, as no virus was detected in any sample collected from a total of 60 hatched chicks. Shivaprasad & Woolcock (1995) reported that the agent associated with the HS syndrome could not be isolated from chicken embryos by conventional routes of egg inoculation. In contrast, we showed that avian HEV could be propagated successfully in embryonated chicken eggs by inoculating 100 GE avian HEV intravenously into 9-day-old embryonated chicken eggs (Meng et al., 2006). Avian HEV was detected in bile and liver samples collected from hatched chicks necropsied at 2–3 days of age with a very high GE titre (Meng et al., 2006). The virus had also been continuously detected in faeces for at least 8 days after hatching.

In this study, however, instead of inoculating embryonated eggs, we directly inoculated each hen with 4000 GE avian HEV, collected their eggs for hatching and then monitored the presence of virus in the hatched chicks. In the five positive egg-white samples from chickens inoculated with 4000 GE avian HEV, two had an avian HEV genomic titre of $10^2$ GE ml$^{-1}$ and three had a titre of $\geq 10^5$ GE ml$^{-1}$. A typical chicken egg contains more than 10 ml white, thus in

### Table 2. Hatchability of eggs collected from hens experimentally infected with avian HEV

<table>
<thead>
<tr>
<th>Batch*</th>
<th>No. of eggs hatched</th>
<th>Hatchability (%)</th>
<th>No. of eggs unhatched</th>
<th>Fertility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dead embryos</td>
<td>No fertilization</td>
</tr>
<tr>
<td>−1</td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>85</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>82</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>85</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>75</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Eggs collected during the same period were hatched in a batch before (batch −1) and after ( batches 1–5) hens were inoculated intravenously with avian HEV infectious stock.
this study, avian HEV virus could enter the egg white with a relatively high dose to infect chicken embryos and chicks; however, no virus was detected in samples from the 60 hatched chicks with the same methods as described by Meng et al. (2006). Taken together, the present and previous results (Meng et al., 2006) suggest that avian HEV in eggs cannot survive the early embryonation phase (1–9 days of embryonation) and is thus unable to transmit further to chicks. A suitable animal model for HEV vertical transmission study still needs to be investigated.

Acknowledgements

This work is supported in part by a grant (to X.-J. M.) from the National Institutes of Health (AI 50611).

References


