EVIDENCE FOR WIDESPREAD INFECTION OF WILD RATS WITH HEPATITIS E VIRUS IN THE UNITED STATES

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Abstract. Hepatitis E is an important medical pathogen in many developing countries but is rarely reported from the United States, although antibody to hepatitis E virus (anti-HEV) is found in > 1% of U.S. citizens. Zoonotic spread of the virus is suspected. Sera obtained from 239 wild rats trapped in widely separated regions of the United States were tested for anti-HEV. Seventy-seven percent of rats from Maryland, 90% from Hawaii, and 44% from Louisiana were seropositive for anti-HEV. Rats from urban as well as rural areas were seropositive and the prevalence of anti-HEV IgG increased in parallel with the estimated age of the rats, leading to speculation that they might be involved in the puzzling high prevalence of anti-HEV among some U.S. city dwellers. The discovery of anti-HEV in rats in the United States and the recently reported discovery that HEV is endemic in U.S. swine raise many questions about transmission, reservoirs, and strains of HEV in developed countries.

Hepatitis E is the first or second most important cause of acute clinical hepatitis in many developing countries of Asia, the Middle East and North Africa. Hepatitis E can occur sporadically or in epidemics and the peak clinical attack rate usually occurs in young adults. Hepatitis E is caused by hepatitis E virus (HEV), an unclassified virus that is enterically transmitted. Antibody to HEV (anti-HEV), indicative of past infection, has been detected in only 5–60% of the general population of developing countries where the disease is endemic. The peak age-specific acquisition of anti-HEV occurs in young adults. The relatively low prevalence of antibody in young children and the relatively late acquisition of infection in some populations are unusual patterns for a virus that is believed to be transmitted principally by the fecal-oral route. Although this pattern is consistent with a cohort effect, studies from India suggest that this is not the explanation.

In industrialized countries, clinical hepatitis E is rarely reported and the few cases that do occur are generally among individuals who acquired their infection in a developing country. It is therefore perplexing that anti-HEV has been detected in these industrialized countries in 0.4–5.0% of healthy populations (generally blood donors), even in the absence of known risk factors. In some studies, even higher prevalences of anti-HEV have been found in specific populations in the United States. Although the specificity of some tests used for measuring anti-HEV has been questioned, it is unlikely that false-positive results can explain the relatively high prevalence of anti-HEV in populations with no significant clinical hepatitis E.

It has been proposed that animal reservoirs of HEV exist in some regions and that human infections may represent, in part, a zoonosis. The successful transmission of HEV to swine, rats, and sheep in the former USSR and in Asia has been reported, as has the transmission of HEV to several non-human primate species. Antibody to HEV has been detected in swine, wild rats, mice, and monkeys from areas where hepatitis is endemic and in domestic swine and colony-raised macaques in the United States. Recently, a novel HEV strain (swine HEV) was recovered from naturally infected swine in the United States and transmitted to specific pathogen-free swine under experimental conditions. Interestingly, an HEV strain recovered from a case of clinical hepatitis E in the United States was found to be closely related to swine HEV, suggesting that the patient’s HEV infection might have been zoonotic. Indeed, it was subsequently shown that the U.S.-2 strain of human HEV and the swine strain could be experimentally transmitted to swine and to non-human primates, respectively, and thus could cross species barriers.

However, since populations previously reported to have an unusually high prevalence of anti-HEV in the United States would not be likely to have significant exposure to swine, we have looked for evidence of other potential animal reservoirs. In this study, we tested sera of wild rats trapped in different regions of the United States for serologic evidence of HEV infection and performed an epidemiologic analysis of the results.

MATERIALS AND METHODS

Collection of rat sera. Wild rats were trapped in three geographic regions: the inner city of Baltimore, Maryland, urban and rural regions of the islands of Oahu and Hawaii, Hawaii and New Orleans, Louisiana. Eighty-three sera were collected in 1997 from wild rats in 7 different locations within the city of Baltimore, Maryland. Rats were collected using standardized, live trapping protocols in central alleys of residential neighborhoods. After trapping, rats were brought to a central laboratory, anesthetized, identified to species, weighed, and external body measurements were recorded. Blood was collected by cardiac puncture, allowed to clot at room temperature for 1 hr, centrifuged, and stored at −70°C. All the rats tested were identified as Rattus norvegicus.

One hundred forty-seven sera were similarly collected in 1986 from 3 different species of wild rats in urban and rural areas.
species. The serum samples had been stored at 2°C were stored at −20°C. The sera trapped along the levee in New Orleans, Louisiana. The sera were stored were stored at −20°C.

Enzyme-linked immunosorbent assay for anti-HEV in rat sera. The ELISA for detection of anti-HEV in rat sera was performed essentially as described for the detection of anti-HEV in primate and swine sera, using as antigen a truncated (55 kD) recombinant HEV capsid protein expressed from baculovirus in SF-9 insect cells. The recombinant HEV capsid protein was expressed from the sequence of the SAR-55 strain of HEV, a prototype human HEV strain recovered from a case of hepatitis E in Pakistan. For detection of anti-HEV in rat sera, the original secondary antibody was replaced with peroxidase-labeled goat anti-rat IgG or anti-rat IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Serum samples from the wild rats were diluted to 1:100 in blocking buffer (phosphate-buffered saline, pH 7.4, containing 0.05 % Tween 20, 10% fetal calf serum, and 0.5% gelatin) and tested in duplicate. The positive control serum was always used at a dilution of 1:10,000, which was just below the end point titer of the serum.

Four seronegative laboratory rats (strain F344/NCR) were inoculated intramuscularly with 10 μg of recombinant HEV capsid protein in Freund’s incomplete adjuvant and given three booster injections with aqueous protein at two-week intervals. Sera were collected prior to immunization and at weekly intervals. Serum samples from 110 normal laboratory-bred rats (R. norvegicus, strain Sprague-Dawley; Harlan Bioproducts, Indianapolis, IN) of various ages and tested for anti-HEV IgG and IgM in duplicate.

For anti-HEV IgM ELISA standardization, we used sera obtained after hyperimmunization of the rats with open reading frame 2 (ORF 2) protein, as for the anti-HEV IgG ELISA. The ELISA cut-off value was determined by testing the 110 sera collected from normal laboratory rats.

Reverse transcription–polymerase chain reaction (RT-PCR). For detection of HEV RNA, total RNA was extracted from selected rat sera with TRIZOL reagent (Gibco-BRL, Gaithersburg, MD), and reverse transcription and amplification were attempted using the two sets of degenerate primers and methodology described previously for swine HEV.

Statistical analysis. Comparison between mean optical density (OD) values of male and female control rat serum samples was performed with the unpaired t-test, and the mean OD values between the different age groups of rats were compared by one-way analysis of variance.

RESULTS

Standardization of the ELISA for detection of anti-HEV in rat serum. The ELISA for detection of rat anti-HEV was standardized with serum samples collected from four rats hyperimmunized with recombinant ORF 2 protein. All four rats developed anti-HEV titers of 10^5 or greater. Sera from the 110 laboratory rats were tested at a dilution of 1:100 to determine the cut-off value for the ELISA. There was not a significant difference between mean OD values obtained from male rats and from female rats (P = 0.129) or from rats of different ages (approximately 7, 12, 16–17, 26, and 34 weeks of age, respectively) (P = 0.217). The cut-off value for the ELISA (0.220) was set at 3 SD above the mean OD value for these normal rat sera (Figure 1).

As an additional evaluation of the ELISA cut-off value, sera from the 99 wild R. norvegicus rats were screened at a dilution of 1:100 and the distribution of ODs was plotted along with the distribution of ODs from the 110 laboratory rats. As seen in Figure 2, the ODs of sera from the laboratory rats and wild rats demonstrated a bimodal distribution, with virtually all of the values from the laboratory rats falling below an OD of 0.2 and the majority of OD values from wild rats falling above this value. The cut-off value of 3 SD above the mean OD value for the laboratory rats coincided with the trough of the bimodal curve, thus confirming its suitability as the cut-off value for the ELISA.

Prevalence of anti-HEV IgG in wild R. norvegicus rats from three geographic regions. We tested 108 sera collected from R. norvegicus rats trapped in Maryland, Hawaii, and Louisiana. Sixty-four (77%) of 83 rats from Maryland, 15 (94%) of 16 rats from Hawaii, and 4 (44%) of 9 from
Louisiana were positive for anti-HEV. Weights were available for most rats trapped in Maryland and Hawaii. Weight can be used as an indirect indicator of age and R. norvegicus rats reach sexual maturity at a weight of approximately 150–200 grams.²³ The prevalence of anti-HEV IgG increased with weight (Figure 3). Only 40% of juvenile rats and 45% of adolescent rats were positive for anti-HEV compared with 80–100% of adult rats. Thus, R. norvegicus rats appeared to become infected as juveniles or young adults and remained anti-HEV positive thereafter.

**Prevalence of anti-HEV IgG in sera from three species of wild rats in Hawaii.** Rattus norvegicus (brown rat) and R. rattus (black rat) have been distributed worldwide by human activities. Rattus exulans is a species that has been spread throughout Polynesia by human migrations and that also occurs in India, Thailand, and Southeast Asia. All three species of rats reside in Hawaii and were represented in the sera obtained from wild rats in that state. As seen in Table 1, all three species were strongly positive for anti-HEV (83–93%). The anti-HEV status of R. rattus rats was compared with their weights (an indirect measure of maturity). Black rats are smaller on average than brown rats. As with the R. norvegicus species, the prevalence of anti-HEV increased with weight (Figure 4). Forty percent of rats less than 60 grams, 66% of those 61–75 grams, but 95% of those ≥ 76 grams in weight were positive for anti-HEV, which is additional evidence for peak infection rates among young rats.

**Prevalence of anti-HEV IgG in sera by trapping site.** Among the animals trapped in Hawaii, 64 were from 5 regions on the Island of Oahu and 83 were from 6 regions on the Island of Hawaii (Figure 5). Ninety-four to 100% (mean = 98 %) of the rats trapped from the various sites on Oahu and 50–100% (mean = 86%) of the rats trapped at the various sites on Hawaii were positive for anti-HEV IgG. In Baltimore, Maryland, 83 rats were trapped from 7 districts (Figure 6). Overall, 77% of the rats were positive for anti-HEV but this reflected a very low prevalence (27%) in one of the 7 sites surveyed. This low prevalence may reflect, at least in part, the relationship between body size and HEV infection. Rats at this site had an average weight of 232 grams, which was more than 100 grams less than the average body mass of rats at the remaining 6 sites ($P < 0.001$). Seventy-five to 100% (mean = 85%) of the rats from the remaining sites were positive for anti-HEV IgG.

**Detection of anti-HEV IgM and the PCR for HEV RNA.** In an attempt to identify the putative HEV-related agent, the serum samples collected from R. norvegicus rats trapped in Baltimore were tested for anti-HEV IgM and for HEV RNA. Of the 83 sera tested, only one was weakly positive for anti-HEV IgM and four were borderline positive. Forty-seven serum samples were tested by RT-PCR, including five positive or borderline for anti-HEV IgM, 16 positive only for anti-HEV IgG, and 17 negative for both anti-HEV IgG and IgM. Under the experimental conditions used, HEV RNA was not detected in any of the 47 sera.

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**Table 1**

Prevalence of IgG antibody to hepatitis E virus (HEV) open reading frame 2 in serum of wild rats according to the species and to the geographic origin

<table>
<thead>
<tr>
<th>Location</th>
<th>Species</th>
<th>Number of samples</th>
<th>Anti-HEV positive</th>
<th>Percent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maryland</td>
<td>Rattus norvegicus</td>
<td>83</td>
<td>64</td>
<td>77</td>
</tr>
<tr>
<td>Louisiana</td>
<td>R. norvegicus</td>
<td>9</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>Hawaii</td>
<td>R. rattus</td>
<td>113</td>
<td>102</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>R. norvegicus</td>
<td>16</td>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>R. exulans</td>
<td>18</td>
<td>15</td>
<td>83</td>
</tr>
</tbody>
</table>

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**Figure 3.** Relationship between IgG antibody to hepatitis E virus (HEV) open reading frame 2 positivity and weight of *Rattus norvegicus*. Sample sizes are indicated above the corresponding bars.

**Figure 4.** Relationship between IgG antibody to hepatitis E virus (HEV) open reading frame 2 positivity and weight of *Rattus rattus*. Sample sizes are indicated above the corresponding bars.

**Figure 5.** Prevalence of IgG antibody to hepatitis E virus open reading frame 2 in sera of wild rats in Hawaii according to the trapping site. The size of the circle is proportional to the prevalence. The islands are not drawn to scale.
These data strongly suggest that many wild rats in the United States are naturally infected by HEV. Serum of wild rats captured in three widely separated regions of the United States contained antibodies reactive with capsid antigen of a human strain of HEV. A high prevalence of anti-HEV IgG was detected in three different species of rats trapped in urban as well as in rural sites. Previously, anti-HEV was detected in sera from rodents in the central Asian regions of the former Soviet Union where HEV is endemic and the existence of animal reservoirs may explain the persistence of HEV in that environment. Since HEV is frequently spread through contaminated water, humans and animals might be infected by the same source.

In the United States, where hepatitis E is not endemic, natural infection of swine by a unique strain of HEV was recently demonstrated. The observation that an HEV strain was circulating in the U.S. swine population raised the question of whether infection with this virus could explain the unexpectedly high prevalence of anti-HEV in U.S. citizens without known risk factors for HEV infection. However, because the highest prevalence among humans was in large cities, where contact with swine would not be common, we looked for other sources of HEV. Since rats in the former Soviet Union were reported to be seropositive for HEV and rats are ubiquitous city dwellers, we examined rats in the United States for evidence of HEV infection. We found a high prevalence of anti-HEV in wild rats, suggesting that HEV is naturally widespread in these rodents in the United States.

Antibodies to HEV in rat sera were detected by ELISA with antigen derived from the ORF 2 gene of a human strain of HEV from Pakistan. This assay detects anti-HEV with high sensitivity and specificity. In our study, the specificity of the assay was confirmed by the lack of anti-HEV in laboratory rats that were raised in a controlled environment. Since the anti-HEV ELISA based on the SAR-55-derived antigen is able to detect antibody against diverse human strains of HEV, including all of the recognized major genotypes of this virus, one or more epitopes of the ORF 2 antigen must be highly conserved. Indeed, the amino acids of ORF 2 are at least 90% identical among all known strains of HEV.

Anti-HEV of the IgM class was detected in serum of at most five wild rats. In monkeys experimentally inoculated with human HEV strains, the IgM response was low and transient compared with the IgG immune response. In naturally infected swine, anti-HEV IgM appeared earlier than anti-HEV IgG but IgM levels were low and detectable for only 1–2 weeks. In experimentally infected swine, HEV viremia measured by PCR analysis of HEV RNA in the serum was detected for 1–2 weeks and disappeared approximately 1 week before to 1 week after seroconversion. In piglets naturally infected by HEV, seroconversion occurred in very young animals. The same appeared to be true for rats, since the prevalence of anti-HEV IgG increased with the weight, thus age, of rats. In this study, the majority of wild rats trapped in the different areas were adults as shown by their weight. Given the apparent short half-life of anti-HEV IgM, the early age at which infection usually occurs, and the scarcity of young rats in the populations we examined, it was not surprising that we were able to identify only a single rat that was clearly IgM positive.

A major question is whether the HEV strain apparently infecting wild rats is a new strain of HEV unique to rats or whether it is a variant of U.S. swine HEV. Rats are often found in close proximity to swine, thus providing the opportunity for cross-infection. However, urban rats in Hawaii and Maryland are in close proximity with humans, not with swine. Since the ORF 2 protein is so highly conserved, it will probably not be possible to answer this question by serologic means. Rather, the sequence of the virus that is endemic in rats will be required and for this reason an acute case of rat HEV will have to be identified. To identify a possible virus, we initially focused on those five samples that were definitely or marginally reactive in the IgM ELISA as possibly indicating a recent infection. However, we were not able to pass a virus from serum or tissue samples to laboratory rats, nor were we able to amplify HEV sequences from serum using primers designed to amplify all known HEV strains. Since in other HEV infections, the virus is cleared around the time of appearance of anti-HEV and all five samples tested contained anti-HEV IgG, any viremia may have already resolved and any virus still present may have been neutralized. Alternatively, if a totally new strain of HEV was involved, the primers may not be adequate.

To obtain appropriate samples, it will be necessary to catch younger rats and to inoculate sera and tissue extracts from them into laboratory rats that can then be monitored regularly for signs of infection. These studies are in progress.

The origin of the serologic reactivity to HEV in the United States and in other developed countries is still unknown. However, rats are found in virtually all cities around the world and our data showed that wild rats in at least one nonendemic country, the United States, are probably naturally infected by HEV. Whether or not wild rats play a role in the perpetuation of HEV in nature or transmit HEV to human strains of HEV, including all of the recognized major genotypes of this virus, one or more epitopes of the ORF 2 antigen must be highly conserved. Indeed, the amino acids of ORF 2 are at least 90% identical among all known strains of HEV.
humans in nonendemic areas remains to be demonstrated. However, at this time it appears to be a reasonable hypothesis.

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