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The zoonotic LCK-3110 strain of *Rocahepevirus ratti* leads to mild infection in chickens after experimental inoculation

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ABSTRACT

Rocahepevirus ratti [rat hepatitis E virus (HEV)] was originally isolated from rats and found to be non-infectious to nonhuman primates, suggesting humans were not a susceptible host. However, in 2018, rat HEV infections were identified in human patients. High seroprevalence for rat HEV in rats in many countries necessitates studying this emerging zoonotic outbreak. Lack of a human derived rat HEV infectious clone, cell culture systems, and animal models have hindered this effort. In response to the increase in human infection cases by rat HEV, we utilized an infectious clone of the zoonotic rat HEV LCK-3110 strain originally reported from human cases. Capped RNA transcripts of the rat HEV LCK-3110 strain were synthesized, and replication was assessed in both cell culture via transfection and chickens via intrahepatic inoculation. Naive chickens were cohoused together with inoculated chickens. Our results demonstrated that although chickens were susceptible, virus replication was inefficient with only a few of the chickens inoculated with rat HEV having low levels of viremia and fecal virus shedding. However, LCK-3110 HEV was able to transmit between chickens as several naive cohoused chickens became infected as evidenced by viremia, fecal shedding, and the presence of viral protein upon histopathology of the liver. Rat HEV is an emerging zoonotic virus with an ability to spillover across species. Chickens have potential to serve as intermediary hosts, possibly playing a role in rat HEV spread and exposure to humans.

1. Introduction

Chicken is a major protein food source throughout the world, however, undercooked chicken is often contaminated with multiple bacterial species such as *Campylobacter, Salmonella*, and *Clostridium perfringens* (Rouger et al., 2017). Recent studies have highlighted that viruses from poultry, livestock, and pets pose threats to humans (Chen et al., 2021). The close habitat shared between domesticated animals, pets, and humans provide favorable conditions for the evolution and zoonotic adaptation of viruses (Chen et al., 2021). An emerging zoonotic pathogen, hepatitis E virus (HEV), ranks 6th on a list of top 50 spillover zoonotic wildlife viruses with human disease risks (Grange et al., 2021). The *Hepeviridae* family is divided into 4 genera; *Avihepevirus, Chirohepevirus, Paslahepevirus*, and *Rocahepevirus. Avihepevirus* infects avian species, *Chirohepevirus* infects bats, *Paslahepevirus* infects a broad range of mammals including humans, swine, rabbits, deer, etc., and *Rocahepevirus* infects rodents (Purdy et al., 2022). There are also many strains that have acquired an ability to spillover to other species, primarily within the *Paslahepevirus balayani* species. HEV from pigs, camels, rabbits, and deer have been demonstrated to cause disease in humans (Wang and Meng, 2021). The most well studied zoonotic transmission route of HEV to humans is via the consumption of undercooked pork products. Most recently strains from the R*ocahepevirus ratti* (rat HEV)-C1 have been detected in cases of human liver disease (Sridhar et al., 2018). Pigs have also been demonstrated to be susceptible to the LCK-3110 rat HEV strain (Yadav et al., 2024) and have been detected in swine herds (Rios-Muñoz et al., 2024), suggesting they can serve as a transmission vector to humans. The susceptibility of chickens to *Rocahepevirus ratti* HEV strains is yet to be studied.

HEV is an understudied emerging pathogen causing severe disease

Abbreviations: BHK-21, Baby hamster kidney; dpi, Days post infection; HEV, Hepatitis E virus; Huh7 S10–3, Human hepatoma cells; LMH, Leghorn male hepatoma cells; ORF, Open reading frame; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; SPF, Specific pathogen free; wpc, Weeks post contact.

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pathology in pregnant populations (Wu et al., 2020) and immunocompromised humans (Damiris et al., 2022). In general, HEV consists of three open reading frames (ORFs). ORF1 comprises the non-structural polyprotein regulating viral replication. ORF2 and ORF3 are structural proteins translated from the bicistronic sub-genomic RNA (Kenney and Meng, 2019). The virus is seen in two morphological forms in nature: naked and quasienveloped (Yadav and Kenney, 2022). Naked virus is devoid of the ORF3 protein and is more infectious than quasienveloped virions (Kenney and Meng, 2019; Yadav and Kenney, 2022). The host range of HEV has been increasing over the last few decades demonstrating multiple spillover cases to humans and other species (Wang and Meng, 2021). Previously, the major HEV species causing human disease was *Paslahepevirus balayani* (Yadav and Kenney, 2022). However, recent human cases in Hong Kong, Canada, Spain, and France are attributed to *Rocahepevirus ratti* species (Benavent et al., 2023). Rats are a primary natural reservoir for *Rocahepevirus ratti* species of HEV (Reuter et al., 2020). In the last 6 years, 21 cases of rat HEV have been documented in both immunocompromised (12 cases) and immunocompetent (9 cases) humans (Benavent et al., 2023). Lack of knowledge on a transmission source for zoonotic rat HEV transmission events makes it a priority to experimentally test different domesticated livestock animals that may play an important role in emerging HEV outbreaks.

Rocahepevirus ratti-C1 has been demonstrated in various continents of the world including North America, Asia, Africa, and Europe (Reuter et al., 2020). Interestingly, 60 % of *Rocahepevirus ratti* species have been identified in rats from Asia (Benavent et al., 2023). Out of 21 human infections, 16 of them have been reported in Asia (Benavent et al., 2023). Even though the actual transmission source in all reported cases is unknown, rat HEV has been demonstrated in the feces of infected rats suggesting environmental contamination may be a primary transmission route (Benavent et al., 2023). The excretion of rat HEV in urine is debated but could also serve as a potential transmission source if occurring (Benavent et al., 2023; Li et al., 2017). Different risk factors such as contact with infected animals, or the consumption of contaminated food and water has been suggested (Benavent et al., 2023) but the lack of cross species transmission studies has hindered the field of rat HEV.

In the last two decades, successful construction of infectious cDNA clones of *Paslahepevirus balayani* HEV genotypes 1 (human HEV) (Emerson et al., 2001), *Avihepevirus magniiecur* (avian HEV) (Kwon et al., 2011; Park et al., 2015), *Rocahepevirus ratti-C1* (rat HEV) (Li et al., 2015), and *Paslahepevirus balayani* HEV genotypes 3 and 4 (swine HEV) (Zhu et al., 2013; Córdoba et al., 2012) have led to many discoveries on viral pathogenesis, host tropism, and vaccine development (Scholz et al., 2020). However, the previous lack of an efficient cell culture system with HEV led to the use of direct intrahepatic inoculation of RNA transcripts to study the pathogenesis associated with HEV (Huang et al., 2005; Huang et al., 2005). Hence, this study aimed to investigate the spillover ability of a zoonotic *Rocahepevirus ratti* HEV in chickens via direct intrahepatic inoculation of HEV LCK-3110 transcripts into the chicken liver. Our study suggests a possible indirect role of chickens in the transmission of zoonotic rat HEV in the environment and potential for transmission to humans.

2. Materials and methods

2.1. cDNA clone of LCK-3110 Rocahepevirus ratti and A. magnniiecur HEV

The LCK-3110 strain was originally isolated from the feces of a solid organ transplant patient with chronic hepatitis E in Queen Mary Hospital, Hong Kong in 2017. The patient had mild to moderate inflammatory infiltrate comprising small lymphocytes in the portal tracts and was finally cured with ribavirin treatment (Sridhar et al., 2018). The full-length genomic sequence of the LCK-3110 strain of rat HEV (Genbank MG813927.1) was artificially synthesized (Genscript). The plasmid pSP64 poly (A) vector (Promega) was used to clone the full-length rat HEV genome between the SalI and SacI sites as described previously (Yadav et al., 2024). After successful insertion, the plasmid was transformed into stable *E. Coli* (NEB) and grown overnight at 37 °C in the presence of ampicillin. The avian HEV infectious clone was a genotype 2 avian HEV strain (Genbank AY535004) originally recovered from a bile sample from a naturally infected chicken with hepatic splenomegaly syndrome in the United States (Haqshenas et al., 2001) and previously characterized by Huang et al. (Huang et al., 2005).

2.2. Linearization of plasmid DNA

For DNA linearization, plasmid DNA encoding rat HEV was linearized using EcoRI (NEB). Plasmid DNA encoding avian HEV was linearized using Xho1. Five percent of the reaction was subjected to gel electrophoresis with ethidium bromide staining and visualized with ultraviolet light to verify that linearization had occurred.

2.3. In vitro transcription for mRNA synthesis

Viral capped mRNA (LCK-3110 HEV) was made from linearized DNA using the Promega Ribomax Large Scale RNA Production System SP6 (Promega PRP 1300) with anti-reverse cap analogue (ARCA) CAP (TriLink Biotechnologies). Similarly, viral capped mRNA (avian HEV) was made from linearized DNA using the Promega Ribomax Large Scale RNA Production System T7 (Promega PRP 1300). The fidelity of transcripts was assessed and normalized by agarose gel electrophoresis.

2.4. Cell culture

LMH (isolated from chicken liver with hepatocellular carcinoma, ATCC:CRL-2117), BHK-21 (isolated from kidney of golden hamster, ATCC:CCL-10) and Huh7 (human hepatoma cells) S10–3 subclone (Emerson et al., 2010; Yadav et al., 2021) was kindly provided by Dr. XJ Meng. LMH cells were cultured in Waymouth's MB 752/1 medium containing 10 % FBS (Fetal bovine serum). BHK-21 cells were cultured in Eagle's Minimum Essential Medium containing 10 % FBS. Huh7 S10–3 cells were cultured in DMEM (Dulbecco Modified Eagle's Medium) containing 10 % FBS. All cells were grown at 37 C and 5 % CO_2.

2.5. Transfection of LMH, HUH7 S10–3, and BHK-21 cells with in vitro-transcribed capped Rocahepevirus ratti HEV RNA

Cells were seeded to acquire a density of 2×10^6 cells in 12 well plates. Sixteen microliters of 1×10^5 viral RNA copies/ml was utilized for RNA transfection using a Mirus Trans-IT mRNA transfection kit (Mirus bio, MIR 2225). After 48 h of transfection, cells were passaged 1:3 to three new wells and cells were incubated for an additional 3 days.

2.6. Immunofluorescence staining and flow cytometry quantification of in vitro-transcribed capped Rocahepevirus ratti HEV RNA transfected cells

Five days post transfection (dpt), cells were trypsinized and pelleted. Cells were then fixed in 200 μL of 100 % methanol at 4 °C and stored at −80 °C. Cells were centrifuged out of methanol, washed, and resuspended in phosphate buffered saline (PBS). Cells were blocked-in blocking solution (5 % non-fat dried milk, 0.1 % Triton X-100 in PBS; PBST) in a 96-well plate for 30 min at 37 °C. Cells were then washed with PBS once before probing with primary antibody - rabbit anti-truncated ORF2 HEV against P balayani genotype 1 HEV previously shown to cross react with rat and avian HEV ORF2, though more weakly than other P balayani strain ORF2s (Cossaboom et al., 2012; Kenney and Meng, 2015; Sanford et al., 2012) diluted 1:50 in blocking solution for 30 min at 37 °C. After washing twice in PBS, cells were incubated with secondary antibody; goat anti-rabbit-phycoerythrin (PE) (Life Technologies) diluted to 1:200 in PBS for 30 min at 37 °C. Cells were then

washed twice in PBS, resuspended in 200 μL of PBS. Fluorescence was analyzed for 100,000 events using a flow cytometer (BD Accuri C6 Plus, Biosciences, San Diego, CA, USA). Gates were set to exclude dead cells, doublet discrimination based on forward and side scatter profiles, and mock infected cells were used to gate background fluorescence (Yadav et al., 2021).

2.7. Indirect immunofluorescence

At 5 dpt, transfected cells were fixed in 100 % cold methanol, permeabilized with PBST, and blocked with 5 % non-fat milk (Sigma-Aldrich, St. Louis, MO, USA). Immunostaining for ORF2-encoded capsid protein was performed using a 1:200 blocking buffer diluted rabbit anti-truncated ORF2 HEV antibody for 30 min at 37 °C. Cells were washed 3 times with PBST. A fluorescently labeled goat anti-rabbit IgG H&L antibody (Alexa Fluor 594; Abcam, Cambridge, FL, USA) was used at a dilution of 1:400 in PBS to detect bound primary antibodies. 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. For quantification of virus infectivity, wells were manually observed with a fluorescent microscope (Keyence) for specific fluorescence, and the presence of fluorescent foci was recorded. A fluorescent foci was defined as a minimum of one to two cells showing clear intracytoplasmic fluorescence (Yadav et al., 2021).

2.8. Intrahepatic inoculation of chickens with capped RNA transcripts from LCK-3110 rat HEV

All animal experiments in this study were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC 2020A00000068) and virus studies were approved by the Ohio State Institutional Biosafety Committee (IBC 2016R00000082). Specific pathogen free (SPF), white leghorn chickens (obtained from the Ohio State University flock) were anesthetized using isoflurane gaseous anesthesia in the presence of a licensed veterinarian at The Ohio State University. Ultrasonography was used to visualize the liver. Capped LCK-3110 rat HEV transcripts (approximately 1×10^5 viral RNA copies/ml) were intrahepatically inoculated into 10 anesthetized chickens in both the right and left lobe of the liver. As a positive control, 10 chickens were intrahepatically inoculated with the capped RNA transcripts of avian HEV (*Avihepevirus magniiecur,* GenBank: AY535004.1) (Huang et al., 2004) (approximately 1×10^5 viral RNA copies/ml). Eight chickens in the negative control group were each inoculated intrahepatically with 200 μL sterile PBS in each lobe.

Cloacal swabs were obtained by swabbing the cloacal region and colorectum, and sera were collected via wing vein prior to inoculation and weekly thereafter from each chicken. One week post infection, HEV negative chickens were introduced and co-housed with the LCK-3110 rat HEV inoculated chickens. There were five necropsies performed in the study on the 2nd, 3rd, 4th, 5th, and 6th weeks post infection. During necropsy, each chicken, its liver, and its spleen were weighed, and an organ index was calculated by dividing the organ weight by the total carcass weight. Livers were evaluated for evidence of gross lesions such as subcapsular hemorrhage as previously described (Park et al., 2015; Billam et al., 2005). Bile was also collected from the chicken during each necropsy and tested for either rat HEV or avian HEV, respectively.

2.9. RNA extraction and RT-QPCR

RNA extraction was performed using Trizol reagent (Invitrogen) on harvested cell supernatant and cell lysates (prepared by 3 cycles of freeze and thaw) on day 5 from LMH, BHK-21, and Huh7 S10–3 cells. Similarly, RNA was extracted from feces, blood, liver, and bile. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed. Two hundred fifty microliters of each processed sample were used for extracting the RNA. A one step RT-qPCR was carried out using TaqMan Fast Virus 1-step Master Mix (ThermoScientific) using a protocol of 50 °C for 15 min, 95 °C for 2 min and 45 cycles of 95 °C for 5 s and 60 °C for 30 s (Mastercycler RealPlex). A forward primer rat HEV F, 5′-CTTGTTGAGCTYTTCTCCCCT-3′, a reverse primer, 5′-CTGTACCG-GATGCGACCAA-3′, and a probe 5′-FAM- TGCAGCTTGTCTTTGARCCC -Dabcyl-3′ were used for the detection of rat HEV (Sridhar et al., 2018). A 10-fold serial dilution of the in vitro transcribed rat HEV RNA (10^7 to 10^1 copies) was used as the standard for the quantification of the viral genome copy numbers.

For avian HEV, a similar one step RT-qPCR was carried out using a protocol of 50 °C for 30 min, 95 °C for 5 min and three-step cycling of 50 times at 95 °C for 15 s, 60 °C for 75 s and 72 °C for 15 s (Mastercycler RealPlex). A forward primer avian HEV F, 5′-AATGTGCTGCGGGGTGT-CAA-3′, a reverse primer, 5′- CATCTGGTACCGTGCGAGTA-3′ and a probe 5′-FAM- CTCCCAAACGCTCCCAGCCGGA -Dabcyl-3′ were used for the detection of avian HEV (in house designed). A 10-fold serial dilution of the in vitro transcribed avian HEV RNA (10^7 to 10^1 copies) was used as the standard for the quantification of the viral genome copy numbers.

2.10. Histological examinations and immunohistochemistry (IHC)

Liver samples were collected at each necropsy and were fixed in 10 % neutral buffered formalin for routine histopathological examination. Tissues were embedded, sectioned (3.5 μm), and stained with Gill's hematoxylin and eosin (H&E) for light microscopic examination as described previously (Gupta et al., 2012). Formalin-fixed tissue sections were evaluated after performing immunohistochemistry (IHC) for the detection of HEV, as previously described with slight modifications (Gupta et al., 2012). The polyclonal rabbit anti-HEV ORF2 antibody was used as the primary antibody and a horseradish peroxidase conjugated anti-rabbit antibody (BioGeneX) was used for visualization as brown staining. Stained tissues were counterstained with hematoxylin.

2.11. Recombinant protein production for enzyme linked immunosorbent assay (ELISA)

Two nucleotide sequences encoding for the amino acids 391–620 of *Paslahepevirus balayani* genotype 3 ORF2 were codon optimized for bacterial expression and commercially synthesized (gblock; IDT). Amino acids 391–620 which encompassed the region 455–603 of ORF2 known as the protruding domain is the immunodominant epitope in HEV (Santarpia et al., 2020). The average intergenotypic amino acid identity within *Paslahepevirus balayani* strains is 89.5 % while the amino acid identity with LCK-3110 was only 48 % (Sridhar et al., 2021). In addition, cross reactivity of antibodies raised against the capsid protein of HEV gt3 has been demonstrated with rat HEV (Simanavicius et al., 2018). The individual sequences were inserted into a bacterial T7 expression vector pRSETa (Invitrogen). Restriction enzyme digestion and Sanger sequencing verified the insertion. Recombinant protein for ELISA was produced using BL21 (DE3) chemically competent cells via auto-induction (Studier, 2005). Proteins were analyzed via SDS-PAGE and western blot with anti-HEV ORF2 polyclonal rabbit serum. The bacteria were lysed with B-Per™ reagent (Thermofisher) (5 mL/gram) with 1 mM ethylenediaminetetraacetic acid (EDTA). ELISA protein was solubilized and purified with Ni-NTA columns followed by dialysis. Protein was visualized using western blot. Protein was quantified using a Bradford assay.

2.12. ELISA

ELISAs were modified and optimized to detect HEV ORF2-specific IgY antibodies in serum. Five μg/ml of purified protein diluted in carbonate buffer (20 mM Na2CO3, 20 mM NaHCO3, pH 9.6) were bound to Nunc Maxisorp 96 well plates (Thermofisher) at 50 μL per well at 4 °C overnight. One hundred fifty microliters of blocking buffer [4 % nonfat dried milk in PBS with 0.1 % Tween (PBST 0.1 %)] was added to the antigen-coated wells and incubated for 2 h at 37 °C. 50 μL of serum was

heat inactivated at 56 °C for 30 min. Inactivated serum was 2 -fold serially diluted in blocking buffer and added to each well. The plates were incubated for 1 hour at 37 °C. After washing, 50 µL of HRP-conjugated secondary antibody [donkey anti-chicken IgY (Sigma)] in 4 % NFDM/PBST (0.1 %) at a dilution of 1:200 K was added and incubated at 37 °C for 1 h. Wells were washed with PBST (0.1 %) five times between each step. 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Seracare) was added (50 µL) and incubated for approximately 10 min, and the reaction was stopped by adding 50 µl of 0.3 mol/L sulfuric acid. Plates were read at an absorbance of 450 nm using a SpectraMax F5 plate reader (Molecular Devices). All experiments were done under the same conditions with each sample tested three times.

2.13. Virus culture

Huh7 S10–3 cells were seeded in six well plates at 2×10^5 cells per well in 2 ml of DMEM with 10 % FBS and penicillin (100 units/ml) and streptomycin (100 g/ml) and incubated at 37 °C for 24 h. The infections were performed utilizing a 10 % fecal suspension (feces from LCK-3110 rat HEV inoculated chickens collected on day 28) diluted 1:5 in DMEM and 0.45 µm filtered (Thermo Scientific™ Nalgene™ Sterile Syringe Filters.). Culture media was removed, and cells were inoculated with 1 ml of the resulting solution (2×10^5 viral RNA copies). At room temperature, plates were rocked for 1 h and then incubated at 37 °C for 6 h. The inoculum was removed, and fresh culture media was added. Supernatants were collected on day 0, 2, 4, and 6. Supernatants and lysates were tested for rat HEV via RT-qPCR.

2.14. Statistical analyses and reproducibility

All quantitative data are presented with the mean and standard deviation. Two-way Analysis of Variance (ANOVA) and Student's unpaired two-tailed t-test were used to determine the statistical significance between the groups. GraphPad Prism 9.4.1 was used to do statistical

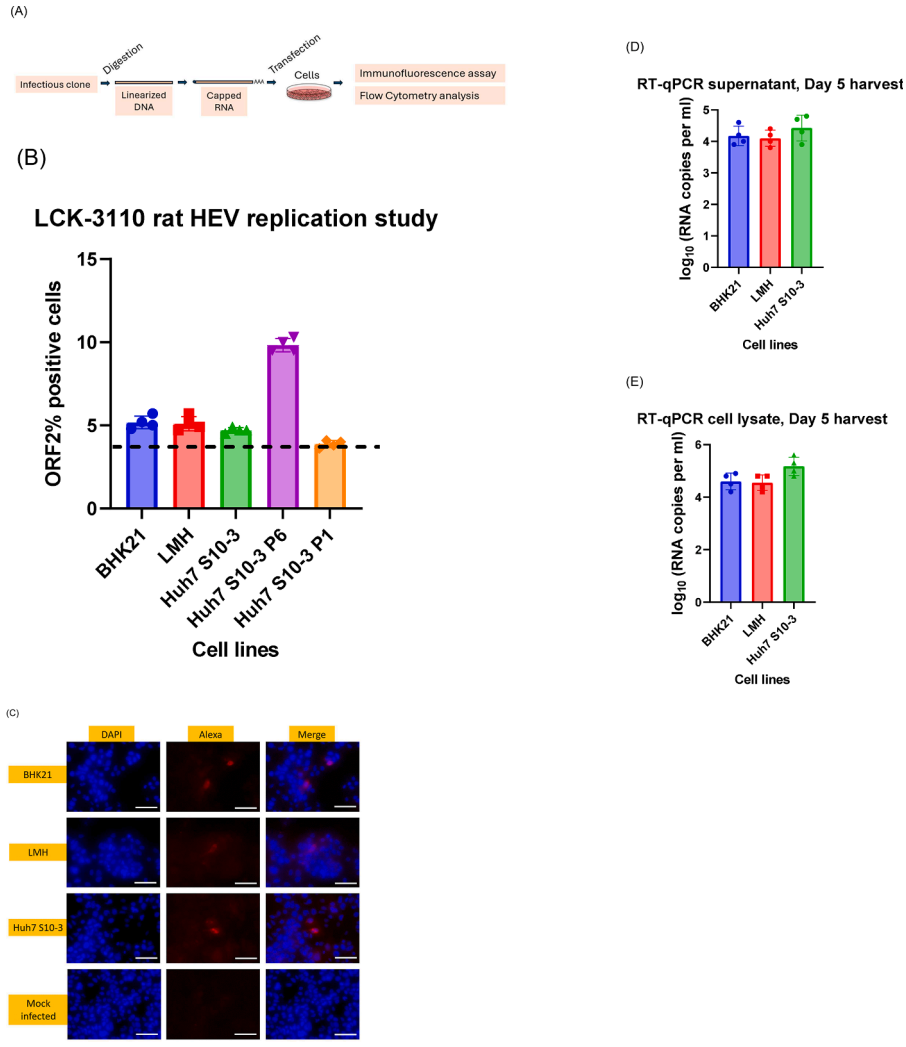

Fig. 1. LCK-3110 rat HEV replication.
(A) Workflow of HEV capping and transfection of target cells. LMH, Huh7, and BHK-21 cell lines were transfected with in vitro transcribed capped HEV RNA (rat HEV). (B) Flow cytometry quantification of LMH, Huh7, and BHK-21 cells transfected with capped RNA transcripts of rat HEV. Huh7 S10–3 cells were also transfected with cell culture adapted Kernow-C1 genotype 3 P6 strain and non-cell culture adapted Kernow-C1 genotype 3 P1 strain. P6 and P1 belonging to the *Paslahepevirus balayani* species were used as a control to determine the assay cutoff for the replicative ability of rat HEV (represented by the dotted line). The assay was performed in the cells harvested on day 5 post transfection. Samples were fixed in methanol and probed with rabbit anti-ORF2 followed by goat anti-rabbit alexa fluor 594 antibodies. Each bar (mean ± SD) represents separate transfections stained in parallel and displays the mean of two independent biological experiments with three replicates per sample. (C) Immunofluorescence detection of HEV ORF2 antigen in methanol fixed LMH, Huh7 S10–3, and BHK-21 cells 5 days post transfection. Cells are stained with goat anti-rabbit IgG H&L combined with anti-rabbit Alexa fluor 594 (red), and 4′, 6-diamidino-2-phenylindole (DAPI) (blue). Scale bar = 30 µm. (D) RT-qPCR data from the supernatants collected from day 5 of replication assay. (E) RT-qPCR data from the cell lysates collected from day 5 of replication assay.

analyses. $p < 0.05$ was considered significant.

3. Results

3.1. Detection of HEV antigen in LMH, HUH7 S10–3, and BHK-21 cells transfected with capped RNA transcripts from the full-length cDNA clone of rat HEV LCK-3110 strain

A full-length genomic cDNA clone of the rat HEV LCK-3110 strain was constructed and successfully cloned into the Psp64 poly (A) vector. The RNA transcripts made from the full-length cDNA clone were found to be replication competent in vitro and in vivo (Yadav et al., 2024). The capped RNAs were transfected into cell lines of chicken liver, human liver, and baby hamster kidney cells to assess the replication ability of the rat HEV. Transfected cells were fixed and probed for ORF2 expression. ORF2 (structural protein of HEV) serves as an indicator of complete viral replication because it is translated from subgenomic RNA during the late stages of HEV replication (Kenney and Meng, 2015; Graff et al., 2006). Productive replication was assessed by immunofluorescence assay (IFA) and flow cytometry quantification of the fixed cells (Fig. 1A). A polyclonal antibody against *Paslahepevirus balayani* ORF2 capsid protein which cross reacts against multiple HEV species (HEV contains only one serotype) was utilized to detect cells expressing the ORF2 protein as an indicator of positive replication. As depicted in Fig. 1B, LMH and BHK-21 were comparatively more permissive for rat HEV replication than in the Huh7 S10–3 cells, though not statistically different. Approximately, 5 %, 5 %, and 4.5 % ORF2-positive cells were observed in LMH, BHK-21, and Huh7 S10–3 cells, respectively. Additionally, Huh7 S10–3 cells were transfected with the non-cell culture adapted *Paslahepevirus balayani* Kernow C1 P1 gt3 HEV strain and Kernow C1 P6 gt3 strain to build a cut-off point to demonstrate the ORF2 percentage positive cells. Detection of ORF2 protein expression in cells via IFA indicates successful replication of the rat HEV LCK-3110 capped RNA transcript in LMH, BHK-21, and Huh7 S10–3 cells (Fig. 1C).

Cell supernatants (Fig. 1D) and cell lysates (Fig. 1E) harvested on day 5 demonstrated viral loads from LMH, Huh7 S10–3, and BHK-21 cells via RT-qPCR. These results demonstrate the replication competence of the input RNA transcripts from the infectious cDNA clone that replicated in the LMH, BHK-21, and Huh7 S10–3 cells.

3.2. Capped RNA transcripts of the rat HEV LCK-3110 cDNA clone were infectious when injected intrahepatically into the livers of specific pathogen free (SPF) chickens

Infectivity or pathogenesis studies with wild type virus are limited with HEV and sometimes results in less interpretable data due to the very low HEV infectious titers obtained in vitro (Debing et al., 2014). Previously, we have used rat HEV LCK-3110 RNA transcripts inoculated intrahepatically to generate infectious virus in gnotobiotic pigs (Yadav et al., 2024). These intrahepatic inoculation procedures have been successfully used for pathogenicity studies with other HEV strains (Córdoba et al., 2012; Huang et al., 2005; Huang et al., 2005). SPF chickens were intrahepatically inoculated with LCK-3110 rat HEV capped viral RNA and tested for replication competence (Fig. 3A). All chickens were negative for viral shedding and seronegative for avian HEV antibodies prior to inoculation and chickens in negative controls remained negative throughout the entire study.

Rat HEV RNA was detected in the cloacal swabs of 40 % of the chickens at 2 wpi (Table 1). Fecal positivity started at 1 week post inoculation (wpi) and was detected sporadically in weeks 4 (1/8) through 6 (1/6) post inoculation with no fecal shedding detected in week 5. Viral RNA in feces (up to 40 %) and serum (up to 37.5 %) were detected only in a few specific birds. Rat HEV RNA was also detected in the bile starting at 2 wpi through week 5, again only in some birds (Table 1). The highest titer seen in cloacal swabs of rat HEV infected chickens was at 3 wpi (2×10^3 RNA copies/ml). Similarly, the highest titer seen in blood (1.2×10^2 RNA copies/ml), bile (1.8×10^3 RNA copies/ml) and liver (1.6×10^3 RNA copies/ml) was also during 3 wpi. In comparison, as a positive control, SPF chickens were inoculated intrahepatically with the capped RNA transcripts of avian HEV VA (Virginia) strain (kindly provided by Dr. XJ Meng, Virginia Tech). Avian HEV infection was evident in SPF chickens as demonstrated by fecal viral RNA shedding (detected in up to 100 % of birds), viral RNA in blood (up to 100 %), and RNA in bile (100 %) starting from 1 wpi (Table 1). The highest titer seen in cloacal swabs of avian HEV infected chickens was at 3 wpi (2×10^5 RNA copies/ml). Similarly, the highest titer seen in blood (1.1×10^4 RNA copies/ml), bile (1.4×10^5 RNA copies/ml) and liver (2×10^5 RNA copies/ml) was also during 3 wpi. Only 50 % (5 of 10) of rat HEV inoculated chickens seroconverted in comparison to 90 % (9 of 10) of avian HEV inoculated chickens (Table 2). Negative control chickens did not seroconvert or have detectable HEV RNA throughout the study.

Table 1
Detection of avian HEV and rat HEV RNA in specific pathogen free chickens experimentally inoculated with capped RNA transcripts from infectious cDNA clones of avian HEV WT strain and rat HEV LCK-3110 strain.

Group	Number of positive samples/total number tested at indicated wpi								Overall no. of infected chickens in each group [a]
	Sample	**0**	**1**	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	
Avian HEV	Feces	0/10	6/10	7/10	7/8	7/8	6/6	5/6	10/10
	Serum	0/10	4/10	7/10	7/8	6/8	6/6	4/6	
	Bile	–	–	2/2	2/2	2/2	2/2	2/2	
	Liver	–	–	2/2	1/2	2/2	2/2	2/2	
Rat HEV LCK-3110 strain	Feces	0/10	2/10	4/10	2/8	1/8	0/6	1/6	5/10
	Serum	0/10	2/10	3/10	3/8	3/8	2/6	0/6	
	Bile	–	–	2/2	1/2	1/2	1/2	0/2	
	Liver	–	–	1/2	1/2	1/2	1/2	0/2	
Mock[c]	Feces	0/10	0/10	0/10	0/8	0/8	0/6	0/6	0/10
	Serum	0/10	0/10	0/10	0/8	0/8	0/6	0/6	
	Bile	–	–	0/2	0/2	0/2	0/2	0/2	
	Liver	–	–	0/2	0/2	0/2	0/2	0/2	

[a] No. of chickens which had detectable avian HEV or rat HEV RNAs by RT-qPCR in feces, sera, bile, or liver at certain points during experiment.
[b] Two chickens were necropsied at 2 wpi, 3 wpi, 4 wpi, 5 wpi, 6 wpi.
[c] Samples from all chickens in the control group remained negative throughout the experiment.
0, 1, 2, 3, 4, 5, 6 – weeks post infection.

3.3. Sentinel chickens developed infections when co-housed with intrahepatically injected chickens suggesting a natural fecal oral route of transmission could occur for rat HEV

The fecal-oral route is considered as one of the major routes of transmission of HEV (Yadav and Kenney, 2022). To determine if the LCK-3110 rat HEV can transmit efficiently to sentinel chickens, naive chickens were co-housed with chickens that had been intrahepatically inoculated with the rat HEV LCK-3110 strain. Sentinel chickens added to the rat HEV inoculated group at 1 wpi started shedding virus in feces at 3 weeks post contact (wpc) (Table 3). Viremia was evident in up to 50 % (2/4) of the sentinel birds at 5 wpc with detectable fecal shedding from weeks 3–6 post contact. High titers of the viral RNA were detected in

Table 2
Seroconversion in specific pathogen free chickens experimentally inoculated with capped RNA transcripts from infectious CDNA clones of rat HEV LCK-3110 strain and avian HEV WT strain.

Group	Number of seropositive chickens/total number tested at indicated wpi							Overall no. of seroconverted chickens in each group [a]
	0	1	2[b]	3[b]	4[b]	5[b]	6[b]	
Avian HEV-WT	0/10	2/10	8/10	8/8	5/8	4/6	4/6	9/10
Rat HEV LCK-3110 strain	0/10	1/10	3/10	3/8	2/8	2/6	0/6	5/10
Mock[c]	0/10	0/10	0/10	0/8	0/8	0/6	0/6	0/0

[a] No. of chickens which had detectable antibodies against avian HEV or rat HEV by ELISA in sera at certain points during experiment.
[b] Two chickens were necropsied at 2 wpi, 3 wpi, 4 wpi, 5 wpi, 6 wpi.
[c] Samples from all chickens in the control group remained negative throughout the experiment.
0, 1, 2, 3, 4, 5, 6 – weeks post infection.

Table 3
Detection of rat HEV RNA in naive specific pathogen free chickens cohoused with chickens inoculated with rat HEV LCK-3110 strain.

Group	No. of positive samples/total no. tested at indicated wpc								Overall no. of infected chickens in each group [a]
	Samples	**0**	**1**	**2**	**3**	**4**	**5** [b]	**6** [b]	
Rat HEV LCK-3110 strain in sentinel chickens	Feces	–	0/4	0/4	1/4	2/4	2/4	1/2	3/4
	Serum	–	0/4	0/4	0/4	2/4	2/4	1/2	
	Bile	-	-	-	-	-	1/2	2/2	
	Liver	-	-	-	-	-	1/2	2/2	

[a] No. of chickens which had detectable rat HEV RNAs by RT-qPCR in feces, sera, or bile at certain points during experiment.
[b] Two chickens were necropsied at 5 wpc and 6 wpc.
0, 1, 2, 3, 4, 5, 6 – weeks post contact.

these chickens although the load was lower than the intrahepatically inoculated chickens. Bile and liver demonstrated viral RNA at 5 wpc and 6 wpc. The highest titer seen in cloacal swabs of sentinel chickens with rat HEV was at 5 wpc (1×10^3 RNA copies/ml). Similarly, the highest titer seen in blood (1.1×10^2 RNA copies/ml) was at 5 wpc. The highest titer seen in bile (1.1×10^3 RNA copies/ml) and liver (1×10^3 RNA copies/ml) was at 6 wpc. Altogether, seroconversion was seen in 3 of the 4 contact exposed birds (Table 4). Overall, these data confirm the transmission ability of rat HEV to co-housed chickens.

Table 4
Seroconversion in specific pathogen free chickens exposed in contact with the chickens inoculated with the rat HEV LCK-3110 strain.

Group	Number of seropositive chickens/total number tested at indicated wpc							Overall no. of seroconverted chickens in each group [a]
	0	**1**	**2**	**3**	**4**	**5** [b]	**6** [b]	
Rat HEV LCK-3110 strain	0/4	0/4	1/4	1/4	2/4	2/4	2/2	3/4

[a] No. of chickens which had detectable antibodies against rat HEV by ELISA in sera at certain points during experiment.
[b] Two chickens were necropsied at 5 wpc and 6 wpc.
0, 1, 2, 3, 4, 5, 6 – weeks post contact.

3.4. Characterization of the pathogenicity of the rat HEV LCK-3110 strain and comparison with the avian HEV strain

To study rat HEV associated pathogenesis, infectious cDNA clone of LCK-3110 rat HEV was constructed and rat HEV transcripts were intrahepatically injected into chickens (Fig. 2A). There were no significant differences seen in the body weight of chickens between the three groups (Fig. 2B). Liver/body weight ratios, an indicator of liver enlargement, were indistinguishable in the mock and rat HEV inoculated chickens. However, avian HEV inoculated chickens had a significantly higher liver organ index from 3 wpi to 6 wpi in comparison to the other groups (Fig. 2C). Similarly, spleen/body weight ratios, an indicator of spleen enlargement, was higher in the avian HEV inoculated chickens from 4 wpi to 6 wpi (Fig. 2D). Interestingly, no effect was seen in the rat HEV inoculated chickens compared to the negative control chickens, suggesting chickens could be asymptomatic spreaders.

Gross pathological lesions from livers were evaluated during necropsies and were also recorded as photomicrographs. Subcapsular hemorrhages were noted in avian HEV inoculated chickens starting from 14 to 28 dpi (Fig. 3A). By 35 dpi, a very dense and clear hemorrhage was noted. Interestingly, gross lesions were limited to only 50 % of the infected chickens. Thus, as previously described (Billam et al., 2005), liver gross pathological lesions associated with avian HEV inoculated groups could not be consistently reproduced in all experimentally infected SPF chickens. Rat HEV inoculated chickens demonstrated very mild subcapsular hemorrhages only on 21 and 28 dpi (Fig. 3A). Gross lesions were limited to only 3 rat HEV inoculated chickens. The mock infected group did not demonstrate any gross pathology in liver throughout the study (Fig. 3A)

Immunohistochemistry (IHC) identified HEV antigens in the liver (Fig. 3B). HEV antigens in rat HEV inoculated chickens were present at lower abundance than the avian HEV inoculated group from 14 to 42 dpi. At 28 dpi, in the livers were infiltrated by inflammatory cells in rat HEV inoculated chickens, HEV antigens were present rarely around the area containing inflammatory cells (Fig. 3B). Sentinel chickens cohoused with rat HEV inoculated chickens presented with detectable HEV antigen in their livers upon necropsy at day 28 and 35 post exposure (Fig. 3C) Negative control chickens remained negative throughout the experiment and did not demonstrate HEV specific antigens in the liver.

Histopathological analysis of liver sections revealed periportal lymphocytic infiltration in the avian HEV inoculated chicken at 28 dpi (Fig. 3D). Mild focal lymphocytic infiltration was observed in rat HEV inoculated chickens at 28 dpi (Fig. 3D). A very mild aggregation of lymphocytes was seen in rat HEV comingled sentinel chicken livers at 42 dpi (Fig. 3D).

3.5. LCK-3110 virus isolated from the chicken intestinal contents on day 28 was replication competent in human huh7 S10–3 cells

Huh7 S10–3 cells were inoculated with LCK-3110 rat HEV obtained from intestinal contents of rat HEV inoculated SPF chickens on day 28 post infection. We detected rat HEV RNA in supernatants from cell culture (Fig. 4A) inoculated with chicken intestinal contents (approximately 2×10^5 viral RNA copies/mL) on day 2, 4, and 6. RNA detected on day 0 (6 h post inoculation) was considered as background with attachment of the virus to the cell surfaces. RNA loads increased by approximately 1.5 $\log_{10}$ from day 0 to day 6 in cellular supernatants demonstrating successful viral replication (Fig. 4A). IFA of Huh7 S10–3 cells on day 6 post inoculation confirmed the presence of ORF2 protein in the inoculated cells (Fig. 4B). These results exemplify the ability of rat HEV derived from the SPF chicken feces to replicate in human hepatoma cells.

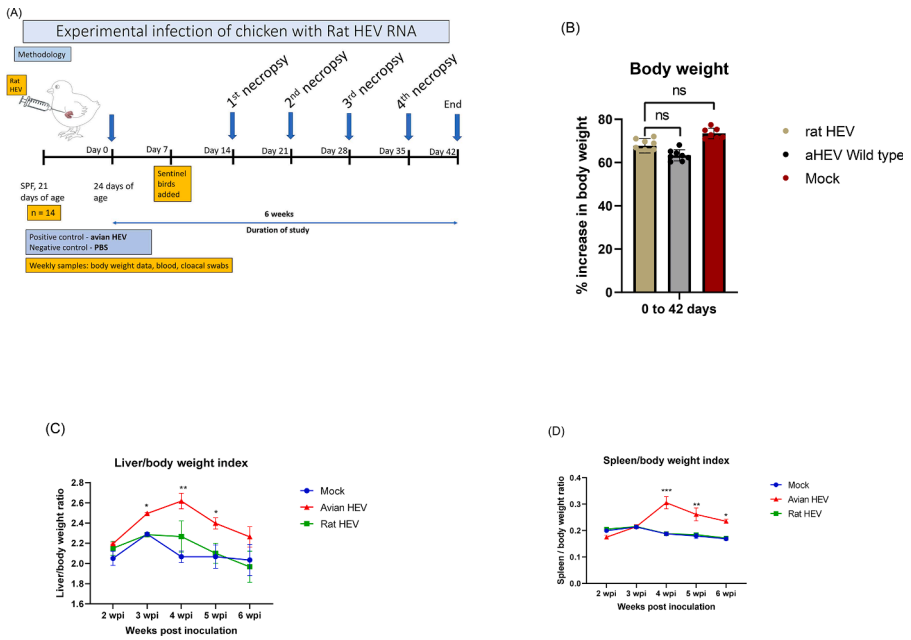

Fig. 2. Infection of rat HEV and avian HEV in chickens via intrahepatic inoculation.
(A) Schematic representation of the experimental design. Capped LCK-3110 rat HEV transcripts inoculated intrahepatically to chickens. (B) Body weight of chickens intrahepatically inoculated with RNA transcripts of rat HEV and avian HEV. (C) Comparison of the liver organ index and (D) spleen organ index of the rat HEV inoculated, avian HEV inoculated and mock inoculated chickens. Two-way Analysis of Variance (ANOVA) followed by the post-hoc test was used to test the significance between groups. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

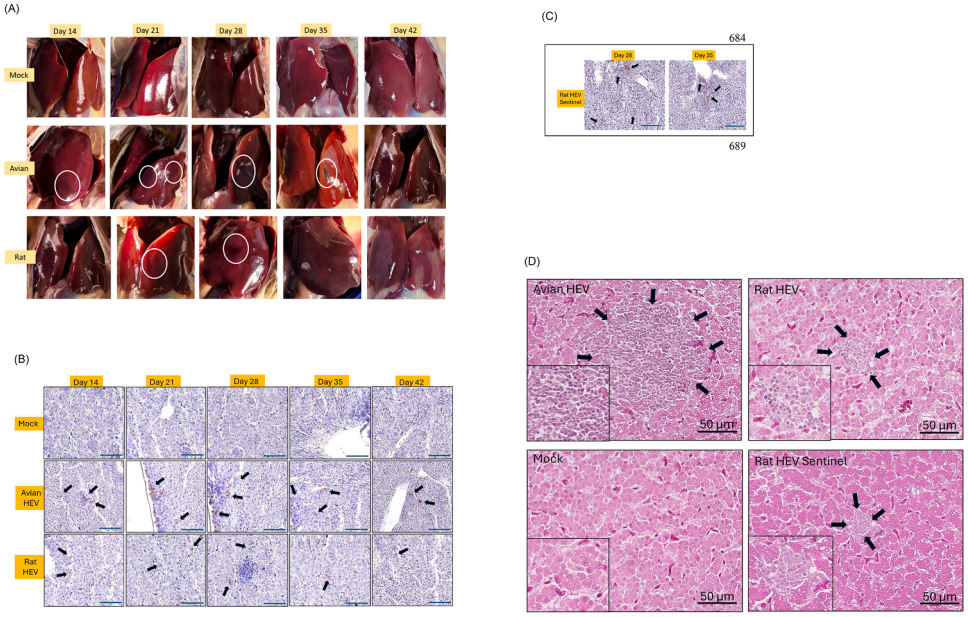

Fig. 3. Comparative gross lesions and immunohistochemistry (IHC) in liver of avian HEV or rat HEV inoculated chickens.
(A) Hemorrhagic rupture and blood spots are seen in the liver of avian HEV or rat HEV inoculated chickens. Rat HEV inoculated chickens demonstrated gross lesions only on day 21 and day 28. Gross lesions are marked by white circles. (B) IHC demonstrates the low presence of rat HEV antigen in the liver of rat HEV inoculated chickens. In contrast, higher avian HEV antigen were observed in avian HEV inoculated chickens from day 14 to day 42. (C) IHC demonstrates the presence of rat HEV antigen in the liver of sentinel chickens cohoused with rat HEV inoculated chickens on days 28 and 35 post exposure. Arrows are indicating to the HEV antigen (brown color) present in the liver tissue. (D) Hematoxylin and Eosin (H & E) staining of liver tissues collected from avian HEV inoculated, rat HEV (necropsied on day 28), and rat HEV sentinel chickens (necropsied on day 42). High infiltration of lymphocytes can be seen clearly in the avian HEV inoculated chickens in comparison to fewer aggregates of lymphocytes in rat HEV inoculated chickens (pointed by arrows). Very few infiltrations of lymphocytes were also noted in the sentinel chickens added to the rat HEV inoculated group. Scale bars = 50 µm.

4. Discussion

Recent advances in reverse genetics have led to the development of intrahepatic transfection procedures that have helped to study the pathogenicity of several single stranded positive sense RNA viruses, including HEV (Emerson et al., 2001; Huang et al., 2007; Pudupakam et al., 2009). Infectious cDNA clones of human, swine HEV, and rat HEV have allowed mechanistic studies and investigation of virulence factors

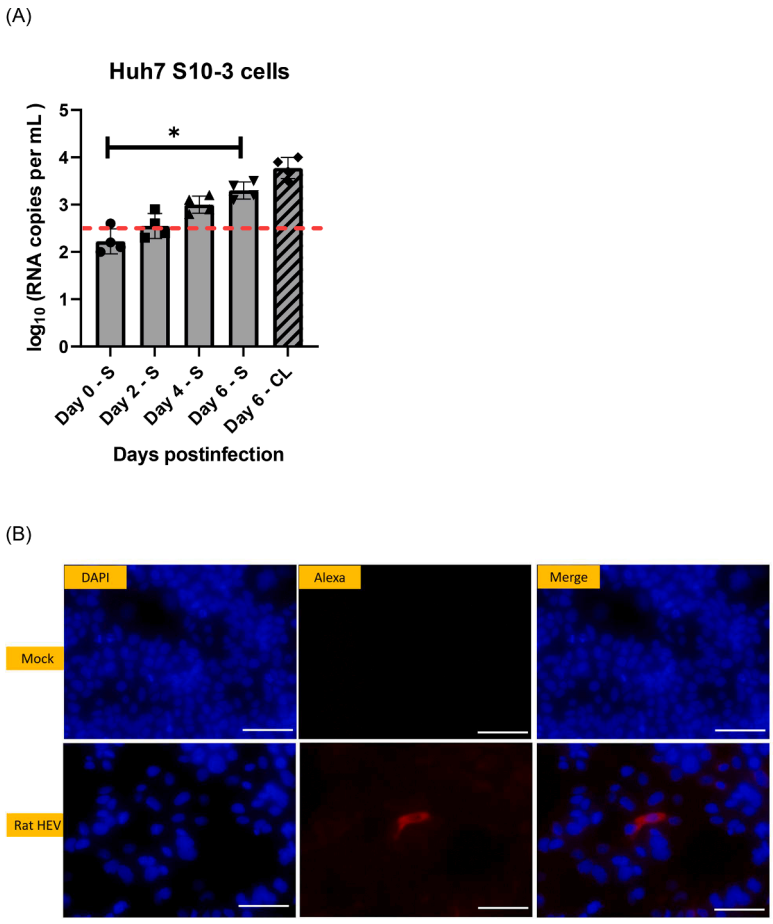

Fig. 4. Isolation of rat HEV from infected chicken feces in cell culture.
(A) Rat HEV viral load in culture supernatant (S) and cell lysates (CL) of Huh7 cell line after inoculation by filtered fecal suspension from rat HEV inoculated chickens. Independent biological experiments, mean ± SD of 4 replicates (*$P < 0.05$), are presented. Red line represents the cut-off value demonstrating the background referring to the attachments of the virus to the cell surfaces. (B) Immunofluorescence detection of HEV ORF2 antigen in methanol fixed Huh7 S10–3 cells 6 days post inoculation. Cells are stained with goat anti-rabbit IgG H&L combined with anti-rabbit Alexa Fluor 594 (RED), and 4′, 6-diamidino-2-phenylindole (DAPI) (blue). Scale bars = 30 μm.

associated with HEV disease (Li et al., 2015; Scholz et al., 2020; Panda et al., 2000; Yamada et al., 2009; Panajotov et al., 2024; Schemmerer et al., 2022). Therefore, the availability of a zoonotic rat HEV infectious clone (LCK-3110) has facilitated an understanding of potential transmission sources of rat HEV to humans. In the present study, we demonstrate the ability of LCK-3110 rat HEV to infect chickens. Chickens are mildly susceptible to the zoonotic rat HEV LCK-3110 strain suggesting a potential role in rat HEV spread in the environment and for potential transmission to humans.

Some important considerations must be applied to our animal model experimental data for real world conditions and several unexpected observations occurred. Our initial infection studies utilized intrahepatic injection of RNA transcripts to generate bird infections which would be an unnatural route in normal production systems, though floor housing with infected birds as sentinels would mimic fecal-oral exposure as in some floor housed production systems. The unexpected seroconversion of one bird prior to detection of viremia or fecal shedding appears to be an outlier in the data suggesting the bird either encountered antigen to generate an immune response prior to active infection (possible oral immune stimulation from consumption of infected feces) or infection occurred more rapidly than expected without detection of viral RNA. Sporadic viremia and fecal shedding of HEV in many different animal models has been noted, specifically with low challenge doses, and rapid seroconversion in less than one week has been observed previously in high dose challenge studies (Sun et al., 2004) making these scenarios a possibility. Our pilot animal studies utilized small numbers of birds at each necropsy timepoint reducing the power of the data obtained at each necropsy.

A similar cell culture study utilizing an infectious clone of a human-derived rat HEV isolate also showed ability to infect human hepatoma cells (Panajotov et al., 2024). They observed much higher viral RNA (10^8 genome copies/ml) compared to our 6-day experiments which topped out at $10^{3.5}$ genome copies/ml. As they noted, replication efficiency in the cells was cell line dependent. A combination of our Huh7 S10–3 subclone and initial infectious dose likely contributed to differences in observed titers between studies or could reflect sequence differences between the studied virus strains. We did not conduct long term studies to determine whether LCK-3110 derived from chicken feces could persist in long term cell culture.

Chickens are a known reservoir of avian HEV which is non-zoonotic and does not infect humans. However, the ability of avian HEV to spillover to wild birds has been demonstrated in multiple studies (Sun et al., 2019). Our findings that LCK-3110 *Rocahepevirus ratti* HEV can transmit subclinically in chickens suggest the transmission of rat HEV may not only be limited to chickens but also other avian species. Future studies determining the adaptability of rat HEV in chicken liver cells and chickens should be conducted. Seropositivity and fecal viral shedding seen in the experimentally inoculated chickens and contact-exposed chickens in our study identifies a need to screen the food supply chain, including chickens on farms and possibly chicken products at

grocery stores. In addition, screening of backyard chickens and chicken flocks in proximity of rat HEV positive rats is necessary.

Pigs have also been demonstrated as susceptible to the LCK-3110 zoonotic rat HEV strain suggesting they could serve as a transmission source to humans (Yadav et al., 2024). In comparison to the pig studies, all experimentally inoculated chickens did not develop infection. This suggests that pigs are more permissive to LCK-3110 rat HEV than chickens. Currently, only two species have been experimentally tested for their susceptibility to LCK-3110 HEV. Further in-depth studies will be required to understand the ability of the LCK-3110 strain to infect other species. To date, only rat HEV strains, LA-B350 and LCK-3110 strains have been studied in pigs. LCK-3110 demonstrated the ability to infect pigs whereas LA-B350 was unable to replicate in pigs (Purcell et al., 2011). Further studies will be required to understand the genetic differences between the above two strains that make one adapted to pigs. These future studies could help predict whether other *Rocahepevirus ratti* strains are high risk to cross species barriers.

In general, zoonotic spillover infections in humans are elaborated by defining the role of intermediate hosts. In the case of rat HEV, even though the primary host is rats, the viral source population involved in the zoonotic transmission of rat HEV to humans could be separate from that of the primary host. For instance, Nipah and Hendra virus transmission from bats to humans involves other species such as swine or horses acting as zoonotic sources. Swine and horses are not considered as the natural hosts because of infection is considered accidental (Eaton et al., 2006). Future studies need to be focused on the length of infectious rat HEV survivability in feces.

Emergence of a zoonotic disease like rat HEV is a complex biological process whereby virus mutation and adaptation to the new host is defined by survival pressures. Our study forms a basis to understand the spillover event of rat HEV to humans. Rat HEV infections reported in immunocompetent patients are typically not fatal, highlighting the similarity of chickens to model the pathogenesis in incidental hosts. The mild phenotype induced in rat HEV inoculated and sentinel chickens observed in the current study could be used to model asymptomatic non-lethal presentation of rat HEV infections in humans. This suggests that chickens can be used as an alternative model for rat HEV transmission studies, thus, improving knowledge on rat HEV survivability and adaptability in humans.

5. Conclusions

Chickens inoculated with RNA transcripts of the LCK-3110 rat HEV strain developed infection demonstrated by virus shedding in feces, viremia, detectable HEV RNA in the bile, and seroconversion. Histopathology confirmed mild infection of chickens with rat HEV and transmission to naïve cohoused chickens. Isolation of infectious rat HEV from chicken feces in human liver cells highlights the necessity to understand this emerging zoonotic rat HEV and expanding host range. Thus, our study indicates that chickens can serve as a possible intermediate host, potentially playing a role in rat HEV spread and transmission to humans.

Author statement

All authors certify that they have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, hasn't received prior publication, and isn't under consideration for publication elsewhere.

Authors declare they have no perceived conflicts of interest or competing interests

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CRediT authorship contribution statement

Kush Kumar Yadav: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Patricia A Boley:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Saroj Khatiwada:** Writing – review & editing, Methodology, Investigation. **Carolyn M Lee:** Writing – review & editing, Methodology, Investigation. **Menuka Bhandari:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Ronna Wood:** Writing – review & editing, Methodology, Investigation. **Juliette Hanson:** Writing – review & editing, Supervision, Methodology, Investigation. **Scott P. Kenney:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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