111 virus.

112

113

114 Results

115

116 Construction of a genomic clone of ratHEV

117 The whole genome of the ratHEV prototype strain R63/DEU/2009 was amplified by
118 RT-PCR in 4 overlapping fragments and cloned. The final genomic clone contained a T7
119 RNA polymerase promotor sequence at the 5'-end and a poly-A sequence at the 3'-end
120 of the ratHEV genome followed by a unique *Xba*I restriction site. A schematic map of the
121 plasmid is shown in Fig. 1A. Restriction analysis of the plasmid resulted in the expected
122 DNA fragments (Fig. 1B). Sequencing of the cloned genome identified 23 point
123 mutations as compared to the original sequence of strain R63/DEU/2009 (GenBank
124 acc.-no. GU345042). Most of the mutations are either synonymous or lead to amino
125 acids, which have also been detected in other ratHEV strains at the respective positions
126 (Supplementary Table S2). Only four unique amino acid exchanges were detected,
127 which are exclusively located within the hypervariable region of ORF1.

128

129 Recovery of ratHEV by inoculation of nude rats with *in vitro*-transcribed RNA

130 The *Xba*I-linearized plasmid was transcribed *in vitro* resulting in capped ratHEV
131 genome-length RNA. A total of 500 µl of the *in vitro*-transcribed RNA (2.6 x 10^{11}
132 copies/µl) was intrahepatically inoculated in the two nude rats LR-1 (6 week old, female)

133 and LR2 (10 week old, male). In addition, the two nude rats VR1 (6 week old, female)

134 and VR2 (10 week old, male) were intravenously inoculated through the tail vein with

135 the same dose of *in vitro*-transcribed RNA. RNA of ratHEV was detected in the stool

136 specimens from both nude rats LR1 and LR2 on day 14 post inoculation (p.i.) with

137 amounts of 4.65×10^{7} and 1.15×10^{8} copies/g, reaching a plateau on day 30 p.i. with

138 amounts of 1.08×10^{11} and 1.94×10^{11} copies/g, respectively (Fig. 2). RNA of ratHEV

139 was detected in sera of LR1 and LR2 on day 49 p.i. with 3.84×10^{7} copies/ml and $4.75 \times$

140 10^{7} copies/ml, respectively. In contrast, ratHEV RNA could not be detected in stool

141 specimens or serum of nude rats VR1 and VR2.

142

143 **Demonstration of infectivity of ratHEV recovered from inoculated nude rats**

144 Stool samples taken from LR1 and LR2 on day 30 p.i. were used to prepare

145 sterile-filtrated 10% suspensions. The two nude rats RR1 (13 week old, female) and

146 RR2 (17 week old, male) and the two 15-week-old female Wistar rats WR1 and WR2

147 were intravenously inoculated with 300 µL of the stool suspension from LR1 or LR2

148 (RR1 and WR1 inoculated with suspension from LR1; RR2 and WR2 inoculated with

149 suspension from LR2). RNA of ratHEV was detected in stool of the rats RR1 and RR2,

150 starting on day 14 p.i. with amounts of 3.89×10^{10} and 4.73×10^{10} copies/g and

151 thereafter keeping amounts of more than 10^{10} copies/g (Fig. 3A). RNA of ratHEV was

152 also detected in sera of RR1 and RR2 at day 28 p.i. with 7.04×10^{7} copies/ml and $1.11 \times$

153 10^{8} copies/ml, respectively. In WR1 and WR2, anti-ratHEV IgG was detected beginning

154 at 14 days p.i. and reaching a plateau at day 28 p.i. (Fig. 3B). The alanine

155 aminotransferase (ALT) levels of rats RR1, RR2, WR1 and WR2 were lower than 40
156 IU/L throughout the whole experiment.
157

158 **Isolation of ratHEV from inoculated nude rats in PLC/PRF/5 cells**

159 The sterile-filtrated 10% rat stool suspension taken from LR1 on day 30 p.i. was
160 inoculated onto PLC/PRF/5 cells. RNA of ratHEV was detected in PLC/PRF/5 cell
161 culture supernatant on day 30 p.i. with amounts of 9.27×10^6 copies/ml and reached a
162 plateau on day 60 p.i. with amounts of 2.59×10^9 copies/ml. Thereafter, high amounts of
163 ratHEV RNA were constantly detected with more than 10^9 copies/ml until 116 days p.i.
164 (Fig. 4A). The capsid protein of ratHEV was detected in the culture supernatant
165 beginning with day 44 p.i. and reached a plateau at day 64 p.i. until the end of the
166 experiment (Fig. 4B). Inoculation of fresh PLC/PRF/5 cells with the cell culture
167 supernatant taken at day 68 p.i. resulted in detection of ratHEV RNA as early as day 16
168 p.i. and capsid protein at day 24 p.i. In addition, the reached amounts of ratHEV RNA
169 (1.87×10^{10}) were considerably higher as compared to the first passage of the virus (Fig.
170 4A and B). No cytopathic effect was observed during the whole experiment.
171

172 **Demonstration of infectivity of cell culture-derived ratHEV for nude rats**

173 The two 20-week-old female nude rats CR1 and CR2 were intravenously inoculated
174 each with 0.5 ml of cell culture supernatant from day 68 p.i. of the first passage. RNA of
175 ratHEV was detected in stool beginning at 12 days p.i. with amounts of 1.35×10^7 and
176 9.97×10^7 copies/g and rose to 6.92×10^{10} and 9.21×10^9 copies/g on days 18 p.i. in rats

177 CR1 and CR2, respectively (Fig. 5). RNA of ratHEV was detected at day 21 p.i. in sera
178 of rats CR1 and CR2 with 8.50 x 10^5 copies/ml and 9.20 x 10^5 copies/ml, respectively.
179
180 **Genome sequence comparison of ratHEV recovered from nude rats and cell**
181 **culture**
182 The entire genome sequences of ratHEV recovered from nude rat faeces (sample taken
183 from rat LR1 on day 30 p.i.) and cell culture supernatant (sample collected from day 68
184 p.i. of the first passage) were analyzed by next generation sequence analysis and
185 compared with the sequence of cDNA clone. The identified mutations and the deduced
186 amino acid exchanges are shown in Table 1. The genome sequences of the cDNA
187 clone and the ratHEV recovered from the intrahepatically inoculated nude rat were
188 identical. In contrast, 9 mutations were found in ratHEV recovered from the cell culture,
189 which resulted in 4 synonymous and 5 non-synonymous mutations in ORF 1 and ORF2.
190 The mutations at positions 295 and 343 are synonymous for ORF1, but
191 non-synonymous for the overlapping ORF4.
192
193
194 **Discussion**
195 RatHEV is a recently discovered virus related to, but distinct from human HEV (Johne et
196 al., 2014a). As laboratory rats can be reproducibly infected with ratHEV and a cell
197 culture propagation system is available for ratHEV, this virus may be used in future as a
198 surrogate virus for human HEV enabling studies on several aspects of virus replication

199 and immune response. However, a system for genetic manipulation of ratHEV was
200 missing so far. The results presented here indicate that the described genomic ratHEV
201 cDNA clone is capable of generation of ratHEV infectious for laboratory rats and cell
202 culture.
203
204 The cDNA clone was constructed similar to recently described infectious cDNA clones
205 from human, rabbit and avian HEV, using T7 RNA polymerase promotor to generate
206 capped *in vitro*-transcribed genomic virus RNA (Huang et al., 2005a, b; Yamada et al.,
207 2009; Cordoba et al., 2012; Cossaboom et al., 2014; Kwon et al., 2011). Several point
208 mutations have been detected in the cDNA clone when compared to the original
209 genome sequence of the used ratHEV isolate as deposited at GenBank. Errors during
210 RT-PCR amplification or the presence of viral quasispecies in the original sample may
211 explain the mutations. As the nucleotide exchanges were either silent or resulted in
212 amino acids, which are also present at the same positions in other ratHEV strains, it
213 was assumed that they do not affect infectivity of the cDNA clone.
214
215 For recovery of infectious virus from the *in vitro*-transcribed RNA, it was directly injected
216 into the liver of nude rats. Similar procedures have been described for human, pig,
217 rabbit and avian HEV cDNA clones (Emerson et al., 2001; Cossaboom et al., 2014;
218 Huang et al., 2005a, b). Nude rats were selected for inoculation as they have been
219 shown to be highly susceptible for ratHEV infection, leading to shedding of high titers of
220 ratHEV for a prolonged time (Li et al., 2013b). The detection of ratHEV RNA in serum

221 and faeces after inoculation of the RNA into the liver of the nude rats was the first
222 indication for successful generation of infectious virus. In contrast, intravenous
223 inoculation of ratHEV RNA was not able to initiate virus replication, presumably because
224 of the lack of its internalization into permissive cells. The virus excreted by the
225 intrahepatically inoculated nude rats was shown to be infectious for nude rats and
226 immunocompetent Wistar rats by intravenous inoculation. Virus shedding, antibody
227 production and (no) clinical signs were similar to that described for rats infected with an
228 organ homogenate of a ratHEV-infected wild rat (Li et al., 2013b). It can be therefore
229 concluded, that the developed system is suitable for generation of infectious ratHEV
230 from the cloned genomic cDNA.
231
232 The recent publication of a cell culture system for ratHEV (Jirintai et al., 2014) prompted
233 us to analyze the infectivity of the generated ratHEV for cell culture. The results of the
234 experiments showed that the virus replicated – after a lag phase of 30 days - with high
235 titers in PLC/PRF/5 cells. After a passage in this cell culture system, the virus growth
236 was observed earlier and with even higher titers. Notably, the reached titers of $>10^9$
237 genome copies/ml are considerably higher than that described for other ratHEV strains
238 (10^7 genome copies/ml, Jirintai et al., 2014), or for human HEV strains (10^7 genome
239 copies/ml, Johne et al., 2014b; and 10^8 genome copies/ml, Okamoto et al., 2011).
240 Genome analysis identified several point mutations in the recovered virus, which may
241 be linked to cell culture adaptation. Although infectivity of the recovered virus for nude
242 rats could still be demonstrated, the biological significance of these mutations has to be

243 assessed in future studies. Direct transfection experiments of cell cultures with in
244 *vitro-transcribed* RNA of the original clone compared to a clone containing the mutations
245 may be performed in order to assess the significance of the mutations for efficient cell
246 culture growth. A direct transfection system would generally be useful for cell culture
247 studies on ratHEV without the need of nude rat inoculation.
248
249 In summary, the generation of a versatile reverse genetics system for ratHEV has been
250 shown here. After introduction of specific mutations into the cloned cDNA, the system
251 may be useful to study the phenotypic effects of the mutations. Since rats are widely
252 used and well characterized laboratory animals, studies on genetically engineered
253 ratHEV may give novel insights into organ tropism, replication and excretion kinetics as
254 well as immunological changes induced by hepeviruses.
255
256
257 **Methods**
258
259 **Generation of a genomic clone of ratHEV.** The complete genome of ratHEV was
260 amplified in 4 fragments by RT-PCR using RNA isolated from the liver of a wild rat from
261 Germany containing the ratHEV prototype strain R63/DEU/2009 (GenBank acc.-no.
262 GU345042). RT-PCR was performed with the QIAGEN LongRange 2Step RT-PCR Kit
263 (Qiagen, Hilden, Germany) and primers listed in Supplementary Table S1. The primer
264 binding sites overlapped with unique binding sites for restriction enzymes within the

265 ratHEV genome, thus enabling subsequent cloning of the RT-PCR products. The
266 5'-end-primer contained a T7 RNA polymerase promotor sequence and the
267 3'-end-primer contained an extension of 17 thymidine residues followed by a unique
268 *Xba*I-site (Fig. 1A). The RT-PCR products were cloned successively into a pUC19
269 vector derivative, which contained a multiple cloning site adapted to the used restriction
270 sites. The sequence of the resulting genomic clone was determined by Sanger
271 sequencing using the PCR primers and additional primers.
272
273 ***In vitro* transcription for generation of capped RNA.** The genomic plasmid was
274 purified using the QIAGEN plasmid maxi kit (Qiagen) and subsequently linearized with
275 *Xba*I. The preparation was further purified by phenol/chloroform extraction and
276 re-suspended in RNAse-free water. *In vitro*-transcription was performed using the
277 mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX, USA), which includes adding of a
278 cap-analogue to the 5'-end of the synthesized RNA, according to the protocol of the
279 supplier. RNA purification was carried out by lithium chloride precipitation.
280
281 **Inoculation of rats and sample collection.** Eight nude rats (Long-Evans-run/run,
282 Japan SLC) and two SPF rats (Wistar, Japan SLC) were used in this study. The rats
283 were individually housed in BSL-2 facilities. All rats were negative for ratHEV RNA and
284 anti-ratHEV antibodies, as determined by nested broad-spectrum RT-PCR Johne et al.,
285 2010a) and ratHEV-specific ELISA (Li et al., 2013b), respectively. To analyze the
286 infectivity of the *in vitro*-transcribed RNA, intravenous injection and intrahepatic

287 inoculation was carried out, respectively. The RNA (2.6 x 10^{11} copies/μl) was injected
288 through the tail vein or through a percutaneous inoculation procedure into five different
289 sites of the liver with approximately 100 μl per injection site. The rats were injected
290 under anesthesia of xylazine and ketamine hydrochlorid mixture. To examine the
291 infectivity of recovered ratHEV, the stool specimen was diluted in phosphate-buffered
292 saline (PBS) to prepare a 10% suspension by shaking at 4 °C for 1 h. Both supernatant
293 of cell culture and 10% stool suspension were clarified by centrifugation at 10,000 x g
294 for 30 min, and then passed through a 0.45-μm membrane filter (Millipore, Bedford, MA,
295 USA). The rats were intravenously inoculated with the stool suspensions or with cell
296 culture supernatant through the tail vein. Serum samples of the nude rats were collected
297 at the end of the experiment for detection of ratHEV RNA. Serum samples of Wistar rats
298 were collected weekly for examination of ratHEV-specific IgG antibodies and ALT values.
299 Stool samples were collected one to two times per week. The experiments were
300 reviewed by the Institute's ethics committee and carried out according to the "Guides for
301 animal experiments performed at NIID" under code 113029 and 114012.
302
303 **Inoculation of cell culture.** The human hepatocarcinoma cell line PLC/PRF/5
304 (JCRB0406) from the Health Science Research Resources Bank was used. Cells were
305 grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v)
306 heat-inactivated fetal bovine serum (FBS; Nichirei, Biosciences INC. Tokyo, Japan),
307 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA), at
308 37 °C in a humidified 5% CO_2 atmosphere. For virus inoculation, confluent cells were

309 trypsinized, diluted 1:3 and cultured in a 25 cm^2 tissue culture flask. Next day, the
310 medium was removed and the cells were washed with PBS. A total of 1 ml of the
311 sample was inoculated onto PLC/PRF/5 cells. After adsorption at 37°C for 1 hour and
312 washing of the cells two times with PBS, the suspension was removed and then
313 replaced by 10 ml maintenance medium consisting of medium 199 (Invitrogen, Carlsbad,
314 CA, USA) containing 2% (v/v) heat-inactivated FBS and 10 mM $MgCl_2$. Further
315 incubation was done at 36°C. The culture maintenance medium was replaced with the
316 new medium every 4 days and used for the detection of ratHEV RNA and capsid
317 antigen. Cells were observed daily by light microscopy for the occurence of a cytopathic
318 effect.
319
320 **Quantitative real-time RT-PCR for detection of ratHEV.** The RNA was extracted
321 using the MagNA Pre LC system with the MagNA Pre LC Total Nucleic Acid isolation kit
322 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's
323 recommendations. To determine ratHEV RNA copy numbers, a TaqMan assay was
324 performed in the 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City,
325 CA, USA) using the TaqMan Fast Virus 1-step Master Mix (Applied Biosystems). The
326 primers (900 nmol/L forward primer 5'-GTG GTG CTT TTA TGG TGA CTG-3', nt
327 4123-4143; and 900 nmol/L reverse primer 5'-CAA ACT CAC TAA AAT CAT TCT CAA
328 ACA C-3', nt 4196-4223), and the probe (250 nmol/L 5'-6FAM-GTT CAG GAG AAG
329 TTC GAG GCC GCC GT-TAMRA-3', nt 4148-4173) were added. One-step quantitative
330 RT-PCR (RT-qPCR) cycling conditions were 15 min at 48°C, a 10 min incubation at

331 95°C, and 50 cycles for 15 s at 95°C and 1 min at 60°C (Li et al. 2013b). The capped *in*

332 *vitro*-transcribed RNA of R63/DEU/2009 was used as standard for calculation of the

333 RNA molecule number. A 10-fold serial dilution of the RNA standards (10^7 to 10^1 copies)

334 was used for the quantitation of viral genome copy numbers in reaction tubes.

335 Amplification data were collected and analyzed with Sequence Detector software

336 version 1.3 (Applied Biosystems).

337

338 **Detection of anti-ratHEV IgG and ratHEV capsid antigen.** Anti-ratHEV IgG was

339 detected by enzyme-linked immunosorbent assay (ELISA) as described previously (Li

340 et al., 2011). An antigen capture ELISA was used to detect ratHEV antigen. Briefly,

341 duplicate wells of flat-bottom 96-well polystyrene microplates (Dynex Technologies Inc.,

342 Chantilly, VA, USA) were coated with 100 µl of a coating buffer (0.1 M

343 carbonate-bicarbonate buffer, pH 9.6) containing a 1:1000 dilution of a hyperimmune

344 serum elicited in a rabbit with ratHEV-like particles (ratHEV-LPs) (Li et al., 2011). The

345 coating was performed at 4ºC overnight. Unbound antibodies were removed, and the

346 wells were washed twice with 10 mM PBS containing 0.05% Tween 20 (PBS-T), and

347 then the blocking was carried out at 37ºC for 1 hour with 150 µl of 5% skim milk (Difco

348 Laboratories, Detroit, MI, USA) in PBS-T. 100 µl of cell culture supernatants were added

349 to the wells and incubated for 1 hour at 37ºC. After the wells were washed 3 times with

350 PBS-T, 100 µl of a guinea pig anti-ratHEV-LPs hyperimmune serum (1:1000 dilution with

351 PBS-T containing 1% skim milk) was added to the wells and the plate was incubated for

352 1 hour at 37ºC. The plate was washed 3 times with PBS-T, and then horseradish

353 peroxidase-conjugated goat anti-guinea pig IgG antibody (Cappel, Durham, NC, USA)
354 (1: 1000 in PBS-T containing 1% skim milk.) was added to each well. After incubation for
355 1 hour at 37°C, the plate was washed 3 times with PBS-T and 100 µl of substrate
356 o-phenylenediamine and H_2O_2 was added. The plate was left for 30 min at room
357 temperature, and then the reaction was stopped with 50 µl of 4 N H_2SO_4. The
358 absorbance at 492 nm was measured with a microplate reader (Molecular Devices
359 Corp., Tokyo, Japan). The cut-off was defined using supernatants of non-infected cell
360 cultures and set as 0.150. Test samples were considered positive when the absorbance
361 was above the cut-off value.
362
363 **Viral genome sequencing.** The whole genome sequence of ratHEV present in
364 selected samples was analyzed by next generation sequencing. Virus particles were
365 enriched from the samples by cesium chloride density gradient centrifugation (Li et al.,
366 2011). A 200 bp fragment library was constructed for each sample preparation using the
367 NEBNext Ultra RNA Library Prep Kit for Illumina v2.0 (New England Biolabs, Ipswich,
368 MA, USA) according to manufacturer's instructions. Samples were bar-coded for
369 multiplexing using NEBNext Multiplex Oligos for Illumina, Index Primer Sets 1 and 2
370 (New England Biolabs, Ipswich, MA, USA). Library purification was done using
371 Agencourt AMPure XP magnetic beads (Beckman Coulter, Pasadena, CA, USA) as
372 recommended in the NEBNext protocol. The quality of the purified libraries was
373 assessed on a MultiNA MCE-202 bioanalyzer (Shimadzu Corporation, Kyoto, Japan)
374 and the concentrations were determined on a Qubit 2.0 flourometer using the Qubit HS

375 DNA Assay (Invitrogen, Carlsbad, CA, USA). A 151-cycle paired-end read sequencing
376 run was carried out on a MiSeq desktop sequencer (Illumina, San Diego, CA, USA)
377 using the MiSeq Reagent Kit v2 (300 cycles). Following preliminary analysis, the MiSeq
378 reporter programme was used to generate FASTQ formatted sequence data for each
379 sample. Sequence data was analyzed using CLC Genomics Workbench Software
380 v7.5.1 (CLC Bio, Aarhus, Denmark). Contigs were assembled from obtained sequence
381 reads by *de novo* assembly. Missing sequences of the 5'-terminal non-coding regions of
382 the genomes were determined using the Rapid Amplification of cDNA Ends Kit
383 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In order to
384 identify point mutations, the generated sequences were compared with that derived
385 from the cDNA clone using DNASIS-Mac v3.0 (Hitachi Solutions, Ltd, Tokyo, Japan).
386
387 **Liver enzyme level.** ALT values in rat sera were monitored weekly by the Fuji Dri-Chem
388 Slide GPT/ALT-PIII kit (Fujifilm, Saitama, Japan). The geometric mean of ALT values
389 during the pre-inoculation period of each animal was defined as the normal ALT value,
390 and a two-fold or greater increase at the peak was considered as a sign of hepatitis.
391
392

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520 **Tables**

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522

523 **Table 1.** Comparison of the genome sequences of ratHEV derived from cDNA,

524 faeces of nude rats and cell culture. Nucleotide and amino acid numbering

525 according to ratHEV strain R63/DEU/2009 (GenBank acc.-no. GU345042).

526

Nucleotide Position	Region	Nucleotide Position	Nucleotide cDNA	Nucleotide Nude rat	Nucleotide Culture	Amino acid Position	Amino acid cDNA	Amino acid Nude rat	Amino acid Culture
295	ORF1	285	T	T	C	95	-*	-	-
343	ORF1	333	C	C	T	111	-	-	-
1552	ORF1	1542	A	A	G	514	-	-	-
1686	ORF1	1676	A	A	T	559	Y	Y	F
1889	ORF1	1879	C	C	T	627	P	P	S
4599	ORF1	4589	A	A	G	1530	D	D	G
5939	ORF2	991	G	G	A	331	A	A	T
6338	ORF2	1390	T	T	C	464	-	-	-
6411	ORF2	1463	C	C	T	488	T	T	I
295	ORF4	269	T	T	C	90	L	L	P
343	ORF4	317	C	C	T	106	T	T	M

529 *- synonymous exchange

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