

1 **Alimentary tract as entry route for hantavirus infection**

2 *Running title:* Gastrointestinal hantavirus transmission

3 *Article type:* Observation

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## 25    **ABSTRACT**

26    Hantaviruses are zoonotic agents that cause hemorrhagic fever with renal and/or  
 27    cardiopulmonary manifestations, reaching fatality rates of up to 50%. A large proportion of  
 28    hantavirus patients also suffer from gastrointestinal complications of unclear cause. Puumala  
 29    hantavirus (PUUV), the predominant endemic hantavirus in Europe, is associated with mild  
 30    forms of hemorrhagic fever with renal syndrome. PUUV is transmitted to humans by  
 31    exposure to aerosolized excrement from infected rodents. In this study we demonstrate that  
 32    PUUV can also infect via the alimentary tract. PUUV retains infectivity in gastric juice for at  
 33    least some time in pH >3 and is able to infect polarized human Caco-2 monolayers. This  
 34    small intestinal cell model exhibited viral association with endosomal antigen EEA-1,  
 35    followed by virus replication and loss of epithelial barrier function with concomitant  
 36    basolateral (serosal) occurrence of viruses. Cellular disturbance and depletion of the tight  
 37    junction protein ZO-1 appeared after prolonged hantavirus infection. Subsequent cell  
 38    rounding and detachment was observed, leading to paracellular leakage. Moreover,  
 39    experimental PUUV infection of Syrian hamsters by the intragastric route led to  
 40    seroconversion and protection against challenge by lethal Andes virus in a dose-dependent  
 41    manner, confirming that PUUV retains infectivity when administered directly to the gut.  
 42    These data are the first report of PUUV infections via the alimentary tract and demonstrate the  
 43    importance of this route of transmission for public health considerations.

44

## 45    **Keywords**

46    Hantavirus, Puumala virus, hemorrhagic fever with renal syndrome, zoonoses, virus entry,  
 47    gastrointestinal infection, gastric barrier, epithelial barrier, tight junction, transmigration,  
 48    endocytosis, hamster model

49 **Importance**

50 Hantaviruses are zoonotic pathogens, transmitted to humans by small animals, which cause  
51 severe hemorrhagic fevers worldwide. To date these viruses are generally thought to be  
52 transmitted by one of two routes: either through the inhalation of aerosolized virus from  
53 infected animal droppings (the predominant route of infection) or by rodent bites. It had been  
54 observed before that Andes hantavirus can infect hamsters by intragastric application, though  
55 the route was less efficient than others tested and the mechanisms were not investigated. In  
56 this study we show that PUUV, a hantavirus endemic to Europe, is capable of surviving in  
57 gastric juice, crossing the gastric barrier and causing a productive infection in the Syrian  
58 Hamster model of PUUV infection. We conclude that the alimentary tract is a productive path  
59 of infection. Our findings provide new insight into the mechanisms of hantavirus infection  
60 and have implications for hantavirus epidemiology and outbreak prevention measures.

61

## 62 INTRODUCTION

63 Hantaviruses are zoonotic viruses harbored by small mammals such as rodents, shrews, or  
64 bats. They can cause disease in humans, leading to hemorrhagic fevers of varied severity. Old  
65 World hantavirus infections usually lead to Hemorrhagic Fever with Renal Syndrome  
66 (HFRS), with case fatality rates of up to 15%, while the clinical course New World hantavirus  
67 infection is mainly linked with Hantavirus Cardiopulmonary Syndrome (HCPS), also known  
68 as hantavirus pulmonary syndrome, and case fatality rates of up to 50%. The symptomatology  
69 of both manifestations is not strict and mixed clinical courses, as well as asymptomatic  
70 infection, can occur (1).

71 In Europe most cases of hantavirus disease are caused by one of two strains of virus. Human  
72 infection with Puumala virus (PUUV), which is harbored by the bank vole (*Myodes*  
73 *glareolus*), typically leads to less severe disease, while individuals infected with Dobrava-  
74 Belgrade virus (DOBV), whose three genotypes *Dobrava*, *Kurkino*, and *Sochi* are carried by  
75 rodents of the genus *Apodemus*, are more likely to exhibit severe symptoms (1). Pathogenic  
76 hantaviruses are generally thought to enter the human body by inhalation of contaminated  
77 droppings from infected host animals, followed by infection of the lung epithelium.  
78 Moreover, in rare cases, rodent bites were reported to be the cause of infection (2).

79 Hantaviruses preferentially use different  $\beta$ -integrins and CD55/DAF for cell entry (3 - 5).  
80 However, the presence of some of the receptor molecules on the basolateral side of the  
81 affected tissues requires effective disruption, or at least penetration, of the cell barrier, as  
82 shown *in vitro* for different tissue types (6). Interference with cellular barrier function of  
83 infected tissues can lead to capillary leakage and is a crucial aspect of hantavirus pathogenesis  
84 (2). As a prototypical member of the genus *Hantavirus*, Hantaan virus, was shown to enter  
85 cells by clathrin-dependent endocytosis after receptor-mediated binding to the cell surface (7).

Besides the typical organ preference of hantavirus disease, several studies have shown that the majority of patients exhibits hemorrhagic gastropathy (8). Moreover, in the Syrian hamster model established for South American Andes virus (ANDV) intragastric inoculation can result in lethal disease, albeit with a higher 50% lethal dose (LD<sub>50</sub>) than the more effective intramuscular, subcutaneous, intranasal, and intraperitoneal delivery routes (9). In rare cases, contact to contaminated food was named as a possible risk factor for human hantavirus infections (10). These findings demonstrate that the gut is affected in human hantavirus disease and that the infection via the alimentary tract is a possible route of transmission.

A gastrointestinal transmission route for HFRS-causing hantavirus has not been explicitly proposed or excluded for either humans or rodent infection. For this reason we conducted both *in vitro* and *in vivo* studies with PUUV, the main European hantavirus responsible for HFRS, to ascertain its viability as a route of infection. *In vitro* we investigated susceptibility of the human small intestinal epithelium for hantavirus infection, based on the polarized Caco-2 cell culture system, an established model for intestinal barrier function, and investigated the resistance of virus particles to gastric juice. *In vivo* we demonstrated that intragastric infection of Syrian hamsters with PUUV can lead to seroconversion and protective immunity against subsequent lethal hantavirus challenge.

## MATERIALS AND METHODS

### *Cell culture*

Caco-2 cells (ATCC HTB-37) were cultivated at 37°C with 5% CO<sub>2</sub> and maintained in minimal essential medium (MEM) with 10% fetal bovine serum, and 1% L-glutamine. For immunofluorescence assays (IFA) Caco-2 cells were grown on coverslips in 24-well plates. For measurement of transepithelial electrical resistance (TER) the cells were seeded on

110 permeable PCF filter inserts with an area of 0.6 cm<sup>2</sup> and a pore size of 0.4 μm (Millipore,  
111 Germany). The cells were grown for 21 days for differentiation to small intestinal properties.  
112 Cell medium was replaced every two or three days. Transepithelial electrical resistance (TER)  
113 was measured by an ohmmeter fitted with chopstick electrodes (EVOM, World Precision  
114 Instruments, USA) before infection and confluent cell monolayers showing epithelial  
115 resistance with above 500 Ω·cm<sup>2</sup> were used for experiments.

#### 116 *Virus cultivation*

117 For *in vitro* studies: PUUV strain Sotkamo was grown on Vero-E6 cells (ATCC CRL-1586;  
118 American Type Culture Collection, Manassas, USA) under standard cell culture conditions.  
119 Viruses were harvested by ultracentrifugation through a sucrose cushion in order to remove  
120 residual cell culture components and titers were determined by focus titration, as previously  
121 described (11). For *in vivo* studies: PUUV strain K27 and ANDV strain Chile-9717869 were  
122 grown on Vero-E6 cells in T-150 flasks and collected from infected-monolayer supernatants.  
123 Cell debris were removed by low speed centrifugation (2,500 rpm in a table top centrifuge),  
124 and virus was twice plaque purified. Virus stocks were aliquoted and stored at -60°C or colder  
125 (12).

#### 126 *Virus inactivation by gastric juice*

127 Gastric juice was taken from adult patients who underwent gastroscopy for other diagnostic  
128 reasons and were not treated by any acid suppressing medication. According to the  
129 Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects)  
130 all patients gave written informed consent.

131 pH was measured and dilution series were performed by solution of NaOH pellets. PUUV  
132 stock was incubated with gastric juice of different pH for given time periods of 0 – 15

133 minutes. Subsequently pH was set to a value of 7 and culture medium was added. The treated  
134 virus solution was serially diluted and titrated on Vero-E6 cells.

135 *Cell infection experiments*

136 Caco-2 cells on coverslips or filter inserts were infected for 1 h at a multiplicity of infection  
137 (MOI) of 0.1 - 1.0. Afterwards remaining inoculum was washed away and the cells were  
138 incubated in culture medium at described conditions. TER measurements in filter inserts were  
139 conducted every 24 h. Samples for microscopy were fixated with 4% methanol-free  
140 paraformaldehyde, afterwards blocked by 25 mM glycine in phosphate buffered saline (PBS).  
141 Samples for quantitative PCR (qPCR) were collected in AVL buffer (Qiagen, Germany) in  
142 case of culture medium or RLT buffer (Qiagen) in case of cells and stored at -80°C. For virus  
143 titration from infection kinetics culture medium was stored at -80°C.

144 *Quantitative RT-PCR*

145 RNA from culture supernatants and cell lysates was extracted according to manufacturer's  
146 specifications by QIAamp Viral RNA Mini Kit and RNeasy Mini Kit, respectively. Reverse  
147 transcription was performed with M-MLV reverse transcriptase (Invitrogen, Germany). Virus  
148 quantification was conducted by real-time RT-PCR, as described before (13).

149 *Fluorescence microscopy*

150 Fluorescence staining and confocal laser-scanning microscopy (CLSM) was performed as  
151 previously described (14). The following antibodies were used: anti-ZO-1 (Zonula occludens  
152 protein-1), anti-EEA1 (early endosomal antigen 1), anti-hantaviral nucleocapsid protein  
153 (1:100), Alexa-Fluor488 goat anti-mouse or -rabbit IgG, and Alexa-Fluor594 goat anti-mouse  
154 or -rabbit IgG (1:500; Invitrogen). Cell nuclei were stained with 4'-6-diamidino-2-  
155 phenylindole dihydrochloride (DAPI, 1:1,000).

156 *Epithelial apoptosis*

157 Occurrence of apoptosis was visualized by TUNEL assay (terminal deoxynucleotidyl  
158 transferase-mediated deoxyuridine triphosphate nick-end labeling, In-situ Cell Death  
159 Detection Kit-Fluorescein, Roche, Germany) in Caco-2 monolayers four days post infection  
160 as previously described (14). Percent of apoptotic events were calculated as ratio of all cells in  
161 a low-power field (200 x magnification). Six pictures per sample containing more than 1,300  
162 cells each were counted for the analysis.

163 *Animal experiments*

164 Female Syrian golden hamsters, age 6-8 weeks (Harlan, Indianapolis, USA) were anesthetized  
165 by inhalation of vaporized isoflurane using IMPAC 6 veterinary anesthesia machine. Once  
166 anesthetized hamsters were challenged intragastrically with 1,000 PFU PUUV, 10,000 PFU  
167 PUUV, or 10,000 PFU  $\gamma$ -irradiated PUUV ( $3 \times 10^6$  rad) diluted in 1mL sterile PBS delivered  
168 by a 3 mL syringe with a 2-inch 18 gauge gavage needle. Forty-two days post PUUV  
169 infection hamsters were challenged intramuscularly (i.m., caudal thigh) with 200 PFU ANDV  
170 diluted in 0.2 mL sterile PBS delivered with a 1mL syringe with a 25-gauge five-eighths-inch  
171 needle. All work involving hamsters were performed in an animal biosafety level 4 (ABSL-4)  
172 facility. Euthanasia was performed on hamsters meeting early endpoint criteria.

173 Research was conducted in compliance with the Animal Welfare Act and other federal  
174 statutes and regulations relating to animals and experiments involving animals and adheres to  
175 principles state in the Guide for the Care and Use of Laboratory Animals, National Research  
176 Council, 2011. The facilities where this research was conducted are fully accredited by the  
177 Association for Assessment and Accreditation of Laboratory Animal Care International.

178 *N-specific ELISA assay:*



179 ELISA plates (Costar, United States) were coated with recombinant PUUV N-antigen in  
 180 carbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked with PBS, 5% skim milk, and  
 181 0.05% Tween 20 (blocking solution) for 1 hour at 37°C, washed once with PBS and 0.05%  
 182 Tween 20 (wash solution), and incubated with hamster sera diluted in blocking solution plus  
 183 2% *Escherichia coli* lysate for 30 minutes at 37°C. Plates were washed 3 times with wash  
 184 buffer and incubated for 30 minutes at 37°C with horseradish peroxidase-conjugated goat  
 185 anti-hamster IgG (Kiegaard & Perry Laboratories [KPL], USA) in blocking solution. Plates  
 186 were washed 3 times with wash buffer and incubated for 10 minutes at room temperature with  
 187 tetra-methylbenzidine substrate (KPL). The colorimetric reaction was stopped by adding Stop  
 188 solution (KPL) and the absorbance at 450 nm was determined. The specific sum optical  
 189 density (OD) was calculated by adding the background subtracted OD values, for dilutions  
 190 whose OD was greater than the mean OD for serum samples from negative-control wells plus  
 191 3 standard deviations. The PUUV N antigen was used to detect not only PUUV but also  
 192 ANDV N-specific antibody responses as previously reported (12). Hamster sera were heat  
 193 inactivated before testing by ELISA.

#### 194 *Statistics*

195 Data are expressed as mean values  $\pm$  standard error of the mean. Statistical analysis was  
 196 performed using 2-tailed Student t test.  $P \leq 0.05$  was considered to be statistically significant.  
 197 GraphPad Prism 6 software was used for the analysis.

198

## 199 **RESULTS**

200 *Susceptibility of hantavirus towards gastric juice.*

201 PUUV survival in the gastric lumen was tested *in vitro* by incubation of virus stocks to human  
 202 gastric juice of varying pHs. The antiviral activity of gastric juice was effective at low pH  
 203 between 1 and 3, with no virus surviving an exposure of as little as 1 minute. PUUV did  
 204 survive exposure at pH values of 4 or 5 with an effective titer reduction below 1 log<sub>10</sub> at pH 7  
 205 (Figure 1A).

#### 206 *Virus replication in Caco-2 monolayers*

207 Prior to beginning experiments we confirmed that Caco-2 cells expressed β1- and β3-integrins  
 208 as well as CD55/DAF, the receptors preferentially used by hantaviruses for entry, on their  
 209 surface (data not shown). Experimental infection of human intestinal cells, Caco-2, with  
 210 PUUV at MOI 0.1 revealed a time-dependent increase of intra- and extracellular viral load by  
 211 quantitative RT-PCR assays (Figure 1B). The intracellular localization of hantavirus in Caco-  
 212 2 cells could also be visualized by CLSM between 24 and 96 h post infection (p.i.).  
 213 Hantavirus antigens were found intracellularly in co-localization with early endosomal  
 214 antigen 1 (EEA1), demonstrating endosomal localization of virus particles (Figure 1C). In  
 215 cells infected with UV-inactivated virus such signals were not present (data not shown).

216 Translocation of PUUV through Caco-2 monolayers after apical infection could be shown by  
 217 qRT-PCR detection of viral RNA in basal culture medium. The basal release of viruses was  
 218 detectable after 24 h and it increased by 2 log<sub>10</sub> over the next 216 h (Figure 1D).

#### 219 *Epithelial barrier dysfunction in infected Caco-2 monolayers*

220 Infection of Caco-2 monolayers at MOI 0.1 revealed stable TER values up to 48 h p.i.,  
 221 however, by 72 h p.i. the TER decreased to 60% of the initial value (Figure 2A). Using  
 222 CLSM, viral antigen was observed intracellularly, in close proximity to the tight junctions,  
 223 which exhibit condensed zonula occludens protein-1 (ZO-1) (Figure 2B). However, clear co-  
 224 localization of PUUV and ZO-1, as evidenced by a lack of yellow signal, did not occur,

225 excluding a direct interaction. Moreover, the ZO-1 staining pattern could indicate a disturbed  
 226 gate function (Figure 2C), by actomysin-constriction mediated tight junction re-distribution,  
 227 which could also lead to a loss of cell-cell contacts. To exclude the possible induction of cell  
 228 death by apoptosis Caco-2 monolayers were stained by TUNEL 240 h p.i. Apoptotic events  
 229 counted in PUUV-infected monolayers did not differ significantly from mock-infected  
 230 monolayers ( $0.46\% \pm 0.10\%$  versus  $0.42\% \pm 0.05\%$ ,  $n=5$ ,  $P = 0.73$ ).

231 Infection of Caco-2 monolayers with higher concentrations of PUUV (MOI 1.0) revealed  
 232 earlier and more pronounced effects (Figure 2C). Here, the condensation of ZO-1 was found  
 233 by 48 h p.i., followed by disappearance of ZO-1 signals, and finally cell detachment and/or  
 234 cell exfoliation leading to epithelial leakage by 96 h p.i.

#### 235 *Administration of PUUV intragastrically leads to productive infection in Syrian Hamsters*

236 Groups of eight hamsters were instilled intragastrically with either 1,000 PFU PUUV or  
 237 10,000 PFU PUUV. To confirm that seroconversion was caused by active viral replication,  
 238 and not input virus, a control group of eight hamsters was injected with 10,000 PFU  $\gamma$ -  
 239 irradiated PUUV. 35 days post challenge the hamsters were bled, and seroconversion  
 240 evaluated by N-ELISA (Figure 2D). 2/8 hamsters in the 1,000 PFU challenge group, and 3/8  
 241 hamsters in the 10,000 PFU challenge group seroconverted. None of the hamsters instilled  
 242 with irradiated virus seroconverted, confirming that viral replication occurred in  
 243 seroconverted animals.

244 Prior infection with PUUV is able to protect animals from lethal ANDV challenge. To  
 245 confirm that hamsters which had seroconverted were truly infected, at 42 days post PUUV  
 246 challenge all hamsters were challenged with 200 PFU of ANDV. 5/5 (100%) of the  
 247 seroconverted hamsters survived ANDV challenge, confirming infection, while 15/19 (79%)

248 of non-seroconverted hamsters succumbed (n=24, P=0.003 according to Fisher's exact test).  
 249 The survival curves for PUUV intragastrically "vaccinated" hamsters are in Figure 2E.

250

## 251 **DISCUSSION**

### 252 *Stomach physiology, survival in the gastric lumen*

253 In the human alimentary tract entering viruses encounter gastric acid and proteolytic enzymes  
 254 with gastric contents falling below pH 4 a maximum 70% of the time (15). pH values in  
 255 human stomach can postprandially easily increase above pH 5.0, even, up to pH 7.0 in young  
 256 children after consumption of food with high buffer capacity such as milk or milk products  
 257 (15, 16). Stomach transit times can vary between 5 minutes and 2 hours, depending on food  
 258 and liquid intake (17). Dyspepsia, which is diagnosed in up to 25% of western population, is  
 259 treated with proton pump inhibitors (PPIs), leading to elevated pH and higher susceptibility to  
 260 gastrointestinal infections (18).

261 In our study PUUV was shown to be able to survive human gastric juice for at least 15 min at  
 262 pH 4, though viral titers were reduced by 3 log<sub>10</sub>. Exposed to gastric juice at pH 5 for 15  
 263 minutes, the reduction of virus titer amounted to 2 log<sub>10</sub>, and at pH 7 less than a 1 log<sub>10</sub> loss of  
 264 viability was observed, suggesting the virus can survive long enough to be viably released  
 265 into the duodenum. Postprandial decreases in gastric pH can lead to reduced activity of gastric  
 266 proteolytic enzymes, like pepsin A or pepsin C, making the environment even more  
 267 conducive to viral survival. Under postprandial or achlorhydric conditions the antimicrobial  
 268 gastric barrier is vulnerable and can be overcome by pathogens (15, 19). Finally, stomach  
 269 conditions are dependent on the host's age, health status (gastric secretion rate), and  
 270 nutritional preferences (buffer capacity of the food) leading to variable risk factors for  
 271 hantavirus survival and therefore infection through the gastrointestinal tract.

272 Intragastric inoculation followed by a systemic infection has been previously demonstrated in  
 273 the hamster model of hantavirus pulmonary syndrome using Andes virus (9). The intragastric  
 274 50% lethal dose (LD<sub>50</sub>) of 225 plaque forming units (pfu), higher than the LD<sub>50</sub> for both  
 275 intranasal (LD<sub>50</sub> = 95 pfu) and intramuscular (LD<sub>50</sub> = 8 pfu) infection, indicating the route of  
 276 infection was the least efficient of the three (9). The fact that the intragastric route leads to  
 277 productive infection at all, however, suggests that it is a possible route of human infection.  
 278 Moreover, the high pH of the rodent stomach (median of pH 3.1 - 4.5 in the mouse) in  
 279 comparison to pH values known from humans (20) implicates an even easier transmission  
 280 among the host animals.

#### 281 *Endocytotic cell entry*

282 The small intestinal tissue, with its major resorptive function, is an entry site for pathogens  
 283 including Norovirus or *Yersinia enterocolitica*. The MALT (mucosa-associated lymphoid  
 284 tissue) is the site for replication and dissemination for several pathogens including HIV (21).  
 285 In our experiments Caco-2-derived human intestinal epithelial cells are capable of being  
 286 infected by PUUV. The virus localized to the endocytic pathway, as visualized by co-  
 287 localization of virus N-protein with EEA1, as already shown for Hantaan virus in Vero-E6  
 288 cells (7), while the membrane ruffling visible at late time points of infection could be an  
 289 additional sign of high endocytic activity. Moreover, interactions of pathogenic viruses with  
 290 tight junction proteins containing PDZ domains (PSD-95/Dlg/ZO-1) or CAR (Coxsackie virus  
 291 and adenovirus-receptor) have been frequently described (reviewed in 22) as have interactions  
 292 between old world hantaviruses and DAF/CD55 (5). The data presented here, including the  
 293 proximity of viral nucleocapsid protein to tight junctions together with ZO-1 ruffling,  
 294 reinforce the idea that PUUV uses similar mechanisms during its infection of intestinal  
 295 epithelial cells.

296 *Epithelial barrier dysfunction*

297 As the result of hantavirus infection, transcytotic viral uptake occurs as seen in our cell  
 298 culture infection model, even at low MOIs. In cell culture experiments using higher viral  
 299 concentrations, cell detachment and severe epithelial damage takes place with preceding  
 300 cytoskeletal rearrangements (data not shown) and tight junction impairment. Finally, cell  
 301 rounding and detachment lead to significant barrier dysfunction. Loss of cells due to apoptosis  
 302 could be excluded, but cell exfoliation or shedding, as well as other cell death mechanisms  
 303 like autophagy or necrosis, could play a role in the pathogenic action of hantaviruses in the  
 304 gut. The latter condition may more accurately resemble clinical cases of hemorrhagic fever,  
 305 where a manifestation of hantavirus disease is compromised endothelial barrier function  
 306 leading to petechiae or even hemorrhages (2). Since disturbance of epithelial barrier function  
 307 in the intestine leads to paracellular antigen influx (leaky gut concept [14]), entry of further  
 308 virus particles and luminal bacterial antigens is possible. This could provoke immune  
 309 activation and gastrointestinal symptoms like diarrhea, vomiting or abdominal pain, which are  
 310 often found in clinical HFRS cases.

311 In the Syrian hamster model of intragastric PUUV infection we were able to confirm that the  
 312 virus is capable of infecting, and replicating within the animal at a challenge dose of 1,000  
 313 PFU, as measured by seroconversion and protection from lethal ANDV challenge. Given the  
 314 limited number of doses tested it is not possible to determine a precise infectious dose that  
 315 infects 99% (ID99) of hamsters, however, given that only 3/8 hamsters infected with 10,000  
 316 PFU PUUV seroconverted the ID99 must be higher than that dose. This would make the ID99  
 317 for intragastric infection at least 10 fold higher than for intramuscular or intranasal  
 318 (unpublished data, Hooper lab), indicating that while intragastric infection is possible; it is  
 319 less efficient than other methods used in the laboratory.

320 *Hantavirus route of infection*

321 Here, we present evidence that the oral route of transmission, potentially by contaminated  
322 food, is plausible for PUUV. Furthermore, it is supposable that encapsulated virus particles  
323 from bronchioalveolar exudate can infect the host via the alimentary tract when swallowed,  
324 which is a common event. The results of our work denote a new aspect of hantavirus  
325 pathogenesis to be included in epidemiological considerations.

326

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337

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403

404 **FIGURE LEGENDS**

405

406 **Figure 1. Infection of intestinal cells by hantavirus**

407 **A) Hantavirus survival in gastric juice.** Infectious dose of  $10^6$  Puumala virus (PUUV)  
408 particles was suspended to pure gastric juice set to pH 1 - 7 with NaOH for 1, 10, or 15 min.

409 After given incubation intervals the gastric juice was neutralized with NaOH and the virus  
410 suspension was used to infect VERO-E6 cells for focus titration of infectious particles.

411 **B) Growth kinetics of hantaviruses in Caco-2 cells.** Caco-2 cells growing on cell culture  
412 slides were infected with PUUV at MOI of 0.1. Viral replication was monitored by qPCR in  
413 culture medium and cells. **C) Intracellular localization of hantavirus in Caco-2 cells.**

414 Confocal laser-scanning microscopy (CLSM) of infected cell monolayers. Caco-2 cells  
415 growing on coverslips were infected with PUUV at MOI of 0.1. The cells were fixed with  
416 methanol-free formaldehyde and stained with antibodies against PUUV nucleocapsid protein  
417 (red) and early endosomal antigen 1 (EEA1, green). Cell nuclei were stained by DAPI (blue).

418 **D) Translocation of hantavirus through polarized Caco-2 monolayers.** Caco-2 cells  
419 growing on filter inserts for at least 3 weeks were infected by PUUV at MOI 0.1. Apical and  
420 basal medium were collected at 24, 120, and 240 h p.i. and investigated for viral replication  
421 by qPCR.

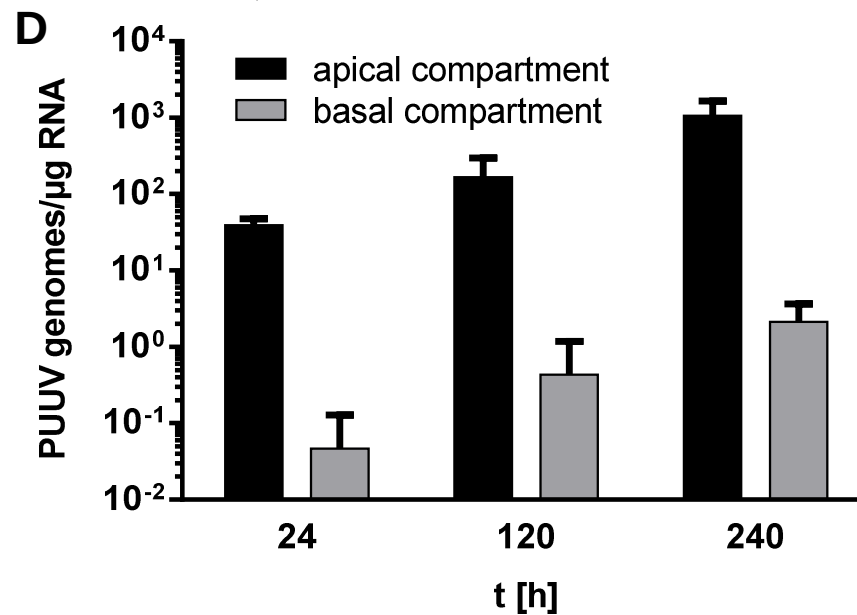
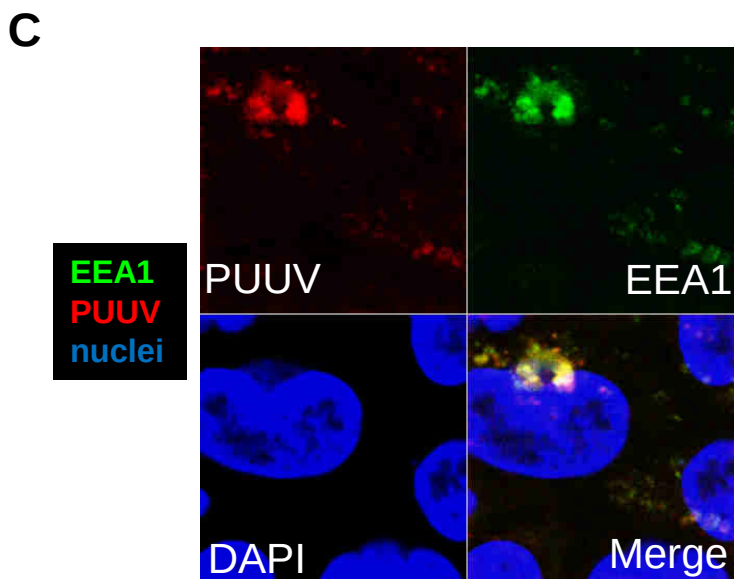
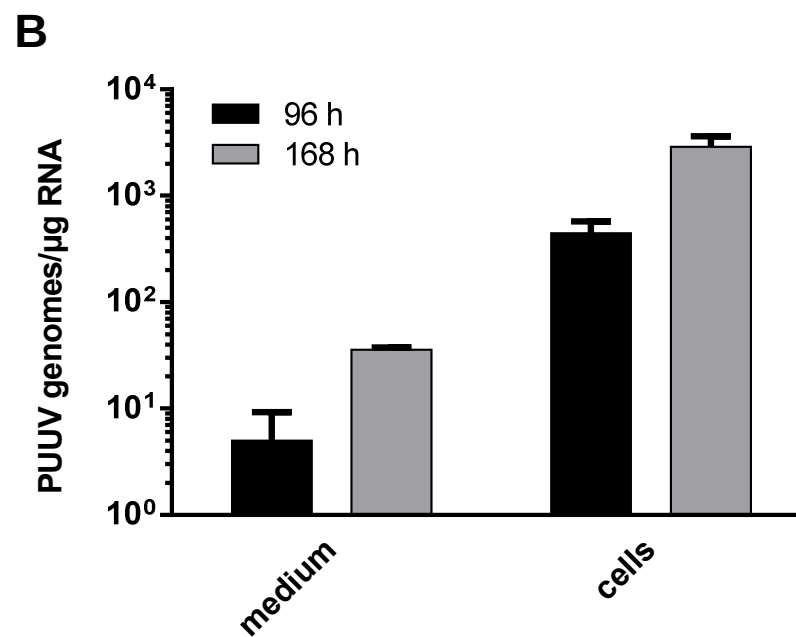
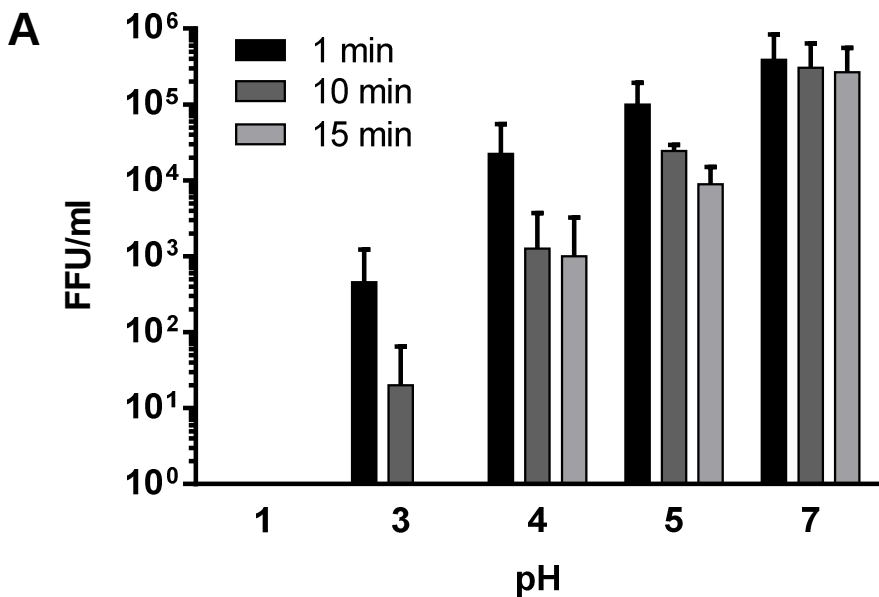
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423 **Figure 2. Epithelial barrier dysfunction and loss of cellular integrity**

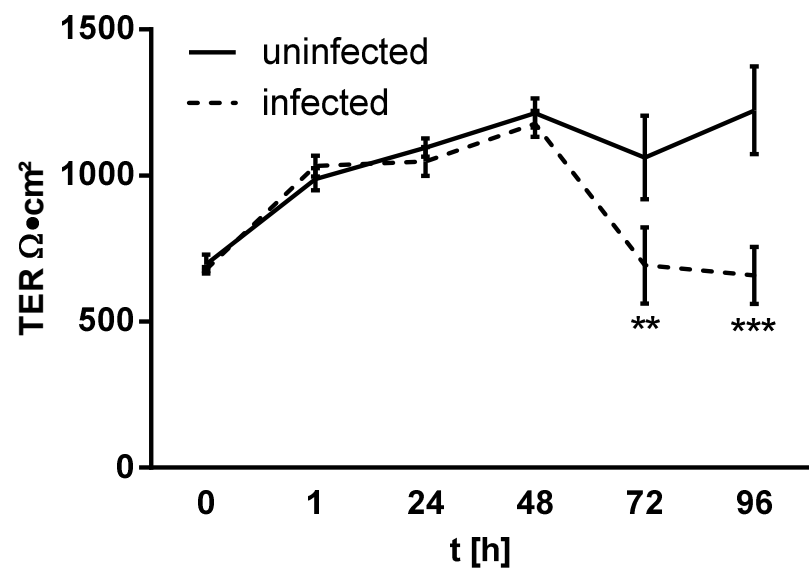
424 **A) Epithelial barrier dysfunction in infected Caco-2 monolayers.** Caco-2 cells growing on  
425 filter inserts were infected by PUUV at MOI 0.1. Transepithelial electrical resistance (TER)  
426 was measured during infection with chopstick electrodes. **B) Tight junction disturbance.**

427 Caco-2 cells grown on filter inserts were infected with PUUV at MOI of 0.1 and analyzed by

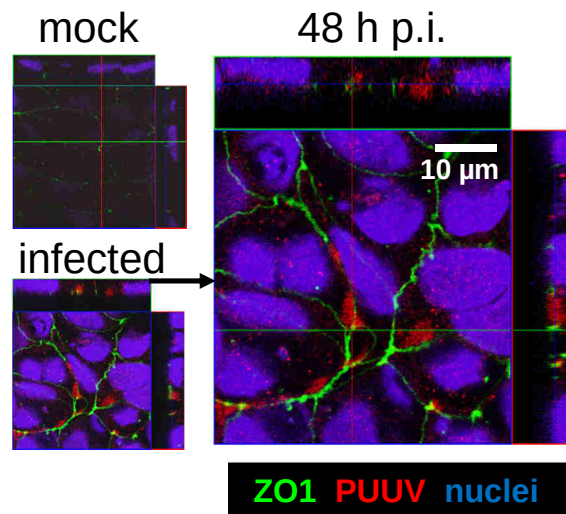
428 CLSM. At 48h cells were fixed and stained for PUUV nucleocapsid protein (red) and zonula  
429 occludens protein 1 (green). Cell nuclei were stained by DAPI (blue). **C) Leaks in Caco-2**  
430 **cells monolayers at high MOI.** Caco-2 cells grown on coverslips were infected with PUUV  
431 at MOI of 1.0. At different time points the cells were fixed and stained with antibodies against  
432 PUUV nucleocapsid protein (red) and zonula occludens protein 1 (green) and analyzed by  
433 CLSM. Mock control is shown at 96 h. **D) Intragastral infection of hamster by Puumala**  
434 **virus.** Syrian hamsters (animal numbers given on the x-axis) were infected with either 1,000  
435 PFU, 10,000 PFU or 10,000 PFU  $\gamma$ -irradiated PUUV. Thirty five days post infection hamsters  
436 were bled to test for seroconversion by N-ELISA. Dots represent hamsters for which  
437 subsequent ANDV challenge was lethal. **E) Survival curves for hamsters “vaccinated”**  
438 **intragastrically with PUUV.** 42 days post intragastric PUUV challenge, the same hamsters  
439 were challenged with 200 PFU ANDV i.m.



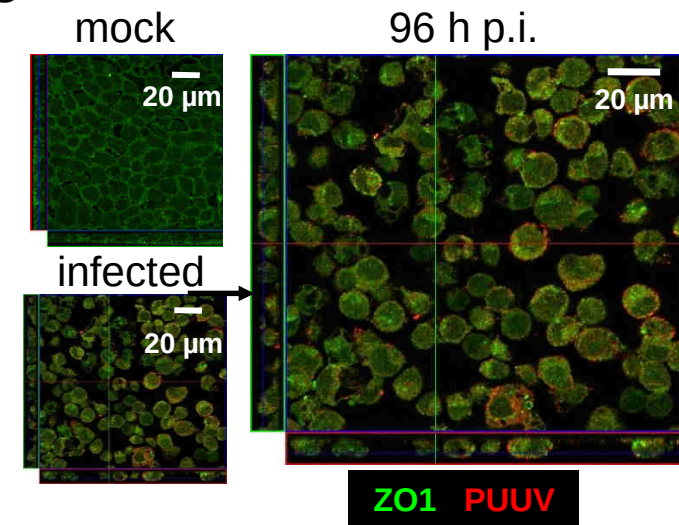
**A**



**B**

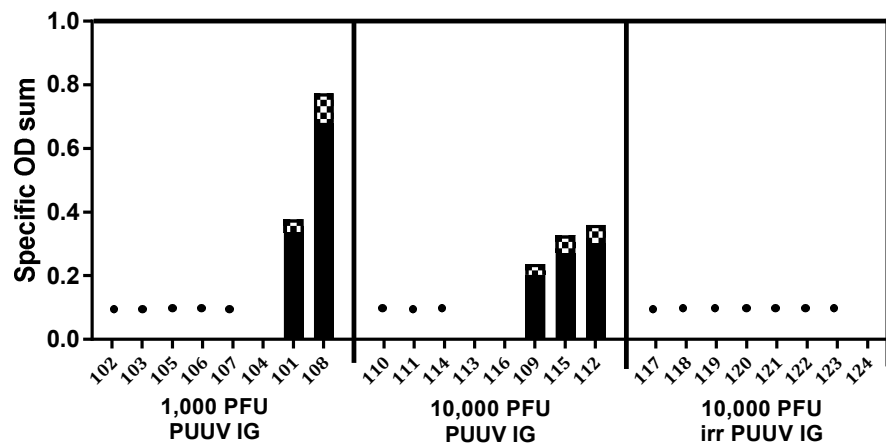


**C**

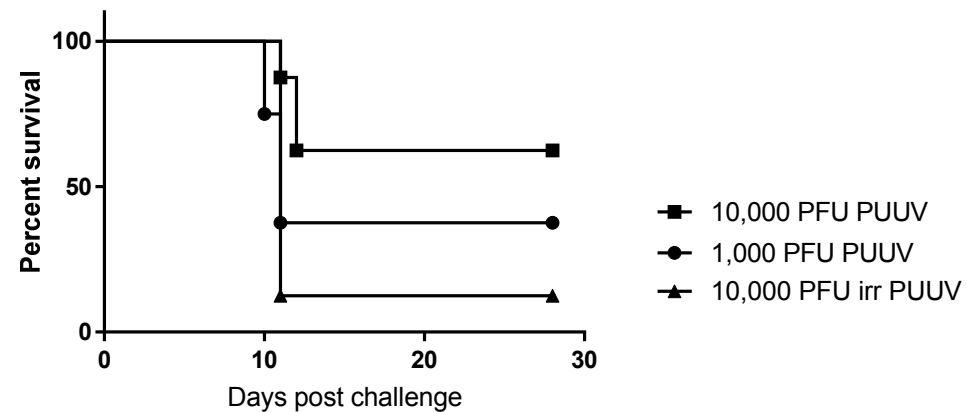


**D**

Day 35 N-ELISA



**E**



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