Human sapovirus propagation in human cell lines supplemented with bile acids

Hirotaka Takagi[a,1,2], Tomoichiro Oka[b,1,2], Takashi Shimoike[b], Hiroyuki Saito[c], Takayuki Kobayashi[d], Tomoko Takahashi[e], Chika Tatsumi[f], Michiyo Kataoka[g], Qiuhong Wang[h,2], Linda J. Saif[h,2], and Mamoru Noda[i]

[a]Management Department of Biosafety and Laboratory Animal, National Institute of Infectious Diseases, Tokyo 162-0052, Japan; [b]Department of Virology II, National Institute of Infectious Diseases, Tokyo 208-0011, Japan; [c]Akita Prefectural Research Center for Public Health and Environment, Akita 010-0874, Japan; [d]Fukuoka Institute of Health and Environmental Sciences, Fukuoka 818-0135, Japan; [e]Iwate Prefectural Research Institute for Environmental Sciences and Public Health, Iwate 020-0857 Japan; [f]Shimane Prefectural Institute of Public Health and Environmental Sciences, Shimane 690-0122, Japan; [g]Department of Pathology, National Institute of Infectious Diseases, Tokyo 162-0052, Japan; [h]Food Animal Health Research Program, Ohio Agricultural Research and Development Center (OARDC), College of Food, Agricultural and Environmental Sciences (CFAES), Department of Veterinary Preventive Medicine, College of Veterinary Medicine (CVM), The Ohio State University, Wooster, OH 44691; and [i]National Institute of Health Sciences, Kanagawa 210-9501, Japan

Human sapoviruses (HuSaVs) cause acute gastroenteritis similar to human noroviruses. Although HuSaVs were discovered four decades ago, no HuSaV has been grown in vitro, which has significantly impeded the understanding of viral biology and the development of antiviral strategies. In this study, we identified two susceptible human cell lines, that originated from testis and duodenum, that support HuSaV replication and found that replication requires bile acids. HuSaVs replicated more efficiently in the duodenum cell line, and viral RNA levels increased up to ∼6 log$_{10}$-fold. We also detected double-stranded RNA, viral nonstructural and structural proteins in the cell cultures, and intact HuSaV particles. We confirmed the infectivity of progeny viruses released into the cell culture supernatants by passaging. These results indicate the successful growth of HuSaVs in vitro. Additionally, we determined the minimum infectious dose and tested the sensitivities of HuSaV GI.1 and GII.3 to heat and ultraviolet treatments. This system is inexpensive, scalable, and reproducible in different laboratories, and can be used to investigate mechanisms of HuSaV replication and to evaluate antivirals and/or disinfection methods for HuSaVs.

sapovirus | bile acids | human duodenum cell line

Sapoviruses (SaVs) are nonenveloped, small, round viruses with a single-stranded, positive-sense RNA genome. They are one of the major enteric viruses detected from acute gastroenteritis worldwide. They belong to the *Sapovirus* genus of the family *Caliciviridae*. Human SaVs (HuSaVs) cause acute gastroenteritis, similar to human noroviruses (HuNoVs) that belong to the *Norovirus* genus within the same family. The suspected transmission route and symptoms are indistinguishable between HuNoV- and HuSaV-associated illness, and it is difficult to control the outbreaks (1). Although significant progress has been made in the propagation of HuNoVs in vitro using three-dimensional cultured Caco-2 cells and a clone of Caco-2 cells, human B cells, and human enteroids (2–6), the establishment of an in vitro cell culture system for HuSaVs has been unsuccessful since their discovery more than 40 y ago.

SaVs have been detected from humans, pigs, wild boars, mink, dogs, sea lions, bats, chimpanzees, rodents, and carnivores (7). Based on the complete capsid sequences, SaVs have been classified into at least 19 genogroups (GI to GXIX) (8, 9). GI and GII SaV strains cause the majority of human gastroenteritis outbreaks and sporadic cases. HuSaVs are further classified into multiple genotypes within each genogroup: GI (GI.1 to GI.7), GII (GII.1 to GII.8 and GII.NA1), GIV, and GV (GV.1 and GV.2) (10–12). Because the efficient in vitro replication of GIII porcine SaV (PoSaV) Cowden strain in porcine kidney cells, and of several human NoVs in enteroids, occurred exclusively when the culture medium was supplemented with bile or bile acids (6,

13), we investigated both nonintestinal and intestinal human cell lines and added bile and bile acids to the cell culture medium to evaluate the HuSaV propagation in these cells.

Results

Certain Bile Acids Are Required for HuSaV Growth In Vitro. To identify the HuSaV-susceptible cell lines, we inoculated the cell monolayers of human cell lines NEC8 (testis), HuTu80 (duodenum), or HCT-8 [HRT-18] (ileocecum) with HuSaV GI.1 stool suspensions in the absence or presence of bile acid sodium cholate (CA), sodium glycocholate (GlyCA), sodium deoxycholate (DCA), sodium glycochenodeoxycholate (GCDCA), or bovine bile (Bile). We washed the cells at 1 d postinoculation (dpi). The culture supernatants were collected at 7 dpi for RNA extraction and the detection of HuSaV RNA by reverse transcription (RT) followed by PCR (RT-PCR). No HuSaV RNA was detected from the three cell lines in the absence of bile or bile acids (Fig. 1). In the presence of bile or bile acids, GI.1 HuSaV RNA was detected from cell lines NEC8 and HuTu80, but not HCT-8 (Fig. 1). For the trials using cell line HuTu80, we also included HuSaV GII.3. Among the bile and four bile acids, GlyCA and GCDCA most effectively promoted GI.1 and GII.3

Significance

Human sapoviruses (HuSaV) are an important cause of diarrhea and foodborne infections worldwide. Despite the discovery of HuSaVs over 40 y ago, no in vitro cell culture system has been established, limiting research on this important pathogen. In this study, we report successful propagation of HuSaV in a human duodenum cell line in the presence of bile acids. Bile acids are biological components in the intestinal tract, and they recapitulate key physiologic factors present in the viral infection site in the intestine. This inexpensive, reproducible and scalable in vitro cell culture system provides a fundamental scientific tool for HuSaV research and development of infection control strategies in the future.
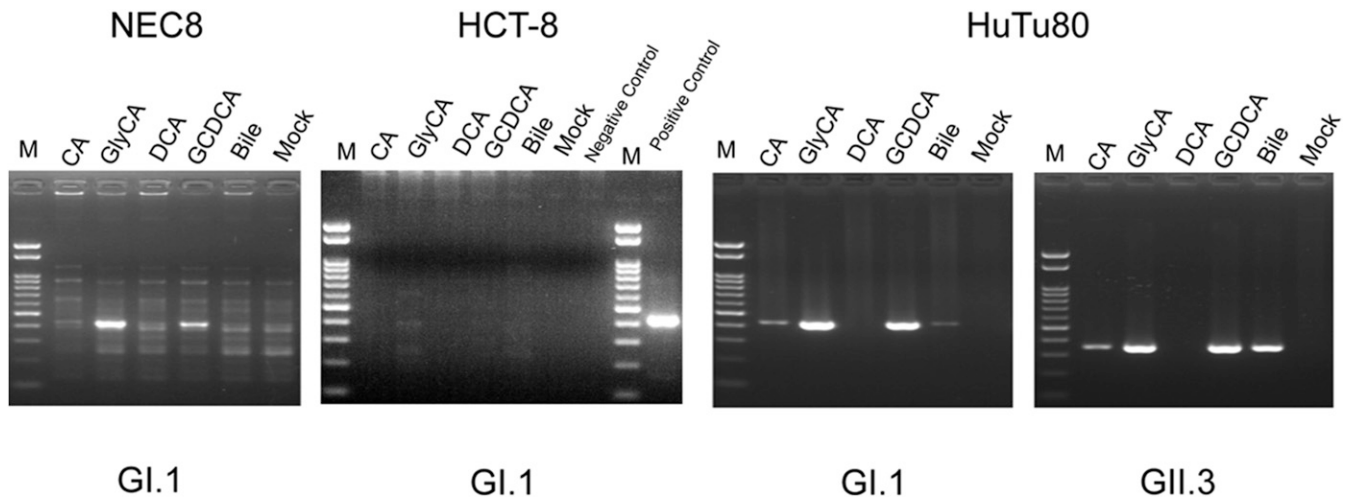
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Fig. 1. Human cell lines NEC8 and HuTu80 but not HCT-8 supported the replication of HuSaV GI.1 and GII.3 in the presence of bile acids in the cell culture medium. HuSaV GI.1 (AK20) or GII.3 (AK11) positive stool suspension was inoculated onto the cell monolayers in the presence of CA, GlyCA, DCA, GCDCA, or Bile, or in the absence of bile acids (Mock). After removing the inoculum and washing the cell monolayers at 1 dpi, we collected the culture supernatants at 7 dpi. The optimal concentration of bile acid for each cell line, as indicated in *Materials and Methods*, was present throughout the culture period. The HuSaV RNA was measured by RT-PCR followed by agarose gel electrophoresis. The GI.1 inoculum and water was included as positive and negative controls, respectively, for HCT-8 cells. The results were confirmed in an independent experiment, and pictures of a representative experiment are shown.

HuSaV replication in cell lines NEC8 and/or HuTu80. In the presence of DCA, no RNA was detected for GI.1 and GII.3 HuSaVs in either NEC8 or HuTu80 cell lines. In this study, we selected GlyCA for subsequent trials.

These results indicated that the combination of appropriate cell lines and certain conjugated bile acids supports HuSaV propagation.

HuSaV Grows Efficiently in Human Intestinal Cells. To investigate the virus growth efficiency, we studied the kinetics of HuSaV replication by quantitation of viral RNA in infected cell lines NEC8 and HuTu80, with the presence of bile acid GlyCA. The culture supernatants were collected at 1, 3, 5, and 7 dpi and 1, 3, 5, 7, 10, and 14 dpi for virus propagation in cell lines NEC8 and HuTu80, respectively, and titrated by RT-qPCR (Fig. 2). In cell line NEC8, GI.1 HuSaV RNA titers increased 1.5 $\log_{10}$ at 3 dpi compared with 1 dpi and increased steadily at later time points. By 7 dpi, the viral RNA titer had increased 2.0 $\log_{10}$. We terminated culture at 7 dpi because all cells detached, but we did not confirm whether these cells were dead. In cell line HuTu80, GI.1 HuSaV RNA titers increased rapidly from 3.5 $\log_{10}$ copies per 100 µL at 1 dpi to 6.6 $\log_{10}$ copies per 100 µL at 3 dpi, then reached the peak titers (7.3 $\log_{10}$ copies per 100 µL) at 5 dpi that persisted (7.4 $\log_{10}$ copies per 100 µL) through 14 dpi, resulting in a ~3.9 $\log_{10}$-increase. These results indicate that GI.1 HuSaV replicated more efficiently in cell line HuTu80 than in cell line NEC8. Therefore, we selected cell line HuTu80 for subsequent trials. In cell line HuTu80, GII.3 HuSaV RNA titers gradually increased and reached the peak titer of 7.7 $\log_{10}$ copies per 100 µL at 10 dpi that persisted through 14 dpi. This peak titer was 3.6 $\log_{10}$ higher than that at 1 dpi and similar to GI.1 HuSaV. Additional HuSaV GI.2 and GII.3 from positive fecal samples were also grown in cell line HuTu80 (*SI Appendix*, Fig. S1 *A* and *B*).

These results indicated that HuSaVs replicate in both cell lines and replicate more efficiently in the duodenal cell line HuTu80. We further confirmed that HuTu80 cell line supports the replication of multiple HuSaV genotypes.

Replication and Production of Progeny HuSaV in Human Intestinal Cells. The replication of HuSaVs in HuTu80 cells in the presence of bile acid GlyCA was confirmed by the detection of double-stranded RNA (dsRNA), nonstructural and structural proteins, and viral particles (Fig. 3 and *SI Appendix*, Fig. S2). At 1, 5, and 10 dpi, the cells inoculated with GI.1 or GII.3 HuSaVs were fixed. Immunofluorescence (IF) assays were performed for dsRNA (the intermediate products during the replication of single-stranded RNA viruses), SaV nonstructural protein NS1-2, and the major structural protein VP1. At 1 dpi, no positive staining was detected. At both 5 and 10 dpi, positive IF staining was detected for dsRNA, NS1-2, and VP1 for both GI.1 and GII.3 HuSaVs as evidence of replication and translation of HuSaV in HuTu80 cells (Fig. 3 *A* and *B*). Based on the dsRNA signals, only a limited percentage (5.6 to ~8.2% [average 7.6%] and 10.4 to ~14.3% [average 12.3%] for GI.1, and 6.4 to ~8.0% [average 6.5%] and 7.1 to ~11.1% [average 8.7%] for GII.3 at 5 and 10 dpi, respectively [n = 3]) of the HuSaV-inoculated cells showed positive signals, and no obvious cytopathic effects were observed, even at 10 dpi (*SI Appendix*, Fig. S2). The parallel examination of viral RNA titers showed that both GI.1 and GII.3 HuSaV RNA titers were increased from <3 to ~4 (at 1 dpi) to >9 $\log_{10}$ copies per 100 µL of culture supernatants (at 5 and 10 dpi) (Fig. 3*C*). In addition, we observed intact SaV-like particles of ~44.1 nm (SD 1.5) (n = 15) for GI.1 and 41.0 nm (SD 1.9) (n = 15) for GII.3 in diameter by electron microscopy (Fig. 3*D*). The recovery rates for GI.1 and GII.3 HuSaV RNA from the cell culture supernatants after ultracentrifugation were 56.8% and 16.8%, respectively (*SI Appendix*, Table S1). The infectivity of progeny GI.1 and GII.3 HuSaVs in passage 0 (P0) and their progeny P1 cell culture supernatants harvested at 10 dpi was confirmed by both viral RNA and VP1 levels in the cell culture supernatants of P1 (Fig. 3 *E* and *F*). Both GI.1 and GII.3 HuSaV RNA titers were increased up to ~6 $\log_{10}$-fold from ≤3 (at 1 dpi) to 9 $\log_{10}$ copies per 100 µL of culture supernatants (at 10 dpi). Consistently, the VP1 levels for P1 and P2 GI.1 and GII.3 HuSaV increased significantly by ELISA at 10 dpi compared with that at 1 dpi.

Together with the observation of intact SaV particles, detection of dsRNA, NS1-2, and VP1, and the successful passaging of the P0 and P1 virus (the passaged virus assigned as P1), we conclude that HuSaVs replicated successfully, produced progeny viruses, and could be passaged in cell line HuTu80 with bile acid supplement.
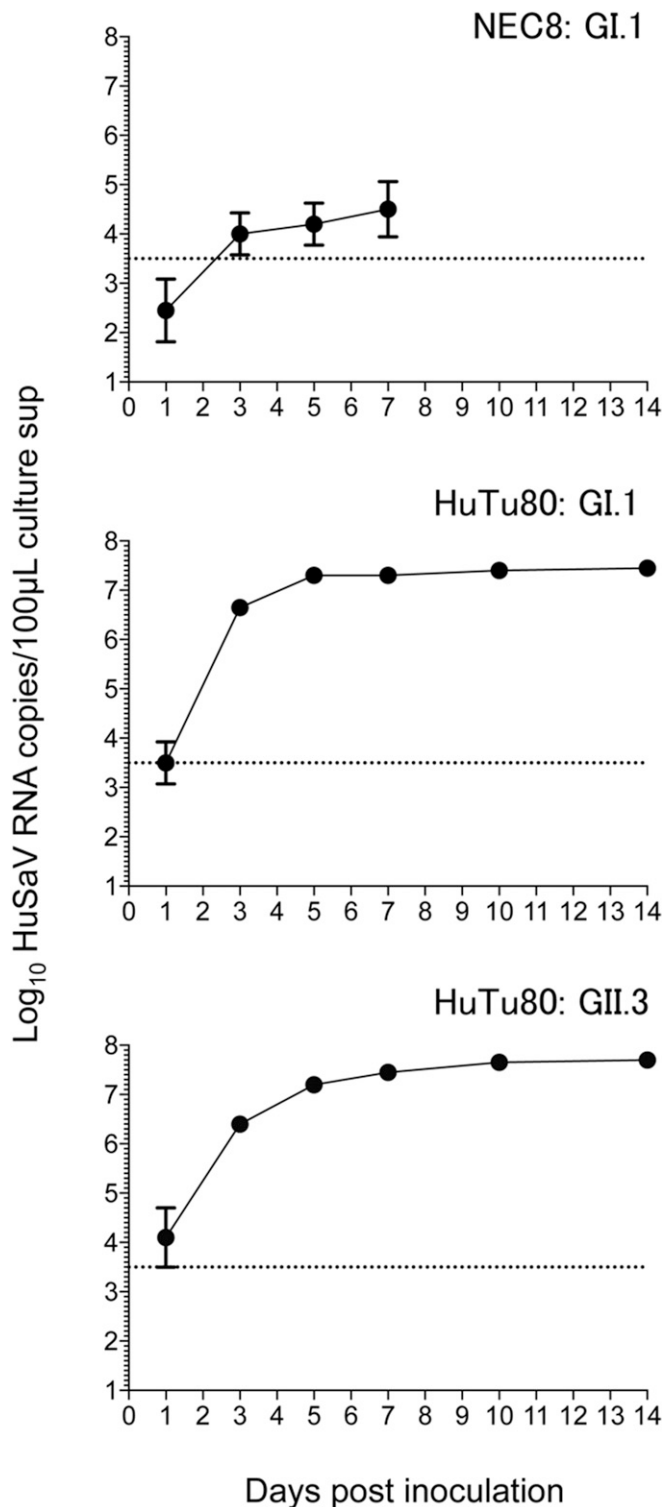
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Fig. 2. Growth kinetics of HuSaV GI.1 and GII.3 in NEC8 and/or HuTu80 cells. HuSaV GI.1 (AK20) or GII.3 (AK11) positive stool suspension was inoculated onto NEC8 or HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers at 1 dpi, we collected supernatants at 1, 3, 5, and 7 dpi or 1, 3, 5, 7, 10, and 14 dpi. GlyCA (500 μM for NEC8 and 1,000 μM for HuTu80) was present throughout the culture period. The SaV RNA was measured by RT-qPCR. Dotted line indicates the lowest quantification limit. Data represent the values of two independent experiments using cells at different passage numbers for each time point. Error bars indicate SDs.

Infectious Dose of HuSaV for Successful Infection as Well as Sensitivities to Heat and Ultraviolet Treatments. Finally, we investigated the limit of virus RNA required for successful infection; 10-fold serially diluted fecal suspensions of GI.1 or GII.3 HuSaV was inoculated onto cell line HuTu80. The GI.1 or GII.3 HuSaV with RNA titers of ~2.0×10^5, 10^4, 10^3, and 10^2, but not 2.0×10^1 and 2×10^0, infected the cells, and viral RNA can be detected at 10 dpi (Fig. 4A).

Substantial RNA titers (at least 2.0×10^5 for GI.1 and 2.0×10^4 for GII.3) were necessary for reproducible infections. Infection was sometimes observed with the inoculation titer of 2.0×10^2 RNA copies for both GI.1 and GII.3 under the conditions tested.

Furthermore, we used cell line HuTu80 to evaluate the thermal and ultraviolet (UV) sensitivity of HuSaVs. GI.1 or GII.3 HuSaV was heat treated at three different temperatures (50 °C, 60 °C, and 70 °C) for three different time periods (10, 20, and 30 min) and then used to inoculate cell line HuTu80 in the presence of GlyCA. Supernatants were harvested at 10 dpi, and viral RNA was titrated by RT-PCR (Fig. 4B). After heat treatment at 70 °C for 30 min and inoculation of the virus into the HuTu80 cells, the GI.1 and GII.3 HuSaVs RNA level decreased to the RT-qPCR lowest quantification limit (3.5 $\log_{10}$ copies per 100 μL). However, after treatment at 50 °C for 30 min and inoculation of the virus into the HuTu80 cells, GI.1 and GII.3 HuSaVs replicated to a level that was similar to non−heat-treated virus. The heat treatment sensitivity was not identical between GI.1 and GII.3 HuSaVs, and GII.3 HuSaV was more resistant than GI.1 HuSaV to 60 °C. Surprisingly, both HuSaV GI.1 and GII.3 were insensitive to UV treatment, even with 5.4 J/cm^2 under our test conditions (Fig. 4C). Along with these results, we further performed UV treatment using passaged (P1) HuSaV stock mixed with feline calicivirus (FCV) and confirmed that the passaged HuSaV was sensitive to UV treatment with 1.8 J/cm^2. Spiked FCV was also inactivated with this condition (*SI Appendix*, Fig. S3).

Discussion

We investigated both human testis (NEC8) and intestinal (HuTu80 and HCT-8 [HRT-18]) cell lines for HuSaV susceptibility, because of our finding that PoSaV also replicates in swine testis origin cells in addition to porcine kidney origin LLC-PK cells. We selected cell lines originated from upper (duodenum) and lower (ileocecal) human small intestinal sites because PoSaV antigen was detected in the small intestine in experimentally infected pigs (14, 15) as well as gastrointestinal symptoms (vomiting and diarrhea) are prevalent among SaV-infected patients (1).

Although conjugated bile acid supplementation allows both HuSaVs and PoSaV Cowden strains to replicate in nonintestinal cell lines (testis and kidney, respectively) from their natural hosts, HuSaVs replicated efficiently in the human duodenum cell line HuTu80. Bile acids are synthesized in the liver and released with bile into the duodenal lumen, and, after they pass through the intestines, most of them are transported in the ileum back into the liver for reuse. This comprises the enterohepatic bile circulation system. The primary conjugated bile acids in duodenum can be dehydroxylated and unconjugated by various bacteria as they pass through the lower small and large intestines (16). PoSaV Cowden strain replicates in the proximal small intestine of experimentally infected pigs (14, 15). Although such in vivo data are not available for HuSaVs, the duodenum of the small intestine may be the infection site of HuSaVs. Our positive HuSaV replication results with cell line HuTu80 and the conjugated bile acids, GlyCA and GCDCA, are consistent with the physiological conditions associated with natural HuSaV infections.
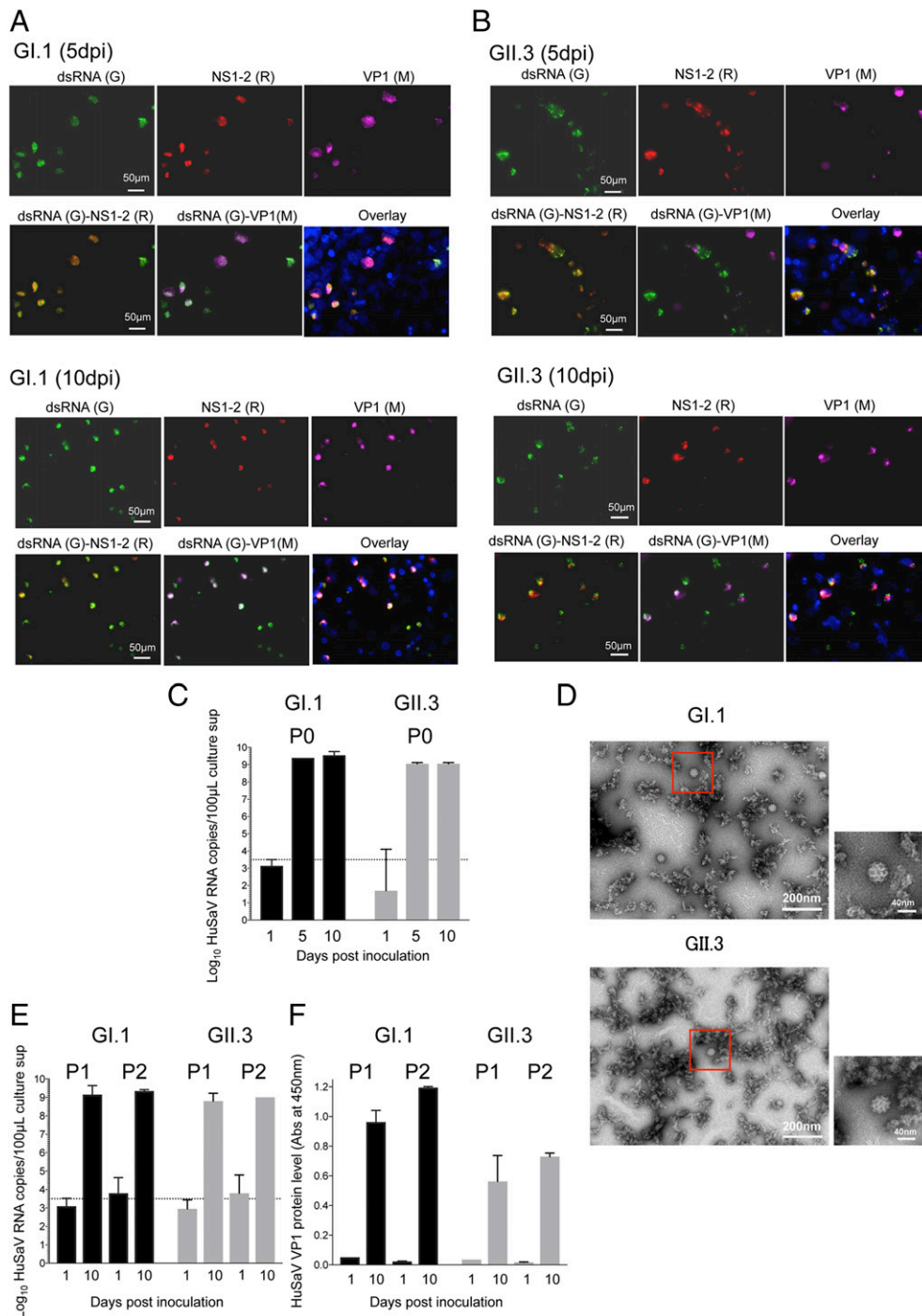
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Fig. 3. HuSaV GI.1 and GII.3 replicated in HuTu80 cells and produced infectious progeny viruses in the culture supernatants. HuSaV GI.1 (AK20) (*A*) or GII.3 (AK11) (*B*) positive stool suspension was inoculated onto HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers at 1 dpi, the cell monolayers were fixed at 5 and 10 dpi; 1,000 μM GlyCA was present throughout the culture period. Cells were permeabilized and blocked before staining with mouse anti-dsRNA antibody, rabbit anti-GI or GII nonstructural protein NS1-2 hyperimmune serum, and guinea pig anti-SaV GI or GII VLPs hyperimmune serum. Subsequently, Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 594-conjugated goat anti-rabbit IgG, and Alexa Fluor 647-conjugated goat anti-guinea pig IgG and DAPI were applied to detect dsRNA (green; G), NS1-2 (red: R), VP1 (magenta: M), and nuclei (blue), respectively. Individual images of dsRNA, NS1-2, and VP1, double overlay images of dsRNA and NS1-2, dsRNA and VP1, and quadruple overlay images of all of the four signals (dsRNA, NS1-2, VP1, and nuclei) were indicated. Images were taken at a magnification of 600× from the regions enclosed by the red boxes in *SI Appendix*, Fig. S2. (*C*) The viral RNA levels of HuSaV GI.1 (AK20) or GII.3 (AK11) in the HuTu80 cell culture supernatants at 1, 5, and 10 dpi were titrated by RT-qPCR. Data represent the mean of the four wells for each time point. Error bars indicate SDs. Dotted line indicates the lowest quantification limit. (*D*) SaV GI.1 (AK20) and GII.3 (AK11) SaV virions were observed in the HuTu80 cell culture supernatants collected at 9 dpi using TEM. Images on the right were cropped from the regions enclosed by the red boxes. The infectivity of the P0 (the initial passage of the virus in cell culture) and P1 (the passaged virus using P0) was confirmed further. The viral RNA (*E*) and VP1 antigen (*F*) levels of HuSaV GI.1 (AK20) or GII.3 (AK11) in the HuTu80 cell culture supernatants at 1 and 10 dpi were titrated by RT-qPCR and ELISA, respectively. Data represent the mean of the two infection experiments for each time point. Error bars indicate SDs. Dotted line indicates the lowest quantification limit.
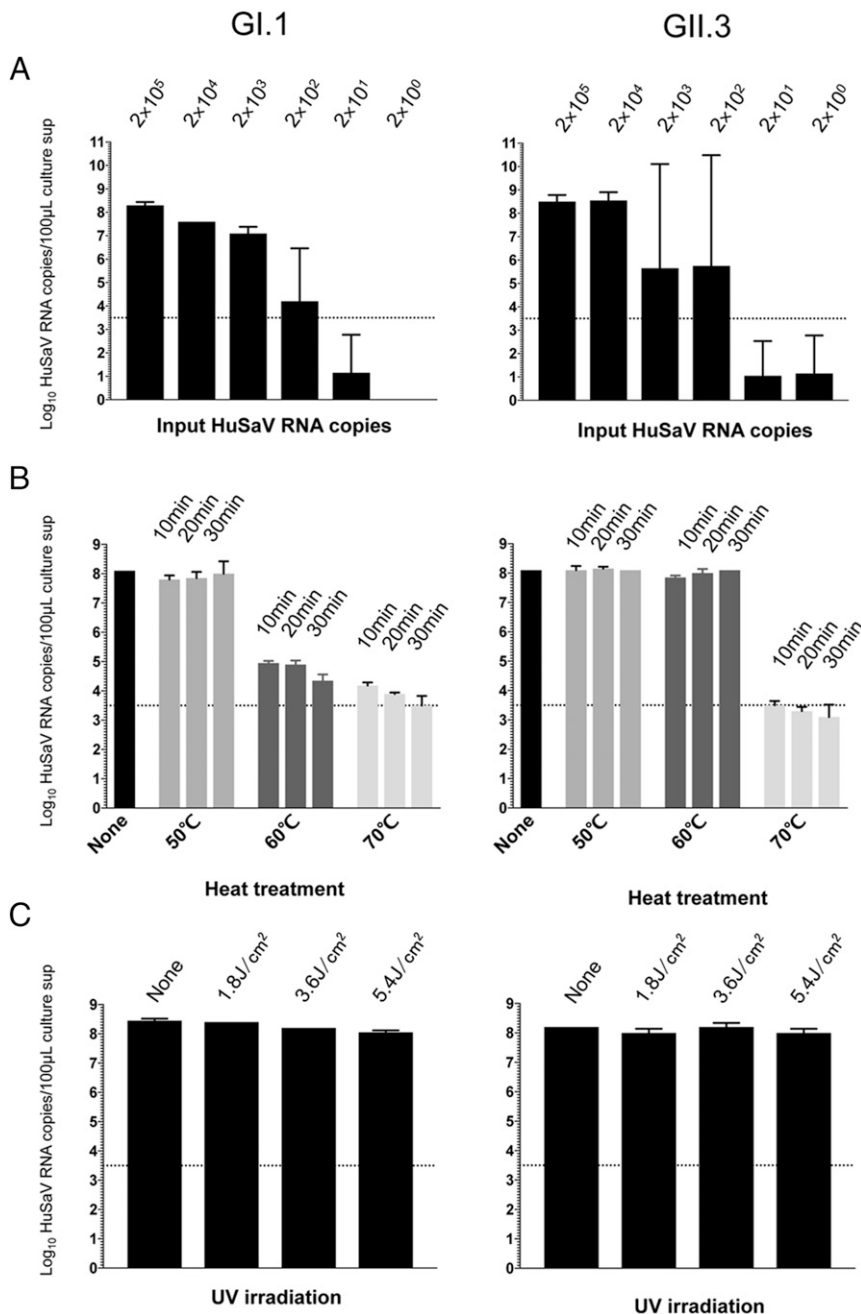
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Fig. 4. The infectious dose and effects of various treatments on infectivity of HuSaV GI.1 or GII.3 in HuTu80 cells. (*A*) Infectivity of serially diluted HuSaV GI.1 (AK20) or GII.3 (AK11); effect of heat treatment (*B*) or UV treatment (*C*) on SaV GI.1 (AK20) or GII.3 (AK11) infectivity in HuTu80 cells. Serially diluted or pretreated SaV GI.1 (AK20) or GII.3 (AK11) positive stool suspension was inoculated onto HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers at 1 dpi, the culture supernatants were collected at 10 dpi; 1,000 μM GlyCA was present throughout the culture period. The SaV RNA was measured by RT-qPCR. Dotted line indicates the lowest quantification limit. Data represent the mean of two independent experiments for each treatment and time point. Error bars indicate SDs.

In this study, we first identified HuSaV-susceptible cell lines NEC8 and HuTu80. In the presence of conjugated bile acid GlyCA, GI.1 and GII.3 HuSaV RNA titers increased up to 6 $\log_{10}$ in cell line HuTu80 (Figs. 2 and 3 *C* and *E*). A notable HuSaV RNA increase occurred within 7 dpi (Fig. 2).

Our IF data indicate that only a small proportion of cells were susceptible to HuSaVs. The characteristics of these cells that contribute to their susceptibility require further investigation. HuTu80 cells are likely a mixture of cells, and the cells susceptible to HuSaVs may be limited. This could be the reason that we did not

see a high percentage of HuSaV-positive cells and obvious virus spread among cells. Our future work will include the cloning of HuTu80 cells and identify the subset of cell population that supports better HuSaV replication than current HuTu80 cells.

The successful propagation of HuSaVs in HuTu80 cells in the presence of GlyCA in two different laboratories (National Institute of Infectious Diseases [NIID] in Japan and The Ohio State University [OSU] in the United States [*SI Appendix*, Fig. S1A]) located on two continents using different HuSaV strains confirms the reproducibility of our findings. The viral RNA titer

increases ranged from 1 $\log_{10}$ to 6 $\log_{10}$ and the propagation efficiencies depended on the samples (Figs. 2 and 3 and *SI Appendix*, Fig. S1).

By comparison, for the in vitro replication of HuNoV, Ettayebi et al. (6) and Sato et al. (4) reported a 1 $\log_{10}$ to 3 $\log_{10}$ increase in HuNoV RNA levels in human intestinal stem cell-derived enteroids and in induced pluripotent stem cell-derived intestinal epithelial cells. In human B cell culture of HuNoVs, Jones et al. (5) reported a 1 $\log_{10}$ to 2 $\log_{10}$ increase in viral RNA levels. Therefore, HuSaV RNA replication in vitro reached a higher level than that of HuNoVs and resulted in infectious virus stock.

Because HuSaV are highly contagious like HuNoV, and suspect foodborne HuSaV outbreaks also have been reported, we evaluated the minimum infectious dose as well as heat and UV resistance of HuSaV using the GI.1 and GII.3 strains. The infectious doses of HuSaV are similar to those of HuNoVs, which were above 2.0×10^2 viral RNA copies (6, 17). HuSaVs cannot be inactivated completely by incubation at 60 °C for up to 30 min (Fig. 4*B*), although inactivation of HuNoVs by incubation at 60 °C for 15 min has been reported using enteroids (6). UV treatment is also used for decontamination of the environment (e.g., wastewater treatment, food-making equipment, and hospital surfaces). Our data demonstrate that HuSaV is relatively stable after heat treatment and resistant to UV irradiation when tested with diluted fecal suspension in our test conditions. In contrast, the passaged HuSaV are sensitive to UV irradiation, similar to FCV (*SI Appendix*, Fig. S3). Combining these results, the unknown factor(s) in the feces would protect the HuSaV from inactivation by UV treatment. These results are important for HuSaV control in public health as well as for foods and in the environment.

Lack of scalability is still hampering the HuNoV field. Importantly, we confirmed the infectivity of progeny HuSaV, by serial passage studies. Because our system is inexpensive and scalable, the preparation of viral pools, development of virus neutralization assays, and evaluation of various inactivation treatments and antivirals become feasible.

In summary, we identified susceptible cell lines for HuSaV propagation in vitro. Like for PoSaV and most HuNoVs, the efficient replication of GI.1 and GII.3 HuSaVs, as well as GI.2, required the presence of bile acids in the culture medium. Successful propagation and production of infectious HuSaVs in human intestinal cells with bile acids will expedite viral gastroenteritis and food safety research.

Materials and Methods

Fecal Specimens. The HuSaV-positive fecal specimens were suspended in sterile Minimum Essential Medium (MEM) with Earle's salt and 0.05% sodium bicarbonate supplemented with 0.5% lactalbumin, 0.2% bovine serum albumin, and antibiotics (60 µg/mL kanamycin and 50 µg/mL gentamicin). MEM powder containing Kanamycin was purchased from Nissui Pharmaceutical Co., LTD.. Lactalbumin was from Sigma-Aldrich. Other reagents were from FUJIFILM Wako Pure Chemical Corporation. The samples were vortexed vigorously and centrifuged at $1,800 \times g$ for 30 min. The supernatants were sterilized through 0.2-µm-pore-size filters. All of these sterilized HuSaV-positive fecal suspensions were aliquoted to individual tubes and stored at −80 °C for long-term storage. The diluted samples were stored at 4 °C and used for cell culture trials. These samples were collected from the feces of subjects with acute gastroenteritis between 2011 and 2017. The tested samples described in this manuscript (GI.1 [AK20], GII.3 [AK11], GI.2 [FS124], GI.2 [D2475], GII.3 [IWTS1], and GII.3 [IWTS2]) were negative for other common human enteric viral pathogens (NoV, rotavirus, astrovirus). Collection and detection of pathogens were performed at Akita Prefectural Research Center for Public Health and Environment, Fukuoka Institute of Health and Environmental Sciences, and Research Institute for Environmental Sciences and Public Health of Iwate Prefecture, and Shimane Prefectural Institute of Public Health and Environmental Sciences, under the regulation of Infectious Diseases Control Law, Food Sanitation Act in Japan. The fecal samples were deidentified prior to use in this study. This study was

approved by the ethics committee of the National Institute of Infectious Diseases and by the Institutional Biosafety Committee at The Ohio State University.

Cell Lines. For HuSaV culture trials, we used three human cell lines: 1) human testicular embryonal carcinoma-derived cell line, NEC8 cells (Japanese Collection of Research Bioresources [JCRB] 0250); 2) human duodenum carcinoma derived cell line, HuTu80 cells (American Tissue Culture Collection [ATCC] #HTB-40); and 3) human ileocecal adenocarcinoma derived cell line, HCT-8 (HRT-18) cells (ATCC #CCL-244).

Cell Culture Conditions. NEC8 cells were grown in Medium199 (Sigma-Aldrich) supplemented with 7% fetal bovine serum (FBS) (Biosera) and the antibiotics (50 µg/mL ampicillin and 100 µg/mL Kanamycin [FUJIFILM Wako Pure Chemical Corporation]). HuTu80 cells were grown in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with GlutaMAX (Gibco, Thermo Fisher Scientific), 5% FBS, and the antibiotics. HCT-8 (HRT-18) cells were grown in RPMI1640 (FUJIFILM Wako Pure Chemical Corporation) supplemented with 5% FBS and the antibiotics.

Supplements for the HuSaV Culture Trials. CA (Sigma-Aldrich), GlyCA (Nakalai Tesque), DCA (Sigma-Aldrich), GCDCA (Nakalai Tesque), and Bile (gall) powder (FUJIFILM Wako Pure Chemical Corporation) were used in HuSaV culture trials. They were dissolved in 20% ethanol solution, filtered through 0.2-µm-pore-size filters, aliquoted, and stored at room temperature until use. The cell toxicity of these five supplements was tested for each cell line, using twofold serial dilutions: CA, GlyCA, DCA, and GCDCA were tested for 1,000 µM to 15.6 µM in a final concentration, and Bile powder was tested for 0.1 to 0.00156% in a final concentration. The cells cultured in 96-well plates were treated with these supplements for 7 d and then fixed and stained for the observation of cell toxicity using a light microscope. The highest concentration of a supplement that did not cause cell toxicity was determined as its working concentration.

HuSaV Culture Trials. Confluent NEC8, HCT-8 (HRT-18), and HuTu80 cell monolayers grown in 12-well plates were used for HuSaV inoculation. Before inoculation, the culture medium was replaced with 0.5 mL of virus growth media that contained the optimized concentrations of the following bile acids for each cell line: CA at 250 µM (NEC8), 500 µM (HCT-8), and 125 µM (HuTu80); GlyCA at 500 µM (NEC8),1,000 µM (HCT-8), and 1,000 µM (HuTu80); DCA at 20 µM (NEC8), 50 µM (HCT-8), and 71.4 µM (HuTu80); GCDCA at 200 µM (NEC8), 500 µM (HCT-8), and 500 µM (HuTu80); and Bile (gall) powder at 0.005% (NEC8), 0.01% (HCT-8), and 0.0125% (HuTu80).

FBS in the virus growth medium was reduced from 7 to 5%, 5 to 2%, and 5 to 3% for NEC8, HCT-8 (HRT-18), and HuTu80 cells, respectively. Five microliters of HuSaV-positive fecal suspensions ($\sim4 \times 10^6$ copies of viral RNA) were added to each well. The plates were incubated overnight in a cell culture incubator (37 °C with 5% CO_2). Then, the cell monolayers were washed twice with L15 medium (Sigma-Aldrich) supplemented with 2% horse serum (GIBCO) and the antibiotics. Finally, 1 mL per well of virus growth media that were optimized for each cell line was added. The media without bile or bile acids was used as controls. These cultures were incubated for 7 d, and HuSaV RNA levels in these cell supernatants were detected by RT-PCR.

For the kinetics of HuSaV RNA replication, confluent NEC8 and HuTu80 cell monolayers in T25 flasks were used. Before virus inoculation, the culture medium was replaced by 3 mL of virus growth medium containing the optimal concentrations of bile acid GlyCA for each cell line. FBS in the virus growth medium was reduced as described above. Twenty or thirty microliters of inoculum (approximately $\sim1.0 \times 10^7$ copies of viral RNA) were added to each flask and incubated overnight. Then the cell monolayers were washed twice with L15 medium supplemented with 2% horse serum and the antibiotics. Finally, 3 mL or 5 mL of virus growth medium that were optimized for each cell line was added to each flask. These cultures were incubated for 7 and 14 d for cell lines NEC8 and HuTu80, respectively. HuSaV RNA levels in these cell supernatants were quantitated by RT-qPCR (see below).

For the detection of SaV nonstructural protein NS1-2 and the major structural protein VP1 as well as dsRNA, which was exclusively generated during viral genome replication, confluent monolayers of HuTu80 cells grown on type I collagen-coated coverslips in 24-well plates were used. Before inoculation, the culture medium was replaced by 0.5 mL per well of the optimized virus growth media containing 1,000 µM GlyCA. Five microliters of inoculum (1.1×10^6 copies of viral RNA) was added into each well and incubated overnight. Then the cells were washed twice with L15 medium supplemented with 2% horse serum and the antibiotics. Next, 0.5 mL

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of virus growth medium that was optimized for cell line HuTu80 was added to each well. These cultures were fixed at 1, 5, or 10 dpi for IF assays (see below). The culture supernatants at each time point were collected and used for SaV RNA titration by RT-qPCR (see below).

RNA Extraction and Complementary DNA Synthesis. Viral RNA was extracted from fecal suspensions or cell culture supernatants using High-Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's instructions. The extracted RNA was used in the following procedure, or stored at −80 °C. The complementary DNA (cDNA) was synthesized as follows: 5 μL of a viral RNA sample was mixed with 0.5 μL of the 20 μM oligo (dT)$_{30}$ primer, which was complementary to the 3′-end poly(A) tail, and 1 μL of 2.5 mM deoxyribonucleotide triphosphates (dNTPs). Then 2 μL of 5× ReverTra Ace RT buffer (Toyobo), 0.1 μL of 10 units per μL RNase inhibitor (TaKaRa Bio Inc.), and 0.3 μL of reverse transcriptase ReverTra Ace (100 U/μL) (Toyobo) were added to the above mixture. This mixture was incubated first at 30 °C for 10 min, then at 42 °C for 60 min. The cDNA synthesis for qPCR was carried out using random hexamers (Takara) and SuperScript III RNaseH (-) reverse transcriptase (Invitrogen) in a final volume of 30 μL as described (18); samples were then stored at −30 °C.

PCR and qPCR. The partial HuSaV genomic sequences were amplified by PCR with forward primers (SaV-1245revF) (19) and reverse primers (SaV-G1R, SaV-G2R, SaV-G4R, SaV-G5R) (20). A final volume of 20 μL of the PCR mixture contained 2 μL of the cDNA, 10 μL of 2× KAPA2G fast ReadyMix with dye (Kapa Bio Science), and 1 μL each of the five primers (10 μM). PCR was performed at 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 53 °C for 20 s, and 72 °C for 5 s, and a final extension at 72 °C for 1 min. The PCR products were purified by using QIAquick gel extraction kit (Qiagen) and were directly sequenced using the Big Dye Terminator cycle sequencing kit (Applied Biosystems). For experiment at laboratory 1 (NIID), the cDNA of the HuSaV genomic RNA was quantified by TaqMan real-time PCR with primers SaV124F, 1F, 5F, and 1245R and FAM-labeled MGB probes (SaV 124TP and SaV 5TP) (18) under the following conditions: 95 °C for 15 min followed by 40 or 45 cycles of a two-step PCR: 94 °C for 15 s, and 62 °C for 60 s.

For experiment at laboratory 2 (OSU), HuSaV GI.2 viral RNA was titrated by one-step real-time RT-PCR using QIAGEN OneStep RT-PCR Kit (QIAGEN) with forward primers (SaV1F, 5′-TTG GCC CTC GCC ACC TAC-3′; SaV124F, 5′-GAY CAS GCT CTC GCY ACC TAC-3′), reverse primer (SaV1245R, 5′CCC TCC ATY TCA AAC ACT A-3′), and probe (SaV124TP, 5′-FAM-CCR CCT ATR AAC CA-MGB-NQF) (18). The reaction was performed at 50 °C for 30 min for RT followed by 95 °C for 15 min for activation of the DNA polymerase. Then 45 cycles were carried out at 95 °C for 15 s and 62 °C for 60 s.

IF Assays. HuSaV-inoculated HuTu80 cells grown on the coverslips were washed once with Dulbecco's phosphate buffered Saline without Mg^{2+} and Ca^{2+} [PBS (-)] and then fixed with 250 μL of 4% paraformaldehyde in phosphate buffer solution (FUJIFILM Wako Pure Chemical Corporation) for 15 min at room temperature. After washing once with PBS (-), the cells were permeabilized and blocked with 250 μL of ImmunoBlock (DS PHARMA BIOMEDICAL) containing 0.1% Triton-X100 for 30 min at room temperature. Then, the cells were incubated with 250 μL of the primary antibodies: mouse anti-dsRNA monoclonal antibody J2 (English Scientific), rabbit anti-SaV nonstructural protein NS1-2 hyperimmune serum for GI (raised against the amino acid residues 1 to 219 of the ORF1 of GI.1 Mc114 [GenBank accession no. AY237422], with 95.4% amino acid identity to the GI.1 strain used in this study), or GII (previously named as anti-A) (raised against the amino acid residues 1 to 231 of the ORF1 of Mc10 [AY237420], with 96.5% amino acid identity to the GII.3 strain used in this study) (21, 22), and the guinea pig hyperimmune serum produced against SaV GI or SaV GII virus-like particles (VLPs) (23, 24) at 1:1,000 dilution in ImmunoBlock for 1 h at 37 °C. After washing three times with 500 μL of PBS (-), the cells were subsequently incubated with 250 μL of the Alexa dye-labeled secondary antibodies: Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 594-conjugated goat anti-rabbit IgG, and Alexa Fluor 647-conjugated goat anti-guinea pig IgG (all from Invitrogen) at 1:1,000 dilution in ImmunoBlock for 1 h at room temperature. Cell nuclei were also stained with Cellstain DAPI Solution (FUJIFILM Wako Pure Chemical Corporation) at this step. After washing three times with PBS (-), cells on coverslips were mounted by a drop of Prolong Gold anti-fade regent (Invitrogen) and covered by glass slides. Imaging was performed using a fluorescence microscope (Olympus IX81, Olympus) and MetaMorph imaging software (Molecular Probe, Molecular Devices, LLC).

Electron Microscopy. For the visualization of HuSaV particles by transmission electron microscopy (TEM), culture trials were performed using confluent HuTu80 cell monolayers in two T150 flasks (21 mL per flask). Twenty or thirty microliters of inoculum of HuSaV GI.1 or GII.3 (approximately ~1.0 × 10^7 copies of viral RNA) were inoculated onto each flask and cultured for 9 d. Bile acid GlyCA was present throughout the culture period.

The collected culture supernatants were centrifuged at 10,000 × g for 1 h at 4 °C to remove cell debris. Then the virus was purified from 33 mL of the supernatants through a 35% (wt/vol) sucrose cushion by ultracentrifugation at 164,100 × g for 3 h at 4 °C using an SW32Ti rotor and an ultracentrifuge (Beckman Coulter). The pellet was dissolved in 300 μL of PBS (-), and 40 μL of the samples were further purified by applying onto 0.15 mL of Saphacryl S-400HR (GE HealthCare Life Sciences Co. Ltd) filled microspin columns and centrifuged at 800 × g for 2 min. The elution was diluted with 10-fold by PBS (-) and then used for TEM. The purified virus particles were stained by 2% uranyl acetate solution and observed with a transmission electron microscope (HT7700, Hitachi Ltd.) at a setting magnification of 30,000. The particle sizes were determined by Hitachi EMIP software. Recovery rate was estimated by qRT-PCR using cDNA prepared from the extracted viral RNA as described above.

Antigen ELISA for the Detection of HuSaV Capsid Protein VP1. HuSaV capsid proteins in the fecal specimens or cell culture supernatants were also quantitated using an antigen ELISA. Briefly, 96-well microtiter plates (Maxisorp; Nunc) were coated with 50 μL per well of rabbit hyperimmune antiserum produced against SaV GI.1 Mc114 or SaV GII.3 C12 VLPs (23, 24) at 1:2,000 or 1:5,000 dilutions, respectively, in 0.05 M carbonate buffer (pH 9.6). The plates were incubated overnight at 4 °C. The wells were washed twice with PBS (-), and then blocked with 250 μL of PBS containing 0.5% casein for 2 h at room temperature or overnight at 4 °C. After washing the wells three times with PBS (-) containing 0.1% Tween 20 (PBS-T), 50 μL of the fivefold diluted cell culture supernatants in PBS-T containing 0.25% casein was added. The plates were incubated for 1 h at room temperature. After washing the wells three times with PBS-T, 50 μL of guinea pig hyperimmune antiserum produced against SaV GI.1 or SaV GII.3 VLPs at 1:3,000 or 1:5,000 dilutions, respectively, in PBS-T containing 0.25% casein was added to each well. The plates were incubated for 1 h at room temperature followed by washing three times with PBS-T. Then 50 μL of horseradish peroxidase-conjugated goat anti-guinea pig IgG (IgG H + L) (Rockland Immunochemicals Inc.) at 1:4,000 dilution in PBS-T containing 0.25% casein was added to each well. The plates were incubated for 1 h at room temperature and then washed three times with PBS-T. Finally, 50 μL per well of 1 mM substrate 3, 3′, 5, 5′-tetramethylbenzidine (Sigma-Aldrich) and 0.01% H$_2$O$_2$ in citrate buffer (pH3.5) was added, and the plates were left in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 μL per well of 1 M H$_2$SO$_4$, and the absorbance was measured at 450 nm with 750 nm as the reference wavelength, using a spectrometer (BioRad Microplate reader Model 680).

Confirmation of the Infectivity of Progeny HuSaV by Passage Studies. P0 virus stock (the initial passage of the virus in cell culture supernatants) of the GI.1 (AK20) or GII.3 (AK11), prepared by infecting HuTu80 cells in T25 flasks as described above, or P1 virus stock (the second passaged virus in cell culture supernatants), was inoculated onto confluent monolayers of HuTu80 cells grown in six-well plates. Before inoculation, the culture medium was replaced by 1 mL per well of the optimized virus growth media containing 1,000 μM GlyCA. Ten microliters of inoculum (2 × 10^7 to ~5 × 10^7 copies of viral RNA) were added to each well and incubated overnight. Then the cells were washed twice with L15 medium supplemented with 2% horse serum and the antibiotics. Next, 2 mL of the virus growth medium was added to each well. The HuTu80 cell culture supernatants at 1 and 10 dpi were titrated by RT-qPCR and VP1-ELISA (see above).

Determination of the HuSaV RNA Level Required for Successful Infection of HuTu80 Cells. The culture trails were performed as described above using 24-well plates. Fifty microliters of 10-fold serially diluted GI.1 (AK20) or GII.3 (AK11) HuSaV suspension (~2 × 10^5 to ~2 × 10^0 copies of RNA) were inoculated onto the monolayers of HuTu80 cells. After removing the inoculum and washing the cells at 1 dpi, we collected the culture supernatants at 10 dpi. HuSaV RNA was titrated by RT-qPCR as described above.

Heat and UV Sensitivities of HuSaV GI.1 and GII.3. Sixty microliters of GI.1 (AK20) or GII.3 (AK11) HuSaV suspension (4.5 × 10^6 copies of viral RNA per 10 μL) inside eight-strip microtube was heat treated at different temperatures (50 °C, 60 °C, and 70 °C) in an ACCU BLOCK Digital Dry Bath (LabNet Co) for 0, 10, 20, and 30 min, or irradiated with UV light at 1.5 mW/cm^2 (UV lamp VL−206.G, CosmoBio) on ice for 20, 40, and 60 min. The infectivity of 50 μL of treated supernatants was tested as described above in HuTu80 cells

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cultured in 24-well plates. The culture supernatants were collected at 10 dpi, and HuSaV RNA was titrated by RT-qPCR as described above.

Data Availability. All study data are included in the article and *SI Appendix*.

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