要　旨

サポウイルス(sapovirus)は、カリシウイルス属に含まれる non-envelope、単鎖(+)RNA ウイルスである。ヒトサポウイルス (human sapovirus; HuSaV) は 18 種類の遺伝子型(GI.1～7、GII.1～8、GIV.1 および GV.1～2)を有し、ヒトノロウイルス (human norovirus; HuNoV) と同属で、かつ急性下痢症を引き起こすため、伝播・兆候・主訴などは集団下痢症事案においてノロウイルスと共に考慮すべき起因ウイルスとなる。実際には幼児や小児などでの集団発生例が多いが、成人での集団発生や食中毒事案の報告も相当数存在する。

一方、人工的な増殖方法の研究において、HuNoV では Caco-2 細胞や B 細胞のような汎用細胞株や、enteroid といった幹細胞により、一部の臨床株での培養報告はあるが、HuSaV では発見から 40 年以上経過しても培養増殖に成功していない。また、これまでヒト急性下痢症の起因ウイルスにおいて、in vitro での汎用性が高く、多様な genotype あるいは血清型の増殖を担保する方法もない。しかしながら、これらの手法を確立することは当該下痢症の発症解明、伝播様式解析、および感染制御手段の研究において非常に重要であり、同様の症状を呈する HuNoV への本手法の将来的な転用・応用の可能性も高い。加えて公衆衛生学上はもちろん、食の安全性確保の観点からも、これまで不可能であったリスクプロファイリングなど実効性の高いオペレーションが可能となるため、非常に重要である。

今回、一部の HuNoV や唯一株化に成功した豚サポウイルス(porcine sapovirus; PoSaV)の報告において、培養時に胆汁酸要求性を示すことから、これに基づき各種胆汁酸適応濃度といくつかの細胞株との組み合わせから HuSaV 増殖系確立の研究を開始した。またウイルス RNA 検出系、既存抗血清を用いた抗原検出 ELISA や蛍

光抗体法による感染細胞の解析についても併せて検討した。本研究の概要は以下のとおりである。

1. HuSaV 細胞培養における胆汁酸要求性

ヒト精巣腫瘍由来細胞 NEC8 (JCRB0250)、腸管癌細胞由来 HuTu80 (HTB-40)および HCT-8 (CCL-244)の 3 種類の細胞株を用いて、4 種類の胆汁酸(コール酸ナトリウム(CA)、デオキシコール酸ナトリウム(DCA)、グリココール酸ナトリウム(GlyCA)、グリコケノデオキシコール酸ナトリウム(GCDCA))および Bile を細胞障害性試験に基づいた各最大許容濃度に従い添加した。

接種 1 日後(1 dpi)に洗浄および培養液交換を行い、7 日間培養した。培養上清中のウイルス RNA を抽出し、RT-PCR にて検出した。HuSaV GI.1 については NEC8、HuTu80 では GlyCA および GCDCA で明瞭な増幅シグナルが検出された。HCT-8 ではいかなる培養条件でも不検出となった。この結果より HuTu80 を用いて HuSaV GII.3 の培養検討を行ったところ、GlyCA、GCDCA および Bile にて明瞭な増幅シグナルが検出された。NEC8 および HuTu80 に共通して GlyCA 含有条件でのシグナルが強いことから、以降の培養検討には GlyCA (1 mM)を用いることとした。

2. 培養細胞間における HuSaV の経時的増殖性比較

HuSaV GI.1 について、NEC8 および HuTu80 を用いて 1 mM GlyCA 含有下で各細胞を T25 フラスコにて単層シート化したものに、陽性検体を 7$\log_{10}$ copies of viral RNA を上限(20 μL)として接種した。接種から一晩培養後に上清を取り除き、洗浄用培地にて 2 回洗浄して維持培地に交換してから経時的増殖性を比較検討した。NEC8 については接種 1、3、5 および 7 日目まで、HuTu80 については接種 1、3、5、7、10 および 14 日目まで、培養上清 100 μL 中のウイルス RNA コピー数を定量した。

NEC8 では 7 日目まででウイルス RNA は $2\log_{10}$ copies に止まった。加えて NEC8 は 7 日を超える維持培養が困難なことから、これ以上の増殖は期待できなかった。一方 HuTu80 では培養 3 日後で $6.6\log_{10}$ copies となり、5 日目には $7.4\log_{10}$ copies に到達し、14 日目まで維持された。

この結果から GI.1 の増殖率は初期値(1dpi)から約 $3.9\log_{10}$ 倍となり、これを踏まえて同様に HuSaV GII.3 の経時的増殖性を確認したところ、GII.3 は GI.1 よりも若干緩やかに増幅するものの、10 日目でピークとなる $7.7\log_{10}$ copies に達し、初期値(1dpi)から約 $3.6\log_{10}$ 倍で GI.1 とほぼ同等となった。

さらに GI.2 1 株および GII.3 2 株を用いた HuTu80 での 10 日間培養における経時的増殖性検討においても明瞭な増殖性が認められた。以上の結果から HuTu80 は少なくとも 3 つの genotype の HuSaV 増殖をサポートすることが明らかになった。

3. HuTu80 における HuSaV の細胞内動態と virion、子孫ウイルス増幅の確認

HuTu80 細胞における HuSaV の挙動に関して、dsRNA、非構造タンパク NS1-2 および VP1 の経時的シグナルと動態について検討した。すなわち、細胞に感染させてから 1、5、10 日目に、各々の特異抗体を用いて IF 法により確認した。1 日目ではシグナル非検出であったが、5 日目及び 10 日目で対象とするすべてのシグナルが確認された。dsRNA に基づく陽性率(n=3)は、GI.1 において 5 日目で 5.6-8.2%(平均 7.6%)、10 日目で 10.4-14.3%(平均 12.3%)であり、GII.3 においては 5 日目で 6.4-8.0%(平均 6.5%)、10 日目で 7.1-11.1%(平均 8.7%)となった。また、すべての感染細胞において細胞変性効果は認められなかった。加えて両 genotype での各段階の培養上清中のウイルス RNA コピー数は $3\text{-}4\log_{10}$ copies/100μL(1 日目)から 5 日目以降 $9\log_{10}$ copies/100μL を超えた。

次に、精製 HuSaV サンプルの透過電子顕微鏡観察によりウイルス様粒子(virion)を捕捉することができ、その平均粒径(各 n=15)は GI.1 で 44.1±1.5 nm、GII.3 で 41.0±1.9 nm であった。各粒子の回収率について、培養上清の超遠心処理前後の RNA コピー数を比較したところ、GI.1 で 56.8%、GII.3 で 16.8%であった。そして陽性検体の培養で得られたウイルスサンプル(P0)を 2 世代まで各 10 日間の継代培養を繰り返し、各培養上清について RNA コピー数と VP1 検出 ELISA シグナルを測定したところ、RNA コピー数は P1 および P2 共に 10 日目で $9\log_{10}$ copies/100μL を超え、VP1 検出 ELISA では P1 から P2 にかけて共にシグナル増大が認められた。以上の事象から、HuSaV は GlyCA 存在下で virion が継代培養できていることが確認された。

4. HuSaV 分離のためのウイルス RNA 必要量と加熱および紫外線に対する感受性評価

HuSaV GI.1 および GII.3 陽性検体の 10 倍段階希釈($5.3\log_{10}$～$1.3\log_{10}$、2×1 RNA copies/100μL)を行い、各々を HuTu80 細胞に接種して 10 日間後の培養上清ウイルス RNA 量を測定したところ、共に $2.3\log_{10}$ copies/100μL を下回ると検出限界以下となることから、分離に要する RNA 量は $2.3\log_{10}$ copies/100μL 以上と考えた。

このことを踏まえ、陽性検体希釈サンプルを用いたドライバスによる加熱処理(50℃、60℃および 70℃)と紫外線(波長 254 nm)照射による GI.1 および GII.3 の経時的感染価減衰を評価したところ、両 genotype にて 70℃･30 分処理で RNA シグナルは検出限界以下となり、50℃･30 分ではシグナル減衰は認められなかった。60℃処理では genotype 間での相違が顕著で、GII.3 で熱抵抗性が強いことが示された。また紫外線照射に対する感受性は、陽性検体では両 genotype ともに 5.4 J/cm^2 の照射でも感染価減衰は認められなかった。一方、両 genotype とも P1 培養上清を同試験に用

いたところ、1.8 J/cm^2 の照射で RNA および VP1 検出 ELISA の両シグナルともに検出されず、照射サンプルに同時にスパイクした Feline calicivirus も 1.8 J/cm^2 の照射でわずかに検出されるにとどまった。

以上のように、まずヒト精巣由来細胞と 2 種類のヒト腸管由来細胞(十二指腸および回盲部)を HuSaV 増殖の検討に使用した。その根拠は PoSaV-Cowden 株の増殖が LLC-PK1 細胞以外の精巣細胞で成立すること、幼豚での in vivo 実験で小腸部位に増殖が認められることによる。その結果、抱合型胆汁酸である GlyCA あるいは GCDCA の存在が HuTu80 細胞での HuSaV 増殖をサポートすることを確認した。ヒト体内における胆汁成分の合成・分泌および再吸収といった一連の代謝から、十二指腸付近の胆汁酸比率は抱合型が主体となることからも、ヒト体内の生理を反映した増殖を示唆していると考える。このことはヒトウイルス性胃腸炎の in vitro における病態生理が模倣され、その発症機構解析や制御手法開発などにも非常に有用である。

続いて HuTu80 細胞による HuSaV 感染粒子(virion)の増殖が世界で初めて確認でき、GI.1 および GII.3 では接種 5-10 日で培養上清中の RNA コピー数がピークとなる一方、IF の結果では HuSaV 感受性細胞は限局的であり、VP1 検出 ELISA の結果からも継代培養の繰り返しによるシグナル増加が示されたことからウイルス RNA とウイルス粒子作出にある種の time-rag の発生が示唆された。このことは HuSaV 株間での増殖速度や viron 回収の検証において非常に重要な情報となった。

HuSaV の in vitro 複製はウイルス RNA および VP1 抗原共に非常に高いレベルに到達しており、継代培養による感染性ウイルスのストック作製が可能となった。これまでヒト胃腸炎ウイルスのほとんどが陽性検体(糞便)をリソースとして使用していることから、有限性、virion 含量が不確定なためのリソース不均一性と研究結果の

確度への影響、医学研究倫理の制約などから解放され、ヒトノロウイルス研究と比較しても非常に重要なブレークスルーとなった。さらに同様の strategy による汎用培養法への研究転換のきっかけともなり得るため、海外の関連研究者も注目している。

HuSaV はヒトノロウイルス同様に集団感染事例や食品媒介を疑うアウトブレイク報告がある一方で、その季節性や感染経路、最小感染量や発症者からの複数 genotype 検出の要因などが解明されていない。また今回の著者らの検出法により、近年生活排水から通年検出される実態が明らかになり、常に一定数の感染者あるいはウイルス排出者の存在が示唆されている。今回の研究成果により、これまでの遺伝子検出や遺伝子定量といった「ウイルスの痕跡トレース」のみならず、感染力を有するウイルスの存在や感染価を導くことが可能となるため、より精度の高いウイルス動態が解明されるとともに、リスクプロファイリングに基づく実効的な感染制御対策立案が可能となった。

さらに、加熱に対する感受性は 60℃・30 分で完全な不活化には至らず、60℃・15 分で不活化されるヒトノロウイルスに対する対策では不十分であることが判明した。また UV 照射については HuSaV 陽性糞便希釈検体と、培養した HuSaV では感受性に大きな相違が確認された。こうした結果も公衆衛生における HuSaV 制御において、きわめて重要な要因となった。

本研究において、HuSaV の汎用培養法を発見したことから、感染経路解明やその対策立案への道が開けたとともに、本研究のさらなる発展による、抗体保有などのヒト血清疫学解析、HuTu80 細胞を用いたウイルス受容体検索、抗ウイルス成分検索など幅広い研究展開が可能となった。

Summary

Sapoviruses are non-enveloped, single-stranded (+) RNA viruses belonging to the family *Caliciviridae,* similar to noroviruses. Many genotypes (likely equivalent to serotypes) of human sapovirus (HuSaV) exist; currently, at least 18 genotypes are known (GI.1-7, GII.1-8, GIV.1, and GV.1-2).
These viruses cause acute gastroenteritis in individuals of all age groups; HuSaV is known to cause infections, especially in infants and children. Human-to-human, food-borne suspected outbreaks due to HuSaV as well as human norovirus (HuNoV) have been frequently reported. However, these infectious diseases cannot be distinguished by symptoms alone.

Cell-based viral infection and propagation systems for porcine sapovirus (PoSaV) have been established; however, such systems are lacking for HuSaVs, although they were discovered more than 40 years ago. HuNoV infection systems using conventional human cell lines, such as Caco-2 and BJAB, and human stem cells, such as intestinal enteroid cells, and fecal specimens as infection sources have been reported; however, their virus propagation efficiency is low, and virus stocks cannot be prepared. Therefore, it is important to construct an in vitro culture system that is convenient and that can facilitate the growth of various types of human gastroenteric viruses to establish infection control methods or to ensure food safety and risk profiling for public health.

In this study, we established an efficient HuSaV propagation system using conventional human cell lines. The effect of bile acid supplementation on HuSaV propagation was also tested because PoSaV and most HuNoVs require bile acids for their propagation in cultured cells.
The summary of this research is as follows:

1. Bile acid requirement for HuSaV susceptibility in cultured human cells
Three cell lines (human testis tumor-derived NEC8 (JCRB0250), human duodenum carcinoma-derived HuTu80 (HTB-40), and human ileocecal adenocarcinoma-derived HCT-8 (CCL-244)) were used along with four bile acids (sodium cholic acid (CA), sodium deoxycholate (DCA), sodium glycocholate (GlyCA), and sodium glycochenodeoxycholate (GCDCA)), or bovine gall (Bile) for determining the maximum permissible concentrations

of these bile acids based on cytotoxicity tests.

One day after inoculation with the HuSaV-positive fecal suspension (1 dpi), the monolayer cells were washed and replaced along with fresh culture medium, followed by culture for another 7 days. Viral RNA was extracted from the culture supernatant and detected via RT-PCR. The PCR signal was positive for HuSaV GI.1 in NEC8 and HuTu80 cells supplemented with GlyCA and GCDCA but was negative in HCT-8 cells, even in the presence of these bile acids. The PCR signal was positive for HuSaV GII.3 in HuTu80 cells supplemented with GlyCA, GCDCA, or bile. GlyCA was used in subsequent culture studies.

2. Efficient HuSaV propagation in human intestinal cells

To quantitatively investigate the HuSaV propagation efficiency, the kinetics of GI.1 HuSaV replication in the infected NEC8 and HuTu80 cell lines in the presence of GlyCA (0.5 mM for NEC81 and mM for HuTu80) were studied. The culture supernatants were collected on 1, 3, 5, and 7 dpi and 1, 3, 5, 7, 10, and 14 dpi from NEC8 and HuTu80 cells, respectively, and titrated using RT-qPCR. In NEC8, GI.1 HuSaV RNA remained at 2 $\log_{10}$ copies up to 7 dpi; it is difficult to maintain and culture NEC8 for more than 7 days. In HuTu80, GI.1 HuSaV RNA increased to 6.6 $\log_{10}$ copies on 3 dpi and reached 7.4 $\log_{10}$ copies on 5 dpi, which was maintained until 14 dpi. The propagation rate of GI.1 was 3.9 $\log_{10}$ higher than the initial value (1 dpi).

HuTu80 was selected for subsequent trials because GI.1 HuSaV propagated more efficiently in HuTu80 than in NEC8. In HuTu80, GII.3 HuSaV RNA titers gradually increased, and reached the peak of 7.7 $\log_{10}$ copies on 10 dpi, which was 3.6 $\log_{10}$ higher than the initial value (1 dpi), similar to GI.1 HuSaV. Furthermore, one GI.2 strain and two GII.3 strains were also propagated in HuTu80 cells. These results indicate that HuTu80 supports efficient HuSaV propagation of at least three genotypes.

3. Confirmation of intracellular replication, translation, and production of infective HuSaV progeny in HuTu80

The replication and translation of GI.1 and GII.3 HuSaVs in HuTu80 cells in the presence of GlyCA were confirmed using immunofluorescence (IF) by detection of double-stranded RNA (dsRNA; as a marker of the intermediate products during the replication of single-stranded RNA viruses), nonstructural protein NS1-2, and structural protein VP1. No signal was detected on 1 dpi, but all the target signals were confirmed on 5 and 10 dpi. The positive rate based on dsRNA (n = 3) was 5.6–8.2% (mean 7.6%) on 5 dpi and 10.4–14.3%

(mean 12.3%) on 10 dpi for GI.1, and it was 6.4–8.0% (mean 6.5%) on 5 dpi and 7.1–11.1% (mean 8.7%) on 10 dpi for GII.3. A small proportion of HuTu80 cells was susceptible to HuSaVs. No cytopathic effect was observed in the infected cells. The viral RNA levels in the culture supernatants of both genotypes increased from 3–4 $\log_{10}$ copies / 100 μL on 1 dpi to 9 $\log_{10}$ copies / 100 μL on 5 dpi and later.

Virus-like particles (virions) in purified GI.1 and GII.3 HuSaV samples were visualized using transmission electron microscopy. The average particle size of GI.1 and GII.3 (n = 15 each) was 44.1 ± 1.5 nm and 41.0 ± 1.9 nm, respectively. When the number of viral RNA copies in the culture supernatant before and after ultracentrifugation were compared, the recovery rate was 56.8% and 16.8% for GI.1 and GII.3, respectively.

The infectivities of progeny GI.1 and GII.3 HuSaVs harvested at 10 dpi were further confirmed by passaging P0 (the virus from culturing the positive fecal sample) and P1 viruses (the passaged virus cultured in the P0 sample). Both P1 and P2 (the virus from the cultured P1 sample) cell culture supernatants exceeded 9 $\log_{10}$ copies / 100 μL at 10 dpi, and the VP1 detection ELISA revealed an increase in signal from P1 to P2. HuSaV successfully replicated, produced infectious progeny viruses, and was efficiently passaged in HuTu80 cells in the presence of GlyCA.

4. Minimum infectious dose and sensitivity of HuSaVs to heat and ultraviolet (UV) treatments

A series of ten-fold diluted GI.1 and GII.3 HuSaV-positive fecal suspensions was inoculated into HuTu80 cells. After removing the inoculum and washing the cells at 1 dpi, the culture supernatants were collected at 10 dpi, and the viral RNA of the culture supernatant was measured. The minimum infectious dose was > 2.3 $\log_{10}$ viral RNA copies for both GI.1 and GII.3 HuSaVs.

Furthermore, the attenuation of infection titers via heat and UV treatments was evaluated. GI.1 and GII.3 HuSaVs were heat-treated at 50 °C, 60 °C, and 70 °C for 10, 20, and 30 min or irradiated with UV light (wavelength 254 nm) on ice for 20, 40, and 60 min. HuSaV infection supernatant was collected as described above.

After heat treatment at 70 °C for 30 min, RNA levels of both the GI.1 and GII.3 HuSaVs decreased to the RT-qPCR detection limit. However, after treatment at 50 °C for 30 min, GI.1 and GII.3 HuSaVs replicated at a level similar to that of a 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 at 60 °C.

Regarding susceptibility to UV irradiation, both GI.1 and GII.3 HuSaV-diluted fecal suspensions were insensitive to UV treatment, even at 5.4 J/ cm^2. Along with these results, UV treatment was performed using passaged (P1) HuSaV stock mixed with feline calicivirus (FCV), and it was confirmed that the passaged GI.1 and GII.3 HuSaVs were sensitive to UV treatment at 1.8 J/cm^2. Spiked FCV was almost inactivated under these conditions.

In this study, human testis-derived cells and two types of human intestinal-derived cells (duodenum and ileocecal region) were selected for analyzing HuSaV susceptibility. This is because the PoSaV-Cowden strain grows in porcine testis cells in addition to porcine kidney cells (LLC-PK1) when supplemented with bile acids, and the PoSaV-Cowden strain infects the small intestine site in young pigs. Accordingly, HuSaV was grown in human testis tumor-derived cells (NEC8) and more efficiently in human duodenum carcinoma-derived cells (HuTu80) in the presence of a conjugated bile acid (i.e., GlyCA and GCDCA). In the human body, conjugated bile acids are the main bile acids in the duodenum. Therefore, it is physiologically consistent that conjugated bile acids are essential for HuSaV propagation in human duodenal cells and would be useful for analyzing infection mechanisms and developing HuSaV control methods.

In the kinetics study, despite the weak HuSaV VP1 signal, the HuSaV RNA signal reached a plateau at 5 dpi but the HuSaV VP1 signal substantially increased at 10 dpi (data not shown). These time lags may correlate with HuSaV virion formation/release speed in the HuTu80 cell culture supernatant. Although these observations could also be attributed to the difference in assay sensitivity (i.e., RT-qPCR is more sensitive than ELISA), this information was important to account for the difference in growth speed among various genotypes and the virion collection timing.

Owing to lack of efficient cell culture systems, most studies on human gastroenteritis viruses, including HuNoVs, have used viral RNA-positive specimens (feces) as resources; however, there are issues such as those related to finiteness, resource heterogeneity due to uncertain virion content, inaccuracy of results, and ethical restrictions in medical research. Efficient in vitro replication and production of HuSaV using HuTu80 cells make it possible to confirm HuSaV infectivity in fecal specimens, and more importantly, could allow preparation of a stock of infectious viruses by passage. Thus, the newly established system

described in this study can help overcome these limitations and establish an efficient growth system for other human gastroenteritis viruses, including HuNoV.

HuSaV-RNA is detected throughout the year in symptomatic and asymptomatic individuals and in wastewater. HuSaV-RNA has also been found in edible bivalves such as oysters and clams. HuSaV distribution is identical to that of HuNoV, and both viruses are spreading globally. However, it is unclear whether detection of viral RNA can reflect the presence of the infectious virus, as current nucleic acid detection systems can only detect traces of viruses. The development of a conventional and efficient culture method for HuSaV using HuTu80 cells will allow analysis of viral dynamics and effective risk profiling from "infectivity."

HuNoV is known to be inactivated upon heating at 60°C for 15 min. In contrast, HuSaV was inactivated completely and was relatively stable even after 30 min of heating at 60°C. These differences might reflect the differences between HuSaV and HuNoV or could be partially responsible for lower propagation efficiency and shorter culture period for the HuNoV culture system. Furthermore, the HuSaV-positive diluted stool sample and cultured HuSaV differed considerably in their UV irradiation susceptibility. Unknown fecal factor(s) could protect HuSaV from inactivation by UV irradiation. These findings are important for developing strategies for HuSaV control in public health settings.

Our findings could serve as reference for performing a wide range of studies, such as detection and removal/inactivation of infectious HuSaV in clinical, food, and environmental resources for risk profiling; development of HuSaV infection control methods; identification of virus receptors and antiviral compounds; and analyses in human serum epidemiological studies such as those involving neutralization antibody retention surveys, among others.

出　典

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Human sapovirus propagation in human cell lines supplemented with bile acids

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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.

Author contributions: H.T., T.O., Q.W., L.J.S., and M.N. designed research; H.T., T.O., T.S., H.S., T.K., T.T., C.T., M.K., and Q.W. performed research; H.T., T.O., T.S., H.S., T.K., T.T., C.T., M.K., and Q.W. analyzed data; and H.T., T.O., Q.W., and L.J.S. wrote the paper.

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The authors declare no competing interest.

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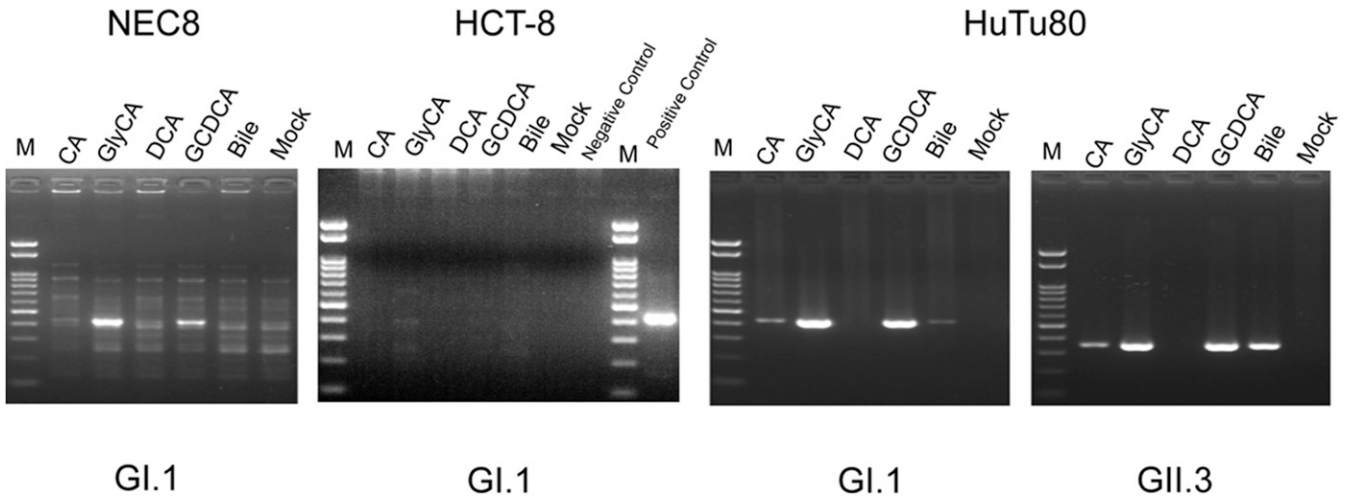

Fig. 1. Human cell lines NEC8 and HuTu80 but not HCT-8 supported the replication of HuSaV GI.1 and GII.3 in the presence of bile acids in the cell culture medium. HuSaV GI.1 (AK20) or GII.3 (AK11) positive stool suspension was inoculated onto the cell monolayers in the presence of CA, GlyCA, DCA, GCDCA, 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.

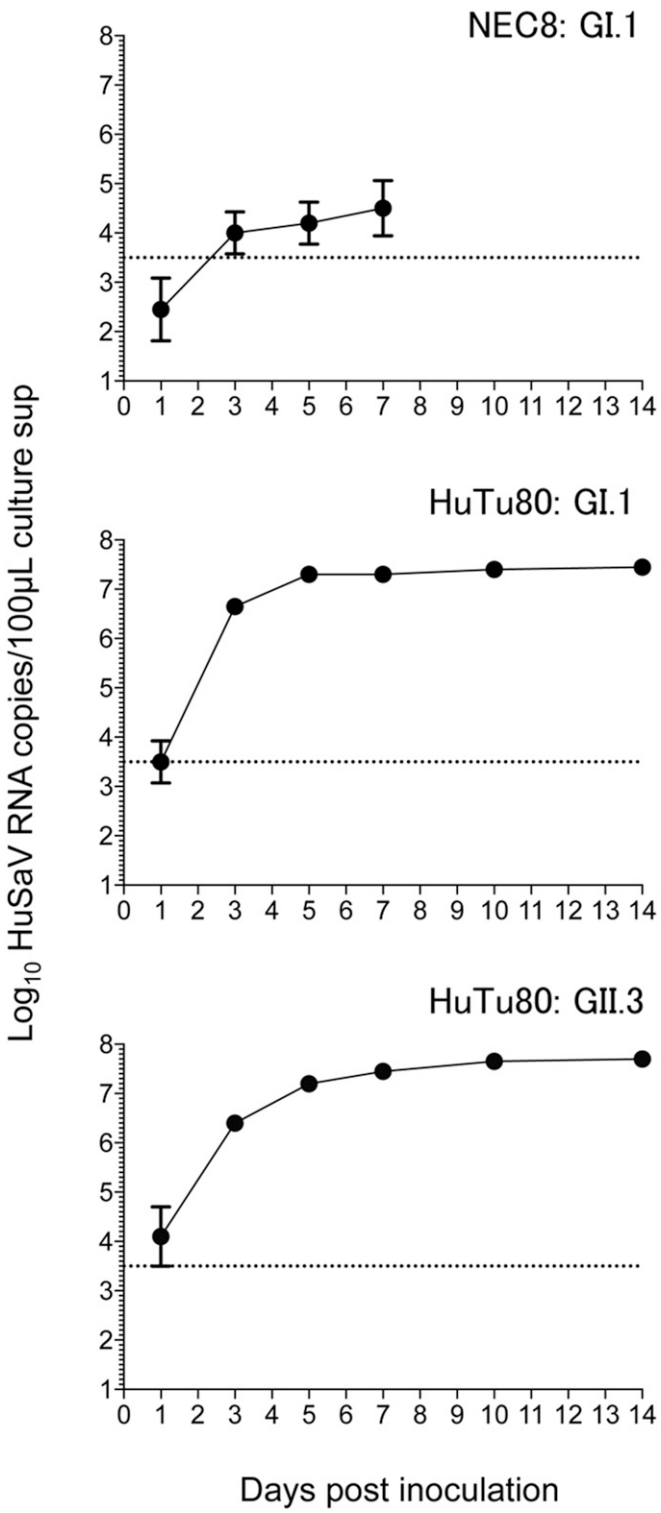

Fig. 2. Growth kinetics of HuSaV GI.1 and GII.3 in NEC8 and/or HuTu80 cells. HuSaV GI.1 (AK20) or GII.3 (AK11) positive stool suspension was inoculated onto NEC8 or HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers at 1 dpi, we collected supernatants at 1, 3, 5, and 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. 4*A*).

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. 4*B*). 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. 4*C*). 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.

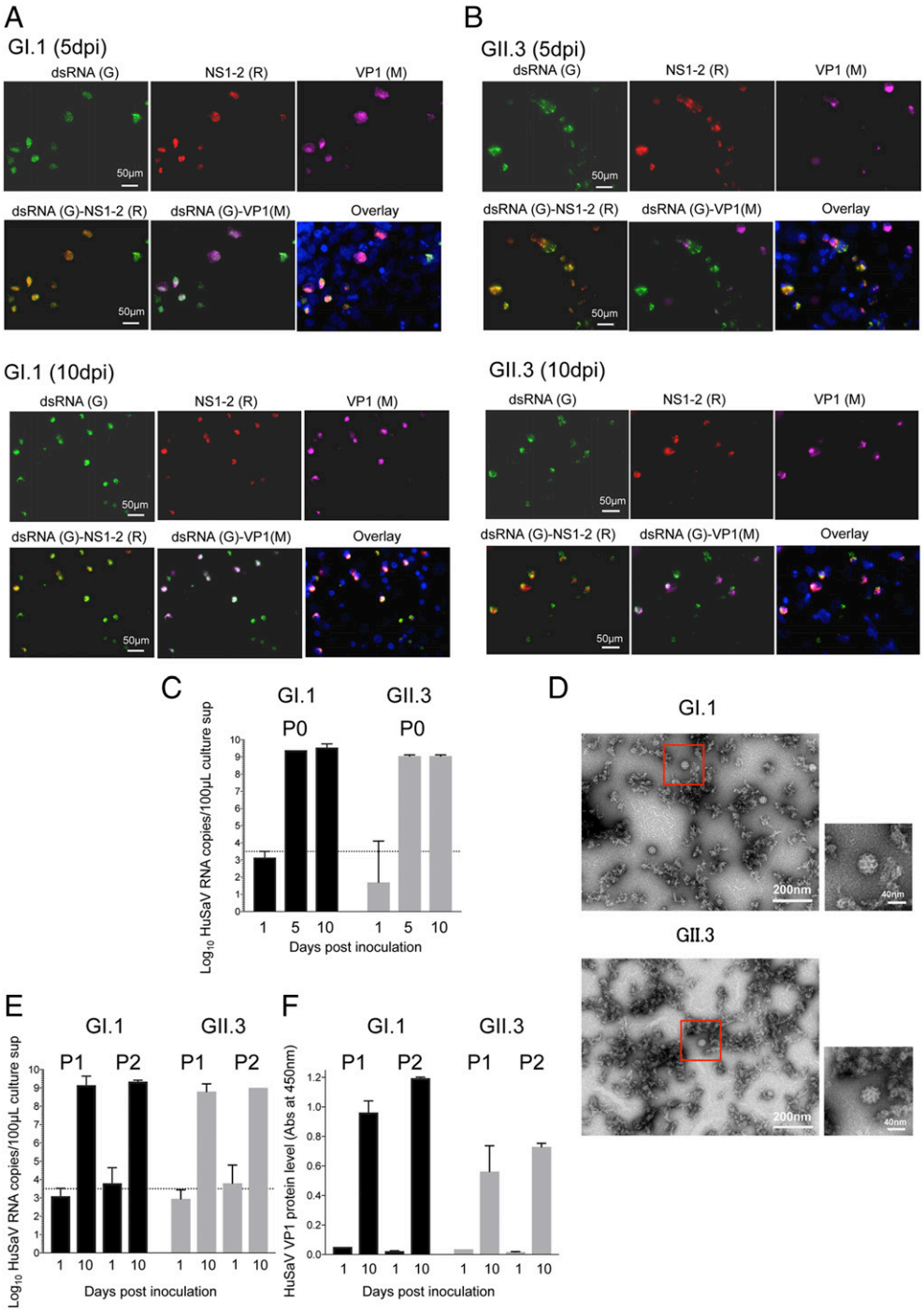

Fig. 3. HuSaV GI.1 and GII.3 replicated in HuTu80 cells and produced infectious progeny viruses in the culture supernatants. HuSaV GI.1 (AK20) (*A*) or GII.3 (AK11) (*B*) positive stool suspension was inoculated onto HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers 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.

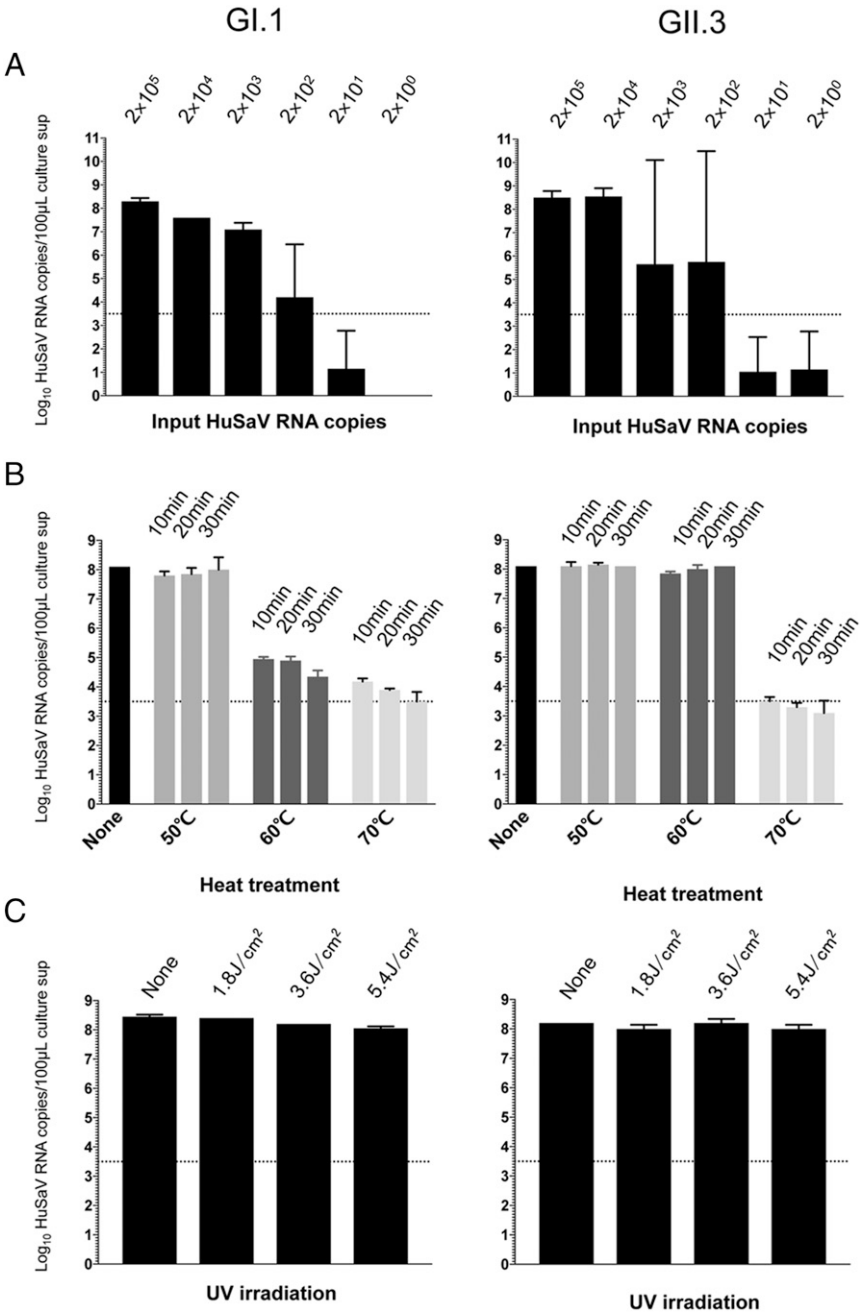

Fig. 4. The infectious dose and effects of various treatments on infectivity of HuSaV GI.1 or GII.3 in HuTu80 cells. (*A*) Infectivity of serially diluted HuSaV GI.1 (AK20) or GII.3 (AK11); effect of heat treatment (*B*) or UV treatment (*C*) on SaV GI.1 (AK20) or GII.3 (AK11) infectivity in HuTu80 cells. 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. S1*A*]) 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 × *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 (~4×10^6 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 ~1.0×10^7 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

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 $\sim 1.0 \times 10^7$ 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_2O_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_2SO_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 $\sim 5 \times 10^7$ 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 ($\sim 2 \times 10^5$ to $\sim 2 \times 10^0$ 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

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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Supplementary Information for

Human sapovirus propagation in human cell lines supplemented with bile acids.

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This PDF file includes:

Supplementary text
Figures S1 to S3
Tables S1

Other supplementary materials for this manuscript include the following:

None

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Supplementary Information Text

Supplemental Material and methods.

Focused imaging of immunofluorescence assay signals.
Images were taken at a magnification of 200X. Imaging was performed using a fluorescence microscope (Olympus IX81, Olympus, Tokyo, Japan) and MetaMorph imaging software (Molecular Probe, Molecular Devices, LLC, San Jose, CA).

HuSaV RNA recovery rate estimation
HuSaV RNA recovery rate was estimated by qRT-PCR(*1*) using cDNA prepared from the extracted viral RNA in the cell culture supernatants after ultracentrifugation (UCF sup), and recovered suspension from ultracentrifugation precipitation (UCF ppt), collected from two T150 flasks (21 mL / flask) cultured for 9 days and used for the visualization of HuSaV particles by transmission electron microscopy (TEM) as described in the main text.

UV sensitivity test using FCV spiked passaged HuSaV stock
The feline calicivirus (FCV) F9 strain was purchased from American Tissue Culture Collection (ATCC VR-2057). Crandell-Rees feline kidney (CRFK) cells obtained from the Japanese Collection of Research Bioresources were cultured in the growth medium (Dulbecco Eagle's minimal essential medium [DMEM: Sigma-Aldrich] supplemented with 5% heat-inactivated fetal bovine serum [Biosera] and antibiotics (50 µg/mL ampicillin and 100 µg/mL Kanamycin). The virus was propagated and titrated in CRFK cells to determine the 50% tissue-culture infectious dose ($TCID_{50}$) using 96-well plates.
Sixty µL of passage 1 (P1) GI.1(AK20) or GII.3 (AK11) HuSaV suspension (4.5×10^6 copies of viral RNA/10µL) spiked with FCV suspension (6.5 $Log_{10}TCID_{50}$/50µL) inside 8-strip microtubes was irradiated with ultraviolet light at 1.5 mW / cm^2 (UV lamp VL−206.G, CosmoBio, Japan) on ice for 20, 40 and 60 minutes. The FCV infectivity of the treated supernatants was titrated in CRFK cells, which were incubated at 36˚C with 5% CO_2, for 4 days. The HuSaV infectivity of 50 µL treated supernatants was tested in HuTu80 cells cultured in 24-well plates. The culture supernatants were collected at 10 dpi and HuSaV RNA and HuSaV VP1 antigen was measured by RT-qPCR or ELISA as described in Materials and Methods in main text.

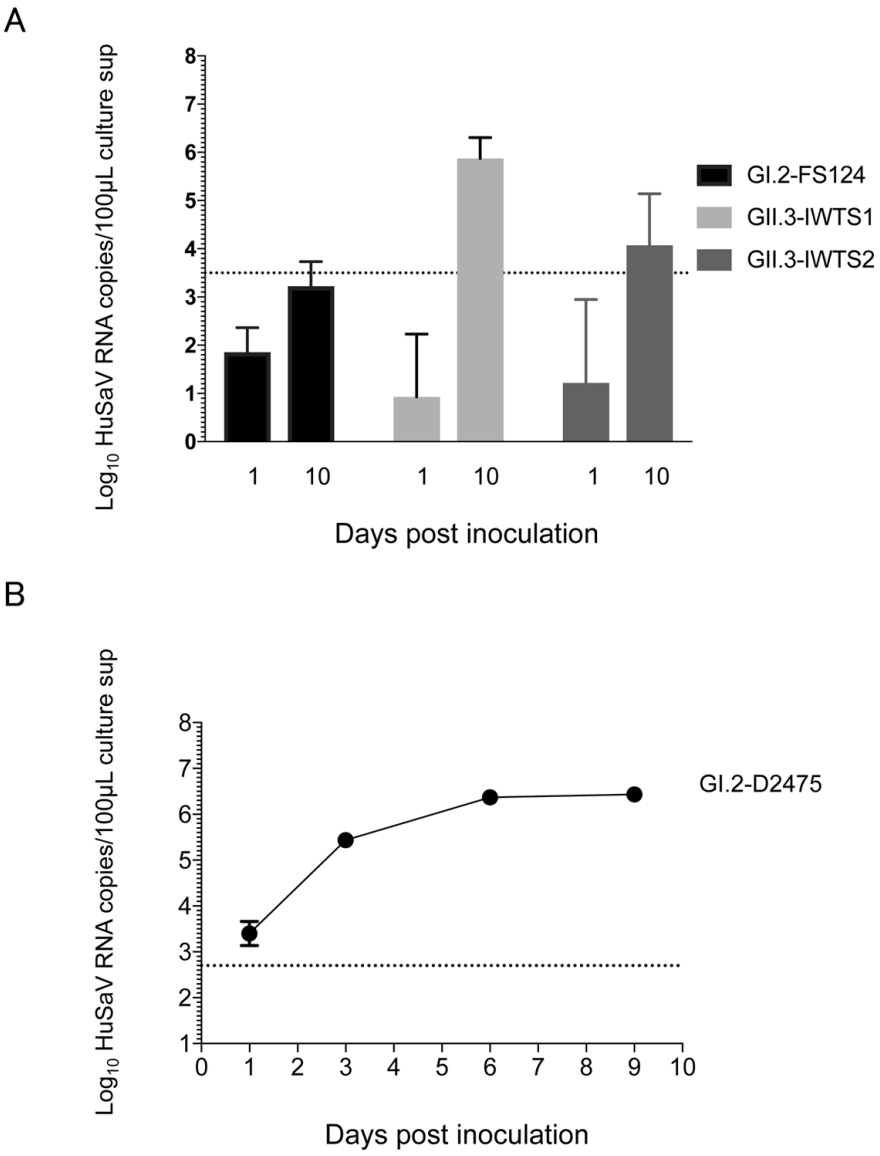
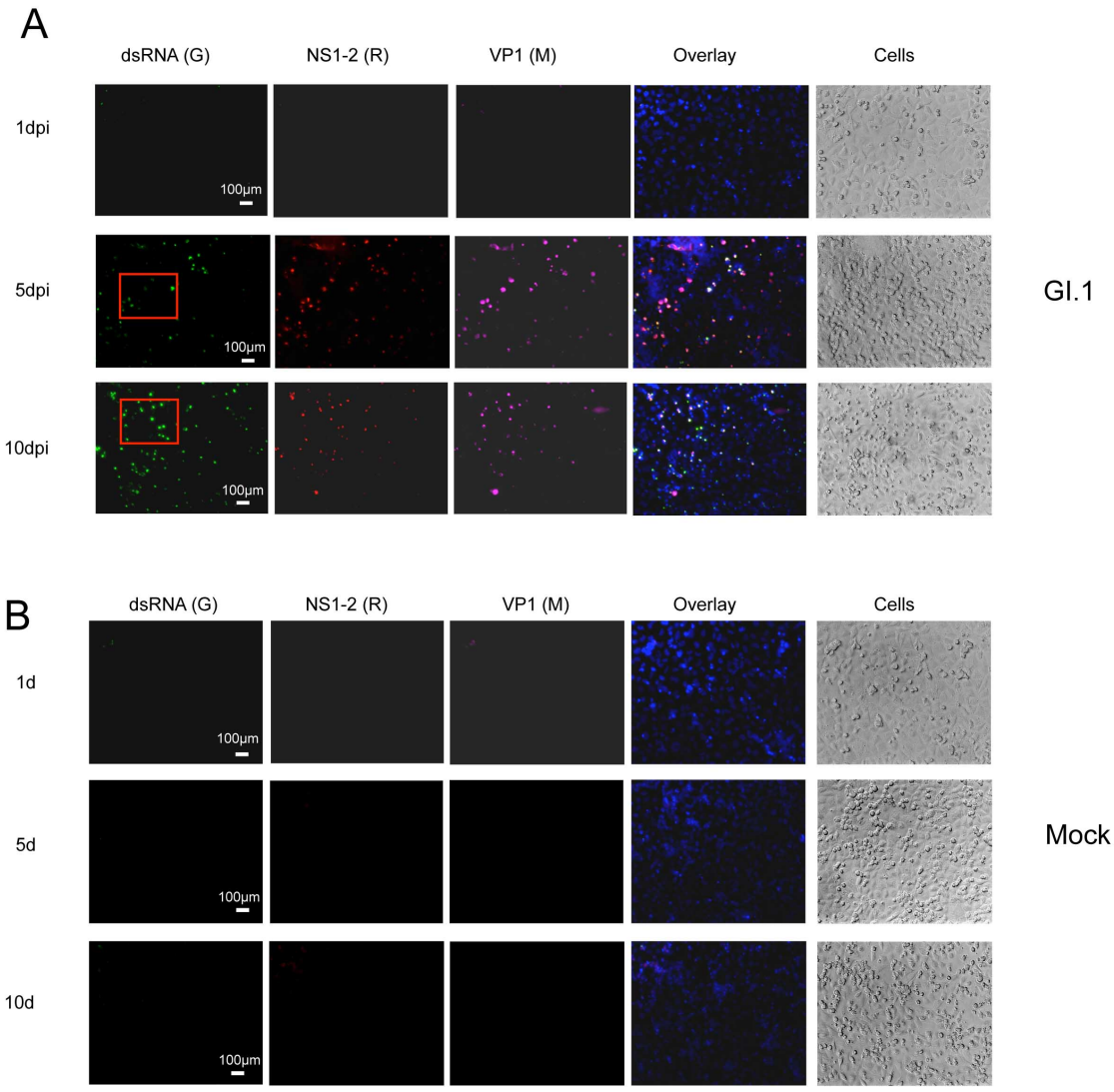


Fig. S1. Infectivity of additional HuSaV positive stool specimens in HuTu80 cells.

Experiment at lab 1 (National Institute of Infectious Diseases [NIID], Japan) (A); One HuSaV GI.2 (FS124) and two additional HuSaV GII.3 (IWTS1 and IWTS2) positive stool suspension (5 µL inoculum containing approx. ~ 1.0 $\times 10^5$ copies of viral RNA per well) was inoculated onto the monolayers of HuTu80 cells, respectively. After removing the inoculum and washing the cell monolayers at 1dpi, we collected the culture supernatants at 10 dpi. The SaV RNA was measured by RT-qPCR. Dotted line indicates the quantification limit. The data represent the mean of two trials. Error bars indicate standard deviations. Experiment at lab 2 (The Ohio State University [OSU], USA) (B) ; HuSaV GI.2 (D2475) inoculation (5 µL inoculum containing approx. ~ 1.0 $\times 10^5$ copies of viral RNA per well) and propagation in 1-day-old HuTu80 cell monolayers were performed in 24-well plates in the presence of GlyCA. The supernatants were collected from duplicated wells and mixed together at 1 (after the removal of the inoculum and the washing step), 3, 6, and 9 dpi. RNA extraction was performed using MagMax RNA isolation kit (ThermoFisher Scientific, USA). The data represent the mean of three trials. Error bars indicate standard deviations. Bile acid (1000µM GlyCA) was present throughout the culture period in all the experiments.


A
dsRNA (G)
NS1-2 (R)
VP1 (M)
Overlay
Cells
1dpi
5dpi
10dpi
100μm
GI.1
B
dsRNA (G)
NS1-2 (R)
VP1 (M)
Overlay
Cells
1d
5d
10d
100μm
Mock

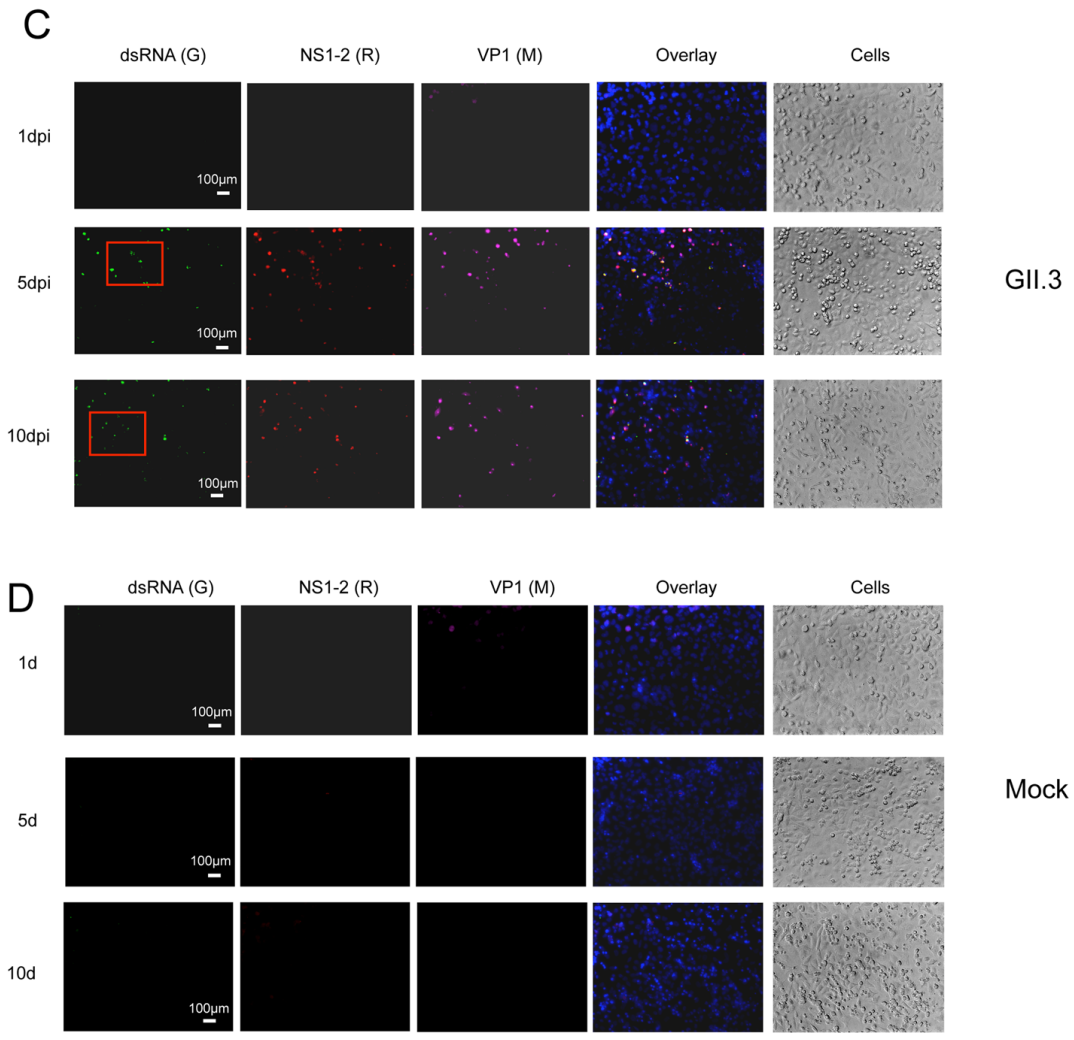


Fig. S2. SaV GI.1 and GII.3 replication in HuTu80 cells.
HuSaV GI.1 (AK20) (A) or GII.3 (AK11) (C) positive stool suspension was inoculated onto HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers at 1 dpi, the cell monolayers were fixed at 1, 5 and 10 dpi, respectively. Mock infected HuTu80 cells fixed at 1, 5 and 10d, respectively, were shown as (B) and (D). GlyCA (1000 μM) was present throughout the culture period. Cells were permeabilized and blocked before staining with mouse anti-dsRNA antibody, rabbit anti-GI or anti GII HuSaV nonstructural protein NS1-2 hyperimmune serum, and guinea pig anti-SaV GI or anti GII HuSaV VLP 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). Individual images of dsRNA, NS1-2, VP1, merged images (dsRNA, NS1-2, VP1, and nuclei) are shown in Overlay. Light microscope images of cells are also shown to visualize the confluent cells in this area. Magnification: 200X.
Infection experiments were performed twice; pictures of a representative experiment are shown. The regions enclosed by the red boxes were further taken at a higher magnification of 600X (see Fig. 3 A and B).

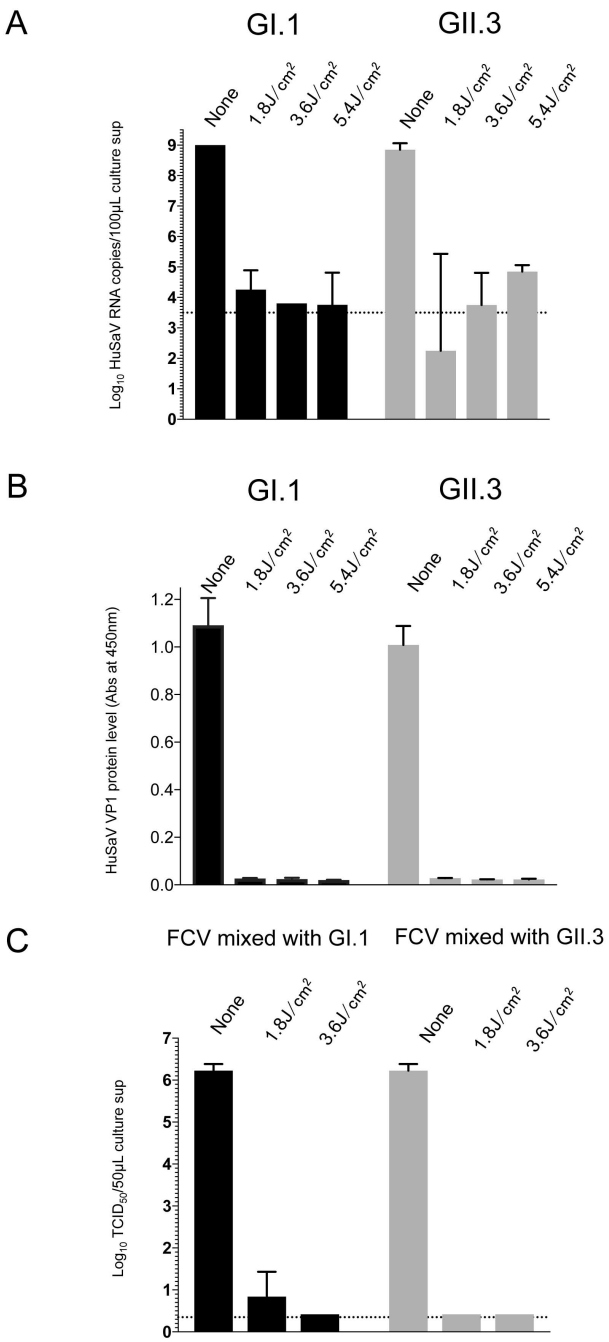


Fig. S3. The effects of UV treatment on infectivity of passaged GI.1 or GII.3 HuSaV and FCV**.**
Pre-treated SaV GI.1 (AK20) or GII.3 (AK11) P1 suspension mixed with FCV was inoculated onto HuTu80 cell monolayers. After removing the inoculum and washing the cell monolayers at 1 day post-inoculation (dpi), the culture supernatants were collected at 10 dpi. 1000 μM GlyCA was present throughout the culture period. The SaV RNA (A) and SaV VP1 antigen (B) was measured by RT-qPCR or ELISA. Dotted line indicates the lowest quantification limit for RT-qPCR.
Pre-treated SaV GI.1 (AK20) or GII.3 (AK11) P1 suspension mixed with FCV was inoculated onto CRFK cell monolayers, and the culture supernatants were collected at 4 days post-inoculation. Then the virus titer (C) was determined.
Data represent the mean of two independent experiments for each point. Dotted line indicates the quantification limit.

Table S1. SaV RNA levels and their recovery rates after ultracentrifugation

	GI.1 (AK20)	GII.3 (AK11)
Cell culture sup	11.1	10.2
UCF sup	7.0	6.3
UCF ppt	10.8	9.5
Recovery rates from cell culture sup	56.8%	16.8%

UCF: ultracentrifugation

Sup: supernatants after ultracentrifugation

Ppt: recovered suspension from ultracentrifugation precipitation

The numbers for Cell culture sup, UCF sup, and UCF ppt are expressed as $\log_{10}$ RNA copies in each total fraction.