Pathogenesis of Korean *Sapelovirus A* in piglets and chicks

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ABSTRACT

Sapelovirus A (SV-A), formerly known as *Porcine Sapelovirus* as a member of a new genus *Sapelovirus*, is known to cause enteritis, pneumonia, polioencephalomyelitis, and reproductive disorders in pigs. We have recently identified α2,3-linked sialic acid on GD1a ganglioside as a functional SV-A receptor rich in the cells of pigs and chickens. However, the role of GD1a in viral pathogenesis remains elusive. Here, we demonstrated that a Korean SV-A strain could induce diarrhea and intestinal pathology in piglets but not in chicks. Moreover, this Korean SV-A strain had mild extra-intestinal tropisms appeared as mild non-suppurative myelitis, encephalitis and pneumonia in piglets, but not in chicks. By real-time RT-PCR, higher viral RNA levels were detected in fecal samples than in sera or extra-intestinal organs from virus-inoculated piglets. Immunohistochemistry confirmed that high viral antigens were detected only in the epithelial cells of intestines from virus-inoculated piglets but not from chicks. This Korean SV-A strain could bind the cultured cell lines originated from various species but replication occurred only in cells of porcine origin. These data indicated that this Korean SV-A strain could replicate and induce pathology in piglets but not in chicks, suggesting that additional porcine specific factors are required for virus entry and replication. In addition, this Korean SV-A strain is enteropathogenic but could spread to the bloodstream from the gut and disseminate to extra-intestinal organs and tissues. These results will contribute to our understanding of SV-A pathogenesis so that efficient anti-sapelovirus drugs and vaccines could be developed in the future.

Keywords: Sapelovirus A, piglets, chicks, pathogenesis, host range restriction

INTRODUCTION

The *Picornaviridae* family, comprising 29 genera, consists of a diverse family of non-enveloped viruses with positive sense single-stranded RNA genomes (Racaniello, 2013; http://talk.ictvonline.org/files/master-species-lists/m/msl/5208). Viruses in this family can cause a wide range of diseases, including intestinal, respiratory, neurological, cardiac, hepatic, mucocutaneous and systemic diseases of various severities in both humans and animals (Racaniello, 2013). Since porcine enterovirus 8 (PEV-8), simian type 2 picornavirues, and duck picornavirus TW90A have a unique genomic organization different from other picornavirus genera (Son *et al*., 2014a), the genus *Sapelovirus* is a newly assigned member of the *Picornaviridae* family (Adams *et al*., 2015). The *Sapelovirus* genus consists of three species: *Sapelovirus A* (SV-A) formerly known as porcine sapelovirus, *Sapelovirus B* formerly named as simian sapelovirus, and *Avian Sapelovirus* formerly known as duck picornavirus TW90A (Adams *et al*., 2015).

SV-A can cause asymptomatic and symptomatic diseases in both field and experimental pigs (Alexandersen *et al*., 2012; Kim *et al*., 2016). The symptomatic disorders include diarrhea, pneumonia, polioencephalomyelitis and reproductive disorders (Alexandersen *et al*., 2012; Huang *et al*., 1980; Lamont & Betts, 1960; Lan *et al*., 2011; Schock *et al*., 2014; Sibalin, 1963). Experimental studies have demonstrated a diverse range of clinical symptoms (Lamont & Betts, 1960; Lan *et al*., 2011; Sibalin, 1963; Yamanouchi *et al*., 1965). These differences largely depend on age, route of infection, and strains inoculated (Alexander & Betts, 1967; Lamont & Betts, 1960; Lan *et al*., 2011; Sibalin, 1963; Yamanouchi *et al*., 1965).

Significant antigenic diversity has been observed in SV-As isolated from different countries and continents (Bohl *et al*., 1960; Dunne *et al*., 1967,1971; Izawa

72 *et al*., 1962; Kadoi *et al*., 1970; L'Ecuyer & Greig, 1966). The genomes of SV-As and
73 other members in the genus *Sapelovirus* have the typical picornavirus genome
74 organization: 5' untranslated region (UTR)-L-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-
75 3C-3D-3' UTR (Tseng & Tsai, 2007; Krumbholz *et al*., 2002; Oberste *et al*.,
76 2002,2003; Son *et al*., 2014a). However, there are significant structural differences in
77 the SV-A genome, e.g, the *cis*-acting RNA element (*CRE*) in the 2c coding region
78 and kissing domain in the 3'UTR are vary between recent Korean and Chinese
79 strains and older English and Chinese strains (Son *et al*., 2014a). These differences
80 in antigenic diversity and structural features could influence the pathogenicity and/or
81 host range restriction of SV-A strains yet we have a limited understanding of the
82 pathogenesis of SV-A (Son *et al*., 2014a).

83 In comparison to other picornaviruses, the SV-A life cycle remains poorly
84 characterized. We have recently demonstrated that SV-A can recognize α2,3-linked
85 sialic acid (SA) on GD1a as a functional SV-A receptor (Kim *et al*., 2016). Alph2,3-
86 linked SA is known to be highly expressed on cells of porcine and avian origin (de
87 Graaf & Fouchier, 2014; Raman *et al*., 2014), indicating that SV-A has the potential
88 to be able to infect both pigs and chickens. Therefore, the objective of this study was
89 to undertake a comparative analysis of the pathogenesis of a Korean SV-A strain in
90 piglets and chicks.

RESULTS

The SV-A strain caused diarrhea and fecal viral shedding in piglets but not in chicks

Chicken cells are typically rich in α2,3-linked SA, whereas porcine cells are abundant in both α2,3- and α2,6-linked SAs (Raman *et al*., 2014). Our previous results have demonstrated that SV-A could recognize α2,3-linked SA as a receptor (Kim *et al*., 2016), suggesting that SV-A might be able to infect and induce pathology in both pigs and chicks. To determine whether SV-A could induce diarrhea and fecal viral shedding in piglets and chicks, 3-day-old piglets obtained from sows by hysterectomy and 3-day-old specific pathogen free (SPF) chicks were orally inoculated with 2 x 10^9 PFU/ml or 5 x 10^8 PFU/ml of SV-A (KS04105 strain), respectively. Compared to mock-inoculation, piglets inoculated with SV-A strain had continuous diarrhea from 1 day post-inoculation (dpi) to 5 dpi (data not shown). However, diarrhea was not observed in mock- or SV-A-inoculated chicks during the entire experimental period.

To assess fecal viral shedding, one-step real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was performed with fecal samples sequentially collected from mock- or SV-A-inoculated piglets and chicks (Chen *et al*., 2014). High viral RNA levels were detected in fecal samples collected from piglets at 1 dpi, reaching a peak at 3 dpi followed by decreasing viral loads from 5 dpi (Fig. 1a). However, SV-A RNA was only detected at 1 dpi from feces of SV-A-inoculated chicks (Fig. 1b), most likely representing virus inoculum passing through the intestines. Collectively, these data indicated that SV-A could induce diarrhea and fecal shedding in piglets but not in chicks.

The SV-A strain caused intestinal and extra-intestinal lesions in piglets but not in chicks

We then assessed the histopathological changes in organs and tissues sequentially sampled from mock- or SV-A-inoculated piglets and chicks. SV-A infection resulted in histopathological changes in the small intestines of virus-inoculated piglets, including villous atrophy and crypt hyperplasia at 1 dpi (Table 1). These mucosal changes were gradually increased in all regions of the small intestine until 5 dpi followed by a decrease at 7 dpi (Fig. 2 and Table 1). Large intestinal lesions including crypt fusion with epithelial cell hyperplasia was observed at 2 dpi, increased until 5 dpi followed by a decrease at 7 dpi (Fig. 2). Lungs from infected piglets showed lymphoid cell infiltration in the peribronchiolar submucosa and perivascular space from 5 dpi to the end of the experiment (Fig. 3). As shown in Table 2, adaptive immune reactions to SV-A infection evident as perivascular cuffing of lymphocytes and gliosis were observed in both gray and white matters of spinal cord (myelitis) and brain (encephalitis) (Fig. 3). These typical host defence reactions were observed from 7 dpi until the termination of the experiment. Neuronophagia and chromatolysis were not frequently found in the spinal cord or the brain. However, spinal cords showed stronger inflammatory reactions than the brain (Table 2). No specific lesion was observed in other organs or tissues collected from piglets regardless of SV-A infection. However, SV-A did not induce any histopathological change in any organ or tissue sequentially sampled from SV-A-inoculated chicks during the entire experimental period (Fig. 4). These data indicated that SV-A could induce pathology in piglets but not in chicks.

Viral antigen was detected only in the intestine of piglets but not chicks

To assess the distributions of SV-A antigen in the organs and tissues, immunohistochemical assay was performed with organs and tissues sampled sequentially from mock- or SV-A-infected piglets and chicks using monoclonal antibody (Mab) specific to SV-A capsid protein. SV-A antigen was only detected in the epithelial cells of villi from SV-A infected piglets at 1 dpi to 5 dpi (Fig. 2 and Table 1). Other organs and tissues collected from mock- or SV-A-inoculated piglets were negative for SV-A antigen (Fig. 3 and Table 2). Consistent with clinical and histopathological observations, SV-A antigen was not detected in any organ or tissue collected from SV-A-inoculated chicks (Fig. 4).

The SV-A caused viremia and replicated in extra-intestinal organs of piglets but not chicks

To assess whether SV-A induced viremia and replicated in extra-intestinal organs of piglets and chicks, qRT-PCR assay was performed with sera and extra-intestinal organs and tissues collected from mock- or SV-A-inoculated piglets and chicks. SV-A RNA levels were relatively low in the sera, spinal cord, lung, and brain in comparison with those in the fecal samples collected from SV-A-inoculated piglets (Fig. 1a). However, SV-A RNA was not detected in the sera and any extra-intestinal organs and tissues collected from mock- or SV-A-inoculated chicks. These data indicated that SV-A induced viremia which was then disseminated to extra-intestinal organs and tissues in piglets but not chicks.

Binding and infection abilities of SV-A to various cells

To determine whether SV-A had a strict tropism for porcine cells *in vitro*, the binding and infection ability of SV-A was examined with various cell lines, including porcine,

human, chicken embryo, canine, simian, hamster and feline. Alexa fluor 594 (AF594)-labelled SV-A attached to all cell lines at various degrees (Fig. 5a). Radio-labelled SV-A was able to bind to all cells examined at similar degrees (Fig. 5b). However, SV-A was only replicated in cells of porcine origin (LLC-PK and PK-15) (Fig. 5c) with similar levels of cytopathic effect (data not shown). The SV-A genome copy numbers robustly increased in cells of porcine origin in time dependent manner but not in cells of other species (Fig. 5d). Our results indicated that only porcine cells were permissible for SV-A infection.

DISCUSSION

All viruses initiate infection by binding to specific receptor(s) on the surface of susceptible host cells (Neu *et al*., 2011). We have previously demonstrated that SV-A could utilize α2,3-linked SA on GD1a glycolipid as a receptor (Kim *et al*., 2016). Indeed, glycolipid associated α2,3-terminal SA is abundant on the cell surface of both avian and porcine species (de Graaf & Fouchier, 2014; Raman *et al*., 2014). However, our results in this study revealed that SV-A infection was limited to cells of porcine origin. It could not infect cells of other origins including chickens and humans. Moreover, SV-A was replicated in piglets but not chicks, confirming that SV-A could not cause interspecies transmission at least between pigs and chickens. Our results also suggested that other factors in addition to glycolipid associated α2,3-terminal SA might be required for efficient SV-A replication in cells of non-porcine origin. We have previously observed that chymotrypsin or trypsin treatment to cells has no effect on virus infection (Kim *et al*., 2016), suggesting that cell surface associated proteins do not play accessory roles in SV-A infection. The nature of post-binding block to infection in cells from non-porcine origin is as yet unknown but it is possible the SV-A infection of avian cells induces the innate response leading to the restriction of viral replication.

Although previous studies have indicated that SV-A can cause enteritis, pneumonia, polioencephalomyelitis and reproductive disorders (Alexandersen *et al*., 2012; Huang *et al*., 1980; Lamont & Betts, 1960; Lan *et al*., 2011; Schock *et al*., 2014; Sibalin, 1963), the pathogenicity and/or host range restriction of SV-A have been poorly characterized. In the current study, the most significant lesions were found in the intestines during the early infection period, where severe villous atrophy was found to be associated with high viral RNA loads in the fecal samples and strong SV-

A-antigen reactivity in the intestinal epithelial cells. In contrast, the spinal cord and brain mainly showed signs of adaptive immune response (non suppurative inflammation) and comparatively low viral RNA copy numbers without clear evidence of SV-A-antigen positive cells. These data indicated that the Korean SV-A strain (KS04105) used in this study was enteropathogenic. Opposite to our data, a recent study has reported that neuroinvasive English SV-A strain's replication in the spinal cord could lead to severe adaptive immune responses (non suppurative polioencephalomyelitis) without causing lesions in other tissues (Schock *et al*., 2014). This English SV-A strain (G5) is phylogenetically closer to English V13 strain than to other SV-A strains circulating recently in Korea and China (Son *et al*., 2014a; Schock *et al*., 2014). The molecular basis involved in the differences in virulence and tropism among SV-A strains remains unknown. However, there is clear evidence showing that variation in RNA structures can contribute to viral virulence (Son *et al*., 2014a). An additional explanation for discordance in tropism may be due to different age of animals used in studies. The English SV-A study used grower pigs (Schock *et al*., 2014), whereas this study used neonatal piglets. Further studies are required to examine the predilections of age and inoculation routes as well as antigenic and genomic differences so that our understanding on variations in SV-A pathogenesis and/or host range restriction can be improved.

Similar to other enteric viruses (Blutt & Conner, 2007; Park *et al*., 2007), SV-A RNA loads were detected in the sera of piglets orally inoculated with SV-A strain in this study, suggesting that SV-A might be able to penetrate the gut barrier from the lumenal side through destruction of enterocytes in the villi. This result also implied that SV-A could reach other organs and tissues via cell free transmission. However,

the mechanism by which SV-A reaches the blood and spreads to other organs and tissues remains to be determined.

In conclusion, this study demonstrated that the Korean SV-A strain could be replicated and induced pathology in piglets but not chicks. Our data also indicated that this Korean SV-A strain could reach the bloodstream from the gut, and be disseminated to extra-intestinal organs and tissues. These results will improve our understanding on life cycle and pathogenesis of sapeovirus so that affordable, useful, and efficient drugs could be developed for anti- sapelovirus therapy.

METHODS

Cells and viruses. LLC-PK, PK-15, and human cervical cancer HeLa cells [American Type Culture Collection (ATCC)] were maintained in Eagle's medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Chinese hamster ovary (CHO) cells were kindly provided by Dr. Sun-Young Im, Chonnam National University, South Korea. Crandall-Reese feline kidney (CRFK), Madin-Darby canine kidney (MDCK), African green monkey kidney Vero and MA-104, human embryonic kidney 293T (HEK293T), and human lung adenocarcinoma cell line A549 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MA-104 cells (ATCC) were cultured in alpha minimum essential medium supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human lung fibroblast WI-38 cells (ATCC) were maintained in DMEM supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary chicken embryo cells [including primary chicken embryo fibroblast (CEF), kidney (CEK), and intestine (CEI) cells from 12-day-old specific pathogen free (SPF) White Leghorn fetus] were grown in M199 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

SV-A strain KS04105 used in this study were isolated from fecal samples of diarrheic piglets of South Korea (Son *et al*., 2014a,b). This strain was passaged eight times in LLC-PK cells, including isolation, adaptation, and triple plaque purification. Isolated viruses were confirmed as PSVs based on immunofluorescence assay (IFA), reverse transcription-polymerase chain reaction and transmission electron microscopy (Kim *et al*., 2016; Son *et al*., 2014a,b).

Reagents and antibodies. AF-594 succinimidyl ester purchased from Molecular Probes (catalog number, A-20004) was dissolved in dimethyl sulfoxide (DMSO). The Mab against SV-A capsid protein was kindly provided by Dr. M. Dauber (Friedrich-Loeffler Institute, Germany). Fluorescein isothiocynate-conjugated goat anti-mouse IgG antibody and peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated streptavidin was obtained from Jackson Immuno Research Lab. SlowFade Gold Antifade Reagent (Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen.

Animal experiments. To evaluate the pathogenicity and host range restriction of SV-A, 3-day-old piglets (n = 27) from sows by hysterectomy and 3-day-old SPF White Leghorn chicks (n = 27) were used. Twenty-four piglets and 24 chicks were orally inoculated with 20 ml (1 x 10^8 PFU/ml) or 5 ml (1 x 10^8 PFU/ml) of the KS04105 strain individually. As negative controls, piglets and chicks were inoculated with the same volume of medium for mock-infection. All animals were fed with sterilized commercialized milk or feed. After the inoculation, clinical signs including diarrhea, pneumonia, and convulsion were evaluated daily as described previously (Park *et al*., 2013). Animals were euthanized at specified times (Table 1).

Necropsy was immediately performed after euthanasia. During necropsy, organs and tissues including each intestinal segment, spinal cords, brain, lung, and liver were excised from piglets or chicks. They were immediately placed in 10% buffered formalin for histological examination. Formalin-fixed samples were embedded in paraffin, sectioned, stained with Mayer’s hematoxylin and eosin, and examined microscopically (Park *et al*., 2013). All samples collected for qRT-PCR analysis were

281 immediately snap-frozen in liquid nitrogen and kept at -80℃ until use.

282

283 **Immunohistochemistry.** The distribution of SV-A antigens in tissues was evaluated
284 through immunohistochemical examination using paraffin-embedded sections and a
285 Mab against SV-A capsid protein as described previously (Park *et al*., 2007). Briefly,
286 paraffin-embedded sections of each organ and tissue were deparaffinized and
287 rehydrated through a graded series of alcohol in 0.1 M PBS and then treated with 0.1%
288 trypsin-0.1% calcium chloride in PBS for 1 hr at 37˚C. Trypsinized sections were first
289 treated with 3% H_2O_2 to quench endogenous peroxidase and then incubated with a
290 Mab against SV-A capsid protein at 4˚C overnight. All sections were stained with
291 peroxidase-labelled streptavidin-biotin (SAB-PO) using a Histofine SAB-PO kit for
292 the mouse Mab. Antigen localization was visualized by incubating the sections with
293 3,3'-diamonobenzidine-H_2O_2 solution. The sections were then weakly counterstained
294 with hematoxylin. To calculate the number of antigen-positive cells in the organs or
295 tissues, 10 fields per section were analyzed, using a 40x objective and a 10x
296 eyepiece, yielding a final magnification of 400x.

297

298 **Virus purification by CsCl gradient centrifugation.** SV-A strain KS04105 grown in
299 LLC-PK cells was purified using cesium chloride (CsCl) gradient centrifugation as
300 described previously (Kim *et al*., 2016). Briefly, infected cell cultures were harvested
301 at 72 hr post-inoculation and freeze-thawed three times. Cell debris was spun down
302 at 2,469 x g for 10 min at 4℃. A total of 500 ml of virus-containing supernatants was
303 concentrated by centrifugation at 245,853 x g for 20 hr at 4℃ using a SW40 rotor
304 (Beckman). The viruses in the pellets were resuspended in TNE buffer (50 mM Tris-

HCl, 100 mM NaCl, 100 mM EDTA, pH 7.5). The suspension was then layered over discontinuous CsCl gradients. After ultracentrifugation, the virus band was collected by puncturing the side of the tube with a needle. The virus solution was then diluted in distilled water and further purified by ultracentrifugation. Purified viruses were dialysed in 0.1 M sodium bicarbonate buffer (pH 8.3) for fluorescence labelling or in TNE buffer for radioactivity assay overnight. Purified viruses were then stored in aliquots at –80℃.

Labelling of viruses with AF-594. Labelling of viruses with AF-594 was performed as described previously (Kim *et al*., 2016). Briefly, purified virus (10 mg at 1 mg ml^{-1}) in 0.1 M sodium bicarbonate buffer (pH 8.3) was labelled with one tenth fold-molar concentration of AF-594 succinimidyl ester (1 mg at 1 mg ml^{-1} in DMSO). Each reaction was mixed thoroughly by vortexing for 30 sec and incubated at room temperature for 1 hr with continuous stirring. Labelled virus was repurified with CsCl as described above, dialysed against virion buffer, and stored in 2-μg aliquots at –20℃.

Dye-labelled binding assay. Dye-labelled binding assay was performed with purified AF-594 labelled-viruses as described previously (Kim *et al*., 2016). Briefly, mock-infected or treated cells were inoculated with multiplicity of infection (MOI) of 1000 of AF-594 labelled-virus and incubated on ice for 5 min followed by incubation at room temperature for 10 min. Cells were washed extensively with cold PBS, fixed with 4% formaldehyde, and washed three times with cold PBS. Dishes were mounted with SlowFade Gold Antifade Reagent containing DAPI solution for nucleus

staining. Infected cells were observed under a LSM 510 confocal microscope and analysed using LSM software (Carl Zeiss).

Labelling of viruses with ^{35}[S]methionine/cysteine. Labelling of viruses with ^{35}[S]methionine/cysteine (PerkinElmer) was carried out as described previously (Kim *et al*., 2016). Briefly, confluent monolayers of cells were infected with SV-A strain KS04105 at a MOI of 0.1 PFU/cell at 37℃ for 4 hr. The medium was replaced with RPMI 1640 lacking methionine and cysteine (Sigma-Aldrich). After starving cell for 2 hr, cells were supplemented with 1 Mbq ^{35}[S]methionine/cysteine (PerkinElmer) ml^{-1}. At 72 hr following virus infection, each labelled virus was purified by CsCl density-gradient centrifugation as described above.

Binding assay of ^{35}[S]methionine/cysteine labelled virus to various cell lines. Binding of ^{35}[S]methionine/cysteine labelled virus to various cell lines was assayed as described previously (Kim *et al*., 2016). Briefly, cells (4×10^4) were plated into 96-well plates. Purified ^{35}[S]methionine/cysteine-labelled virus (50,000 c.p.m.) was incubated with cells on ice for 45 min. Cells were washed three times with ice-cold PBS followed by cell lysis with 0.1% sodium dodecyl sulphate and 0.1 M NaOH. Total radioactivity in the cell lysate was determined by liquid scintillation counting.

Infectivity assay. Infectivity assay of SV-A strain KS04105 in various cell lines was carried out as described previously (Kim *et al*., 2016). Briefly, confluent monolayers of each cell line on confocal dish were infected with SV-A strain KS04105 at a MOI of 1 PFU/cell and incubated at 37℃ for 1 hr. Cells were washed three times with

PBS and replaced with maintenance medium. Cells were incubated at 37℃ for 15 hr prior to being fixed with 4% formaldehyde in PBS. They were subjected to immunofluorescence assay as described below.

Immunofluorescence assay. Immunofluorescence assay was performed as previously reported (Kim *et al*., 2016). Briefly, fixed cells on confocal dish were permeabilized by 0.2% Triton X-100, incubated at room temperature for 10 min, and washed with PBS containing 0.1% new born calf serum (PBS-NCS). Mab against SV-A capsid protein (1:40 dilution) was added and incubated at 4℃ overnight. Cells were then washed three times with PBS-NCS. FITC-conjugated secondary antibody (diluted to 1:100) was then added. Nuclei were stained with propidium iodide. Cells were then examined through confocal microscopy.

SV-A qRT-PCR. SV-A genome RNA levels in the feces, sera and each organ or tissue were quantified by qRT-PCR as described previously with slight modifications (Chen *et al*., 2014; Park *et al*., 2013). Briefly, all tissues and fluid samples collected from experimental animals were individually weighed, homogenized or vortexed at a 1:10 dilution in 0.01 M PBS and centrifuged (tissues 13,000 × *g* for 3 min; fecal samples 5,000 × *g* for 10 min). The supernatants along with the remaining bulk samples were collected and stored at −80˚C for analysis. To quantitate SV-A genome copy numbers, cells were infected without or with SV-A strain KS04105 at a MOI of 1 PFU/cell and incubated at 37℃ for 4, 8, 15, 72 hr post-infection as described above. Each infected cell culture was freeze-thawed three times, and cell debris was spun down at 2,469 x g for 10 min at 4℃. After extracting total RNA from supernatants,

each real time RT-PCR reaction was performed using a Rotor-Gene Real-Tme Amplification system (Corbett Research, Mortlake, Australia) and SensiFAST SYBR Low-ROX One-Step Mix (Enzynomics Inc, Korea) in a final volume of 20 µl containing 10 µl of SensiFAST SYBR Low-ROX One-Step Mix (Enzynomics Inc, Korea), 0.2 µl of reverse transcriptase, 0.4 µl of RiboSafe RNase Inhibitor, 0.8 µl of PSV1 primer (GGCAGTAGCGTGGCGAGC at positions between 153-170 of the 5'UTR), 0.8 µl of PSV2 primer (CTACTCTCCTGTAACCAGT at positions between 242-260 of the 5'UTR), 4 µl of template, and 3.8 µl RNase free dH_2O. Reverse transcription was carried out at 42°C for 15 min followed by the activation of hot-start DNA polymerase at 95°C for 2 min and 45 cycles of 95°C for 10 sec, 60°C for 14 sec, and 72°C for 10 sec. Quantitation of virus RNA copies was carried out using a standard curve derived from 10-fold serial dilutions of *in vitro* transcripted complementary RNA (cRNA) amplified in separate PCR tubes. Rotorgene 6000® (Corbett Research) software was used to calculate the amount of SV-A specific RNA in the samples. The threshold was defined automatically in the initial exponential phase, reflecting the highest amplification rate. With regard to the crossing points resulting from the amplification curves and threshold, a direct relation between cycle number and log concentration of RNA molecules initially present in the RT-PCR reaction was evident. By linear regression analysis of these data, Rotorgene 6000® software was used to set up a standard curve to determine the concentration of RNA present in the samples.

Ethics statement. All animals were handled in strict accordance with good animal practices as described in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985, revised 1996). Our experiment protocol was

approved by the Committee on Ethics of Animal Experiments, CNU with permit number of CNU No. 2012-87.

Statistical analysis. All statistical analyses were performed using SPSS version 11.5.1 for Windows (SPSS, USA). One-way analysis of variance (ANOVA) was used to determine the statistical significance ($P < 0.05$).

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FIGURE LEGENDS

Fig. 1. SV-A viral RNA levels in feces (a), serum (b), spinal cord (c), brain (d), and lung (e) samples obtained from SV-A-inoculated piglets and chicks were determined by SYBR Green real-time RT-PCR. All experiments were performed three independent times. Error bars indicated SD from triplicate experiments. Dashed line indicates the limit of detection.

Fig. 2. Histological changes and antigen distribution in the intestine of piglets inoculated with or without SV-A. Small and large intestinal tissues collected from mock- or SV-A-inoculated piglets were examined histopathologically and immunohistochemically. Bars = 200 μm. Duo, Jej, Ile and Col are abbreviations of duodenum, jejunum and colon, respectively.

Fig. 3. Histological changes and antigen distribution in extra-intestinal organs and tissues of piglets inoculated with or without SV-A. Lung, brain, and spinal cord sampled collected from mock- or SV-A-inoculated piglets were examined histopathologically and immunohistochemically. Panels c, g, k, and o represented higher magnifications of samples shown in panels b, f, j, and n, respectively. Bars = 200 μm (a, b, d, e, f, h, i, j, l and p), 100 μm (m and n), or 50 μm (c, g, k and o).

Fig. 4. Histological changes and antigen distribution in intestinal and extra-intestinal organs and tissues collected from chicks inoculated with or without SV-A. Intestinal, lung, brain, and spinal cords isolated from mock- or SV-A-inoculated chicks were examined histopathologically and immunohistochemically. Bars = 100 μm (a, b, d, e, g, h, j and k) or 50 μm (c, f, i and l). Duo is an abbreviation of duodenum.

Fig. 5. SV-A binds to and infects cells of porcine origin. (a) Binding of AF-594-labelled mock or SV-A (50,000 c.p.m) to various cells from different species was observed by confocal microscopy. (b) Binding of ^{35}[S]Methionine/Cysteine-labelled mock or SV-A (50,000 c.p.m) to various cells from different species was measured by liquid scintillation counting. (c) Infectivity of SV-A to various cells from different species was determined by immunofluorescence assay using a mouse monoclonal antibody against SV-A VP1 protein at 15 hr post infection. (d) Quantification of SV-A genome copy numbers in various cells from different species was determined by SYBR Green real-time RT-PCR. All experiments were performed three independent times. Error bars indicated SD from triplicate experiments. Dashed line indicates the limit of detection.