Activation of COX-2/PGE_2 Promotes Sapovirus Replication via the Inhibition of Nitric Oxide Production

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ABSTRACT

Enteric caliciviruses in the genera *Norovirus* and *Sapovirus* are important pathogens that cause severe acute gastroenteritis in both humans and animals. Cyclooxygenases (COXs) and their final product prostaglandin E_2 (PGE_2) are known to play important roles in the modulation of both the host response to the infection and replicative cycle of several viruses. However, the precise mechanism(s) by which the COXs/PGE_2 pathway regulates sapoviruses replication remains largely unknown. In this study, the infection of porcine sapovirus (PSaV) Cowden strain, the only cultivable virus within the genus *Sapovirus*, markedly increased COX-2 mRNA and protein levels at 24 and 36 hours post-infection (hpi) with only a transient increase of COX-1 levels seen at 24 hpi. The treatment of cells with pharmacological inhibitors such as nonsteroidal anti-inflammatory drugs or siRNAs against COX-1 and COX-2 significantly reduced PGE_2 production as well as PSaV replication. Expression of the viral proteins VPg and ProPol was associated with the activation of the COXs/PGE_2 pathway. We observed that pharmacological inhibition of COX-2 dramatically increased NO production, causing a reduction in PSaV replication that could be restored by inhibition of nitric oxide synthase via the inhibitor N-nitro-L-Methyl-Arginine-ester. This study has identified pivotal role for the COXs/PGE_2 pathway in the regulation of NO production during the

46 sapovirus life cycle, providing new insights into the life cycle of these poorly
47 characterized family of viruses. Our findings also reveal potential new targets for
48 treatment of sapovirus infection.
49

IMPORTANCE

Sapoviruses are one of major etiological agents of acute gastroenteritis in both humans and animals, but little is known about sapovirus host factor requirements. Here, using only cultivable porcine sapovirus (PSaV) Cowden strain, we demonstrate that PSaV induced the vitalization of cyclooxygenases (COXs) and prostaglandin E_2 (PGE_2) pathway. Targeting of COX1/2 using nonsteroidal anti-inflammatory drugs (NSAIDs) such as the COX-1/2 inhibitor indomethacin and the COX-2 specific inhibitors NS-398 and celecoxib or siRNAs targeting COXs, inhibited PSaV replication. Expression of the viral proteins VPg and ProPol was associated with the activation of the COXs/PGE_2 pathway. We further demonstrate that the production of PGE_2 provides a protective effect against the antiviral effector mechanism of nitric oxide. Our findings uncover a new mechanism by which PSaV manipulates the host cell to provide an environment suitable for efficient viral growth, which in turn can be new targets for treatment of sapovirus infection.

64 Diarrhea is the second largest cause of mortality in children worldwide (1). Viruses
65 within the genera *Norovirus* and *Sapovirus* in the family of *Caliciviridae* are significant
66 cause of gastroenteritis in humans and animals with noroviruses alone causing ~200,000
67 deaths per annum in children <5 (2, 3). Despite their socioeconomic impact, the
68 fastidious nature of viruses within these genera has significantly hindered our
69 understanding of their life cycle and development of vaccines and therapeutics (4, 5).
70 Porcine sapovirus (PSaV) is the only cultivable member of the *Sapovirus* genus and
71 replicates in the presence of porcine intestinal contents or bile acids (4, 5). Therefore,
72 PSaV serves as a robust model for studies on the sapovirus life cycle and for the
73 development of therapeutic interventions (6).

74 The coexistence of viruses and their hosts imposes an evolutionary pressure on
75 both the virus and the host immune system. Therefore, viruses have evolved diverse
76 strategies to create a suitable environment conducive for their existence by either
77 activating or suppressing cellular pathways to facilitate replication. The
78 cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) pathway is one of the several host
79 pathways that participate in the modulation of the host response to the infection and the
80 replicative life cycle of viruses (7). For example, the activation of COX-2/PGE_2
81 pathway results in increased replication of cytomegalovirus (8, 9), but PGE_2 inhibits

82 viral replication of parainfluenza 3 virus and adenovirus (10, 11). COXs convert
83 arachidonic acid released by phospholipases A2- and C-mediated hydrolysis of plasma
84 membrane phospholipids following exposure to diverse physiological and pathological
85 stimuli into prostaglandins (PGs), prostacyclines, and thromboxanes (12, 13). Three
86 forms of COXs have been identified to date with COX-1 and COX-2 being the most
87 widely studied. COX-1 is constitutively expressed and known to synthesize various PGs
88 including PGE_2 that participate in a diverse range of normal physiological processes
89 such as cytoprotection of the gastric mucosa, the regulation of renal blood flow, bone
90 metabolism, nerve growth and development, wound healing, and platelet aggregation
91 (12, 13). In contrast, COX-2 is rapidly induced by various stimuli including viral
92 infection, and catalyzes the synthesis of various PGs including PGE_2 that have various
93 activities including pro-angiogenic or anti-apoptotic properties (12, 13). Some of the
94 biological effects of PGE_2 on immunity and inflammation are exerted through the
95 binding to G-protein coupled receptors on the plasma membrane called E prostanoid
96 receptors (14). PGE_2 is recognized as the major prostanoid produced in immune and
97 nonimmune cells, and acts as potent regulators of cell-cell interaction, antigen
98 presentation, cytokine production, differentiation, survival, apoptosis, and cell migration
99 (14).

This study sets to examine the potential role of COXs/PGE_2 pathway in the regulation of the sapovirus life cycle. We demonstrate that COXs/PGE_2 pathway is induced during PSaV replication and that this induction occurs following the expression of the viral VPg and protease-polymerase (ProPol) proteins. We further demonstrate that the production of PGE_2 provides a protective effect against the antiviral effector mechanism of nitric oxide (NO), uncovering a new mechanism by which enteropathogenic viruses manipulate the host cell to provide an environment suitable for efficient viral growth.

MATERIALS AND METHODS

Cell and virus. LLC-PK porcine kidney cells obtained from the American Type Culture Collection (ATCC, USA) were maintained in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The tissue culture-adapted PSaV Cowden strain was recovered from the full-length infectious clone pCV4A, and was propagated in LLC-PK cells with the supplement of bile acid (15).

Chemicals and antibodies. Celecoxib, NS-398, SC-58125, SC-236, nimesulide and SC-560 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Glycochenodeoxycholic acid (GCDCA), dimethyl sulfoxide (DMSO), L-NAME, MDL-12330A and indomethacin were from Sigma-Aldrich (St. Louis, MO, USA). COX-1 siRNA, COX-2 siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology, Inc., (CA, USA). Monoclonal antibody (Mab) against mouse COX-1 and polyclonal antibody against rabbit COX-2 were obtained from Abcam (Cambridge, MA, UK). Mouse Mab against hemagglutinin (HA) tag was purchased from OriGene (Rockville, MD, USA). Synthetic PGE_2 was purchased from Tocris Bioscience (Ellisville, MO, USA). The anti-PSaV capsid Mab and the anti-PSaV VPg polyclonal

126 antibody were previously described (16). Secondary antibodies used were horse radish
127 peroxidase conjugated goat immunoglobulin against rabbit IgG (Cell Signaling, Beverly,
128 MA., USA) and mouse IgG (Santa Cruz), and fluorescein isothiocyanate (FITC)
129 conjugated goat immunoglobulin against rabbit IgG (Jackson Immuno Research Lab,
130 PA., USA).
131
132 **Cytotoxicity assay.** The cytotoxicity of the chemicals used in this study was
133 determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
134 (MTT) assay (17, 18) as per the manufacturer's instructions. Briefly, cells in 96 well
135 plates were incubated with medium containing different concentration of various
136 chemicals for 24 hr. After removal of the media, 200 µl of MTT solution was added to
137 each well and incubated for 4 hr at 37℃ in a CO_2 incubator. Each well was added with
138 150 µl of DMSO and incubated at room temperature for 10 min. The absorbance was
139 read in an ELISA reader at 570 nm. The percentage of cell viability was calculated
140 using the following formula: $[OD_{(sample)} - OD_{(blank)} / (OD_{(control)} - OD_{(blank)}] \times 100$. Non-
141 toxic concentrations of each chemical were used in this study.
142
143 **Treatment of inhibitors and chemicals into LLC-PK cells.** LLC-PK cells

144 were grown in 6 or 12 well plates to attain the desired confluency. The chemicals and
145 inhibitors were dissolved in DMSO to make a 10 mM stock concentration. When
146 appropriate, a series of dilutions were made by diluting the appropriate volumes of
147 chemical or inhibitor stocks in EMEM. Treatment groups were typically as follows:
148 mock-treatment, pre-treatment, post-treatment, and pre-post-treatment. Confluent LLC-
149 PK cells were pre-treated with various concentrations of the inhibitors for 24 h. The
150 cells were washed with phosphate-buffered saline (PBS, pH 7.4) and inoculated with
151 PSaV at a multiplicity of infection (MOI) of 1 fluorescent focus unit (ffu)/cell. For post-
152 treatment groups, different concentrations of inhibitors were added in the maintenance
153 media after the virus adsorption step. For the pre-post treatment groups, LLC-PK cells
154 were pre-treated with different concentrations of inhibitors for 24 h. After removal of
155 inhibitors, the cells were washed twice with PBS and inoculated with PSaV. The
156 inhibitors were again added at the end of virus adsorption period.
157
158 **Plasmid constructs and cell culture.** Each of the regions coding for the PSaV
159 proteins, NS1, NS2, NS3, NS4, NS5, NS6-7, VP1, and VP2 (19) was amplified from the
160 full-length infectious clone pCV4A by PCR assays with primer pairs containing SalI
161 and NheI restriction enzyme sites (Table 1). Each forward primer specific for above

gene(s) had N-terminal HA tag sequences (Table 1). Each amplicon was purified by PuriGel (Invitrogen, Waltham, MA, USA) following the manufacturer's instruction and subcloned into pUNO cloning vector (InvivoGen, San Diego, CA, USA). All amplified regions were verified by Sanger sequencing. Plasmid constructs inserted with different viral genes or empty vector were individually transfected in LLC-PK cells grown in 6 well plates using the Lipofectamine® 2000 reagent (Invitrogen) following the manufacturer's instruction. Cell were harvested at different post-transfection points, and subjected to quantitative real-time PCR and Western blot analysis using the anti-HA antibody.

***In vitro* transcription and RNA transfection.** LLC-PK cells were seeded in 6-well or 24-well plates and transfected with 1 μg of capped *in vitro* transcribed PSaV RNA using Lipofectamine 2000® (Invitrogen). The capped *in vitro* transcripts were derived from the full-length PSaV cDNA clone pCV4A (20) by the mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA) following the manufacturer's instruction. Transfections were performed for 4 h and media was replenished with eagle's minimum essential medium (EMEM) supplemented with or without 200 μM GCDCA. After 6 days post transfections, cells were lysed, harvested and subjected to immunoblotting

and qPCR to analyze COX-1, COX-2 and PSaV VPg levels.

siRNA transfection. LLC-PK cells were cultured in 6 or 12 well culture plates at 70-80% confluency and transfected with siRNA (80 pmole of COX-1, COX-2, and scrambled control siRNA) using the Lipofectamine® 2000 reagent (Invitrogen) following the manufacturer's instruction. Cells were then infected with PSaV at a MOI of 1 ffu/cell. After 1 h, unabsorbed viruses were removed and the cells were maintained in EMEM with 2.5% FBS and 100 μM GCDCA. Cells were harvested at different time points and subjected to qPCR, median tissue culture infective dose ($TCID_{50}$) and Western blot analyses.

Preparation of cell extract and Western blot analysis. Confluent LLC-PK cells in 6 well plates infected with or without PSaV, treated with or without chemicals or inhibitors, transfected with or without siRNAs, or transfected with or without each gene construct of PSaV were harvested at different time points. The cells were washed twice with PBS and lysed with a cell extraction buffer (Invitrogen) supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Total cell lysates were denatured and resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gels. The

198 resolved proteins were transferred in nitrocellulose blotting membrane (Amersham
199 Protran, GE Healthcare Life science, Germany) and immunoblotted with primary
200 antibodies specific to COX-1, COX-2, glyceraldehyde 3-phosphate dehydrogenase
201 (GAPDH), HA or PSaV VPg. Secondary antibodies against rabbit or mouse IgG were
202 applied after the primary antibody. Immunoreactive bands were developed using the
203 enhanced chemoluminescence reaction kit (DoGen, Seoul, South Korea) and images
204 were taken using the Davinch-Western Imaging System (Young Ltd., Kang-nam, Seoul,
205 South Korea). To confirm equal protein loading, transferred blotting membranes were
206 also incubated with an antibody against GAPDH and its reactivity was compared with
207 the intensity of target bands. The quantification of the protein density for COX-1 and
208 COX-2 were performed using Image Studio Lite (LI-COR Biotechnology, Lincoln, NE,
209 USA) and was normalized to the corresponding density of GAPDH of the same samples.
210
211 **RNA isolation.** For quantifying intracellular RNA levels of signaling molecules,
212 mock- or PSaV-infected, chemical or inhibitor treated, or siRNA transfected cells were
213 washed twice with PBS, scraped and collected in clean microtubes. Samples were
214 centrifuged at 10,000 rpm for 10 mins and total RNA was isolated using the PureLink
215 RNA mini kit (Ambion Life technologies, Carlsbad, CA., USA) following the

manufacturer's instructions. For quantifying PSaV RNA, mock- or PSaV-infected, chemical or inhibitor treated, or siRNA transfected cells were freeze-thawed three times, and cell debris was spun down at 2,469 x g for 10 min at 4 ˚C. The supernatants along with the remaining bulk samples were collected and stored at −80˚C until used. Total RNA was extracted from supernatants using RNeasy kit (Qiagen) following the manufacturer's instructions. The RNA concentrations were spectrophotometrically determined at 260 nm using the BioPhotometer plus (Eppendorf, Hamburg, Germany).

Quantitative real-time PCR. cDNAs were prepared by using 1 μg of RNA and reverse transcribed using random hexamers (Promega, Madison, WI., USA). The oligonucleotide primers used in the quantitative real-time PCR were designed from the published sequences of COX-1, COX-2, and PSaV VPg (Table 1). Reactions were set up in 25 μl volumes containing 10 pmol of forward and reverse primers, cDNA, and TOPreal qPCR 2X PreMIX (Enzynomics, Daejon, South Korea). For COX-1 and COX-2, the amplification profile was as follows: 1 cycle of initial denaturation at 95ºC for 10 min, 45 cycles of denaturation at 95ºC for 10 s, primer annealing at 55ºC for 30 s and extension at 72ºC for 45 s. The amplification profile for VPg included 1 denaturation at 95ºC for 10 min, followed by 40 cycles of denaturation 95ºC for 10 s, primer annealing

234 at 60ºC for 20 s and extension at 72ºC for 20 s. Relative expressions of COX-1 and
235 COX-2 levels were calculated by the using $2^{-\Delta\Delta}$CT (21). Samples were normalized to
236 the quantity of β-actin gene. The copy number of the VPg gene was calculated using 10-
237 fold dilutions of known amount of pCV4A to generate the standard curve.
238
239 **$TCID_{50}$ assay.** The $TCID_{50}$ assay was performed as previously described (6).
240 Briefly, ten-fold serial dilutions of clarified virus supernatants were prepared in EMEM.
241 Of these dilutions, 200 µl was inoculated to monolayers of LLC-PK cells grown on 96-
242 well plates supplemented with 200 µM GCDCA and incubated at 37ºC in a 5% CO_2
243 incubator. Virus titers were calculated at 6 days post-infection and expressed as
244 $TCID_{50}$/ml values by the method of Reed and Muench (22).
245
246 **Determination of the infectivity titer by immunofluorescence assay.**
247 Infectivity assay was carried out as described previously (16). Briefly, confluent
248 monolayers of cells on the confocal dish were treated with various inhibitors or
249 chemicals as described above. Mock or treated cells were infected with PSaV at a MOI
250 of 1 ffu/cell and incubated at 37℃ for 1 hr. Cells were washed three times with PBS,
251 replaced with maintenance medium and then incubated for 36 hr at 37℃ prior to being

252 fixed with 4% formaldehyde in PBS.

253 Immunofluorescence assay was performed as previously reported (16). Briefly,
254 fixed cells in 8-well chamber slides were permeabilized by the addition of 0.2% Triton
255 X-100, incubated at room temperature for 10 min, and washed with PBS containing 0.1%
256 newborn calf serum (PBS-NCS). Chamber slides were added with anti-PSV capsid
257 (1:40 dilution) Mab and then incubated at 4℃ overnight. Cells were then washed three
258 times with PBS-NCS. FITC-conjugated goat secondary antibody (diluted to 1:100) was
259 then added. After washing with PBS, chambers were mounted with SlowFade Gold
260 antifade reagent (Life technologies, Eugene, OR, USA) containing DAPI solution for
261 nucleus staining. Infected cells were observed with a LSM 510 confocal microscope and
262 analysed using LSM software (Carl Zeiss). To calculate the percentage of antigen-
263 positive cells in each well, 1,000 cells in each well were counted, using a 40x objective
264 and a 10x eyepiece, yielding a final magnification of 400x. The percentage of antigen-
265 positive cells between mock- and drug-treated or scrambled RNA- and siRNA against
266 COX-2 gene was compared.

267

268 **Determination of NO concentration.** The concentration of NO was determined in
269 culture supernatant of mock- or PSaV-infected cells in the presence or absence of

chemicals or inhibitors by assaying nitrite, one of its stable end products. Collected supernatants were centrifuged to remove cell debris. The assay was done using the Griess reagent system (Promega, Madison, WI., USA) according to the manufacturer's instruction. Briefly, equal volume of each experimental sample and sulfanilamide solution were incubated at room temperature for 10 min. An equal volume of N-1-naphthylethylenediamine dihydrochloride solution was then added to all wells and incubated for 10 min. The absorbance was read at 540 nm in a plate reader. The nitrite concentration for each sample was determined by comparing it with a generated nitrite standard and calculated by linear regression analysis.

RESULTS

PSaV infection induces COXs expression and leads to the production of PGE_2. To determine whether the COXs/PGE_2 pathway was activated during PSaV replication, we examined the impact of PSaV replication on COX gene expression. COX-2 mRNA and protein levels were markedly piled up at 24 and 36 hours post-infection (hpi) concomitant with the increase in PSaV viral RNA and protein levels, whereas COX-1 levels were transiently increased at 24 hpi only (Fig. 1A–C). The level of PGE_2 in the infected cell culture supernatant was also significantly elevated from 12 hpi (Fig. 1D).

To confirm whether the observed increase in soluble PGE_2 was as a direct result of the PSaV-mediated induction of COX-1 and COX-2, the effect of selective or non-selective COX inhibitors on PGE_2 production was examined (Fig. 2). Importantly, all studies were performed at doses of inhibitors shown not to affect cell viability under the experimental conditions used (data not shown). The non-selective COX-1/2 inhibitor indomethacin and the selective COX-2 inhibitor NS-398 both inhibited PSaV-mediated PGE_2 production in a dose-dependent manner when added either immediately after the removal of the virus inoculum (Post treatment) or during the entire course of the infection (Pre-Post treatment) (Fig. 2A and B). In contrast, due to the reversible nature of the inhibitors, the pretreatment of cells and subsequent removal of the inhibitor prior

297 to the addition of PSaV had no effect on the levels of PGE_2 production (Fig. 2A and B).
298 Similar results were also obtained with a range of other COX inhibitors (Fig. 2C and D;
299 data not shown).

300 To further confirm a direct role for COX-1 and COX-2 in the production of PGE_2
301 during PSaV infection, the effect of COX-1 or COX-2 specific siRNAs was also
302 examined. Transfection of siRNA against COX-1 or COX-2 into LLC-PK cells reduced
303 expression levels of their respective target proteins. Although the inhibition of both
304 intracellular proteins was not complete by siRNA transfection, the levels of PGE_2
305 released from COX-1 and COX-2 siRNA-transfected cells were significantly reduced
306 (Fig. 2E and F). These results confirmed that the inductions of both COX enzymes are
307 responsible for the observed increase in PGE_2 from PSaV-infected cells. In addition, the
308 level of siRNA-mediated reduction of COX-1 and COX-2 was sufficient to significantly
309 reduce the production of PGE_2.

310

311 **Inhibition of both COX enzymes negatively regulates PSaV replication.** To
312 determine the impact of COX induction on PSaV replication, we examined the effect of
313 COX inhibitors and siRNAs on PSaV replication. Cells were treated with either
314 inhibitors (selective or nonselective) or transfected with siRNAs specific for COX-1 or

315 COX-2, and the effect on virus replication was monitored at 36 hpi by examining viral
316 titers as well as viral RNA and protein levels. There was no significant effect of COX
317 inhibitors on PSaV replication when cells were pre-treated but the inhibitors were
318 removed prior to infection (Pre) (Fig. 3A-H; Fig. 4A-D*)*. However, the inclusion of
319 COX inhibitors following the removal of the virus inoculum (Post) or during the entire
320 course of infection (Pre-Post) resulted in a significant reduction in PSaV replication (Fig.
321 3A-H, Fig. 4A-D). The COX-1 inhibitor SC-560 reduced the levels of PSaV RNA and
322 infectious virus by up to ~10 fold at 36 hpi (Fig. 3G-H). In contrast, COX-2 specific
323 inhibitors NS-398 and celecoxib, and non-selective COX-1/2 inhibitor indomethacin
324 showed a more significant effect, typically leading to a ~1000 fold decrease in virus
325 yield and RNA synthesis (Fig. 3A-F). Other specific COX-2 inhibitors (SC-58125, SC-
326 236, and nimesulide) were also tested; however less robust inhibition against PSaV
327 replication was observed (data not shown).

328 The effect of COX-specific siRNAs on PSaV replication was also examined (Fig.
329 3I-L; Fig. 4E and F*)*. Transfection of COX-2 specific siRNA had a more substantial
330 effect on PSaV replication than COX-1 siRNAs, causing an ~1000 fold reduction in
331 viral titer with a concomitant decrease in viral RNA levels and viral protein production
332 (Fig. 4E and F). Infection assays also demonstrated that treatment of cells with either

NS-398 or transfection with COX-2 specific siRNAs resulted in a significant decrease in the number PSaV antigen-positive cells (Fig. 4G). Combined these data suggest that both COX-1 and COX-2 enhance PSaV replication possibly via the increased production of PGE_2.

Supplementation of PGE_2 relieves the COX inhibition of PSaV replication. If the pro-viral effect of COX gene induction on PSaV replication was due solely to the production of increased PGE_2, then the addition of PGE_2 would be expected to reverse the inhibitory effect of COX inhibitors on virus replication. To examine this possibility, the ability of exogenous PGE_2 to restore PSaV replication after treatment with the non-selective COX-1/2 inhibitor indomethacin and the COX-2 specific inhibitor NS-398 was examined. The addition of exogenous PGE_2 led to a dose-dependent restoration of both PSaV infectivity and viral RNA levels in cells treated with either inhibitor (Fig. 5). These results confirmed that PGE_2, the final product of both COX enzymes, mediates the pro-viral effects of COX gene induction on PSaV replication.

Bile acid does not influence COX-2 expression during PSaV infection. PSaV replication in cell culture relies on the presence of bile acids including GCDCA in

351 the cell culture medium through a function that had previously been linked to an effect
352 of bile acids on the innate immune response to infection (15). However, recent studies
353 have indicated that this initial conclusion was incorrect as PSaV remains sensitive to the
354 type I interferon (IFN) response in the presence of bile acids (6) and that bile acids
355 function to promote virus uncoating (23). To determine whether bile acids have an
356 effect on the induction of COX-2/PGE_2 pathway during PSaV replication, the induction
357 of COX-2 and PGE_2 was examined in the presence or absence of GCDCA following the
358 transfection of *in vitro* transcribed and capped PSaV genome (Fig. 6). Transfected RNA
359 was used to bypass any role of GCDCA which may play during viral entry and
360 uncoating (23). We observed that COX-2 and PGE_2 were induced in cells transfected
361 with *in vitro* transcribed and capped PSaV genomic RNA, irrespective of whether
362 GCDCA was present or absent (Fig. 6A). As expected, the COX-2 inhibitor NS-398
363 reduced expression of COX-2 and PGE_2, and replication of PSaV (Fig. 6A-D). These
364 data indicate that induction of COX-2 and PGE_2 was a direct result of PSaV replication
365 and was not due to any supplementary effect of GCDCA.
366
367 **PSaV VPg and ProPol activate COX-2/PGE_2 pathway.** Given our
368 observation that PSaV replication was required for the induction of the COX-2/PGE_2

pathway, we investigated whether the expression of viral protein alone was sufficient. The LLC-PK cells were transfected with plasmid carrying each PSaV gene including NS1, NS2, NS3, NS4, NS5, NS6-7, VP1, and VP2 (19). Expression of each viral protein was confirmed via the detection of a HA tag fused to the N-terminus of each protein (Fig. 7A*)*. Western blotting and qPCR analysis of the levels of COX-1 and COX-2 demonstrated that VPg or ProPol expression significantly enhanced the expression of COX-2 and led to an increase in PGE_2 production (Fig. 7B-D).

PGE_2 blocks the antiviral effect of nitric oxide (NO). Previous studies have indicated that at least one of the effects of the prostaglandin production is the regulation of nitric oxide (NO) production (24). NO is a key molecule involved in the host defense mechanism against various pathogens including protozoans, parasites, fungi, bacteria, and viruses (25). NO also has a regulatory role at many stages of the development of inflammation (26). To determine if the PGE_2 produced during PSaV replication impacted NO production, we first examined the level of NO produced during PSaV replication. PSaV infected cells maintained low level of NO prior to 36 hpi when upon a significant increase in PGE_2 production was observed (Fig. 8A). These data suggested

386 that COX induction and the associated increase in PGE_2 production may play an
387 important role in the inhibition of NO production during PSaV replication.

388 To examine this possibility further, the effect of the COX-2 inhibitor NS-398 on
389 NO production during PSaV infection was also examined (Fig. 8B). Inhibition of COX-
390 2 activity was found to lead to a concomitant increase in NO production during PSaV
391 replication, which could be reversed in a dose-dependent manner by the addition of
392 nitric oxide synthase (NOS) inhibitor, L-NAME (Fig. 8B). The reversal of the effect of
393 COX-2 inhibition by L-NAME also resulted in a subsequent restoration of PSaV
394 infectivity levels as well as PSaV RNA and protein levels (Fig. 8C-E). Collectively,
395 these data suggest that the pro-viral effects of PGE_2 produced by both COX-1 and
396 COX-2 enzymes as a result of PSaV infection are mediated by the inhibition of the
397 antiviral effect of the NO.

DISCUSSION

During viral infection, numerous host inflammatory responses are induced, leading to the production of cellular effectors and soluble factors such as IFNs, PGE_2 and NO (27, 28). As obligate intracellular parasites, viruses must therefore subvert and/or avoid the host response to infection in order to complete their life cycle. As a result, pathogens including viruses have evolved a wide variety of mechanisms that enable the control of cellular pathways, evade the host immune response and hijack signaling pathways to facilitate viral replication and pathogenesis. Here, we investigated the potential role of the COXs/PGE_2 pathway in the PSaV life cycle and the control of infection. We found that the COXs/PGE_2 pathway was activated during the PSaV life cycle and that this activation had pro-viral effects via the inhibition of NO production. Furthermore, we determined that expression of the VPg and ProPol proteins of PSaV was sufficient to induce COX expression and PGE_2 production.

Among the soluble factors produced during the response of host cells to viral infection, NO is well known in the antiviral repertoire (29-32). In the immunological system, iNOS is induced by cytokines at the transcriptional level primarily in macrophages, neutrophils, epithelial cells, and hepatocytes where NO is produced in high concentrations (33, 34). Inducible NOS (iNOS), the enzyme responsible for the

production of NO, can be regulated by the COX/PGE_2 pathway (31). NO production is known to restrict the VSV infection (29), an effect that is similarly thought to be offset by the induction of PGE_2 production during VSV replication in vivo (24). Here, we showed that activation of the COX-2/PGE_2 pathway also enhances PSaV replication through an inhibitory effect on NO production.

NO production is significantly enhanced in patients suffering from gastroenteritis (30); this has been observed in norovirus or rotavirus infections in children (32). NO secretion can be triggered *in vitro* and *in vivo* by the rotavirus nonstructural protein 4 (NSP4) (34-36) which in turn may cause diarrhea by elevating intestinal permeability (37, 38), regulating intestinal motility (39) and intestinal ion transport (40, 41). In contrast to rotavirus infection (34-36), little NO production was observed during PSaV replication in the LLC-PK cells, possibly due to the synthesis of PGE_2, suggesting that NO may not be involved in sapovirus-induced diarrhea. However, it is important to note that we cannot fully rule out the potential role for NO in PSaV pathogenesis *in vivo*, where the cellular response to infection is influence by numerous cell types and may not be entirely reproduced in immortalized cells *in vitro*.

In a few instances, viral proteins have been identified to stimulate the activation of COXs/PGE_2 pathway, often by acting either directly or indirectly as a transcriptional

434 transactivator of COX-2 gene expression (42-45). Among the viral proteins for which
435 this activity has been reported, the hepatitis C virus (HCV) protein NS3, a viral serine
436 protease, is known to enhance COX-2/PGE_2 pathway by activating multiple signaling
437 pathways (46). In addition, the severe acute respiratory syndrome coronavirus (SARS-
438 CoV) nucleocapsid protein also activates the expression of COX-2/PGE_2 pathway by
439 binding directly to regulatory elements for NF-κB and CCAAT/enhancer binding
440 protein (45). In the present study, we demonstrated that expression of the PSaV VPg or
441 ProPol proteins in isolation was sufficient to lead to the activation of COX-2/ PGE_2
442 pathway. The mechanism behind this activation remains to be determined and is the
443 subject of future study.

444 In the present study, the levels of COX-1/2 mRNA and proteins were increased in
445 the response to PSaV infection so that we evaluated the effect of COX-1 specific, COX-
446 2 specific or non-selective COX-1/2 inhibitors on PSaV replication. Among these
447 inhibitors tested, COX-2 specific inhibitors NS-398 and celecoxib, and non-selective
448 COX-1/2 inhibitor indomethacin exerted stronger anti-PSaV effects than other inhibitors;
449 having a ~1000 fold decrease effect in virus yield and RNA synthesis. However, other
450 specific COX-2 inhibitors had less strong inhibitory effect against PSaV replication,
451 most likely due to their less potent activity and the fact that higher concentrations

452 required for effective COX inhibition resulted in cell toxicity observed during their use.
453 In addition, a more robust inhibitory effect of COX-2 specific inhibitor NS-398 and the
454 COX-1/2 nonselective inhibitor indomethacin on PSaV replication was observed in
455 post-treatment compared with the pre-post-treatment. The mechanism why post-
456 treatment of these inhibitors has stronger effect than pre-post-treatment remains
457 unknown. The one possibility is that it could be an off-target effect of using high dose of
458 these inhibitors in the LLC-PK cells, although there are no published reports of these
459 kinds of phenomena.

460 In the present study, we observed only a transient increase in COX-1 levels during
461 PSaV infection whereas COX-2 induction was more significantly induced and induction
462 was sustained during the later stage of the viral life cycle. However, the dramatic effect
463 of the non-selective COX inhibitor indomethacin, the COX-2 specific inhibitor NS-398
464 and the effect of COX-specific siRNAs support the hypothesis that both COX-1 and
465 COX-2 enzymes play a role to the PSaV replication.

466 In conclusion, our results demonstrate crucial role for the COXs/PGE_2 pathway in
467 the regulation of NO production during the PSaV replication, which provide an
468 environment suitable for efficient PSaV growth. In addition, our data would indicate
469 that pharmacological targeting of COX-2 could provide a potential targeting strategy for

the control of sapovirus infection facilitating the antiviral effect of NO production. Further studies are required to determine if targeting of the COXs/PGE_2 pathway *in vivo* has a negative impact on PSaV pathogenesis.

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635 **FIGURE LEGENDS**

636 **FIG 1** Induction of COX-1 and COX-2 by PSaV infection. (A and B) The expression of
637 COX-1, COX-2, and PSaV viral RNA in LLC-PK cells infected with PSaV (MOI = 1
638 ffu/cell) was quantified by real-time PCR (qPCR). In the case of COX-1 and COX-2,
639 expression levels were normalized to β-actin and are depicted as the fold induction
640 compared with that of the mock inoculated cells. (C) The levels of the VPg, COX-1,
641 COX-2 and GAPDH proteins were analyzed by Western blot. GAPDH was used as a
642 loading control. (D) The levels of PGE_2 in the supernatants harvested at 36 hours post-
643 infection (hpi) from PSaV-infected LLC-PK cells were determined by ELISA. The
644 levels of PGE_2 in the supernatants were compared between mock- and virus-inoculated
645 groups. Data are presented as means ± standard error of the mean from three
646 independent experiments. Differences were evaluated using the One-Way ANOVA.
647 $^{**}p<0.001$; $^{***}p<0.0001$.

648

649 **FIG 2** Effects of COX-2 inhibitors in PGE_2 production during PSaV infection (A-D)
650 LLC-PK cells were treated with selective COX-2 inhibitors (NS398 and celecoxib),
651 nonselective COX inhibitor (indomethacin), and selective COX-1 inhibitor (SC-560) as
652 indicated prior to the addition of the virus inoculum (MOI = 1 ffu/cell) and then

653 removed (Pre), after the addition of the virus inoculum then left for the duration of the
654 infection (Post) or treated prior to the addition of the inoculum as well as for the
655 duration of the infection (Pre-Post). The levels of PGE_2 in the supernatants harvested at
656 36 hpi were determined by ELISA. The levels of PGE_2 in the supernatants of virus-
657 infected cultures were compared between mock- and chemical-treated groups. (E-F)
658 Confluent of LLC-PK cells was transfected with siRNAs against COX-1, COX-2 or
659 scrambled-siRNA (Scram-siRNA) prior to infection with PSaV (MOI = 1 ffu/cell).
660 Supernatants were collected and ELISA was conducted to determine the PGE_2
661 concentration. The levels of PGE_2 in the supernatants were compared between mock-
662 and siRNA-transfected groups. (Inset) Western blot analysis for COX-1, COX-2 and
663 GAPDH was conducted with LLC-PK cells transfected with COX-1, COX-2 or
664 scrambled-siRNA (Scram-siRNA). Data are presented as means ± standard error of the
665 mean from three independent experiments. Differences were evaluated using the One-
666 Way ANOVA. $^{**}p<0.001$; $^{***}p<0.0001$.
667
668 **FIG 3** Inhibition of COXs attenuates PSaV replication. (A-F) LLC-PK cells were pre-
669 treated (Pre), post-treated (Post) or pre-post-treated (Pre-Post) with non-cytotoxic doses
670 of NS-398, indomethacin, celecoxib, and SC560. At 36 hours post-inoculation (hpi)

671 with PSaV (MOI = 1 ffu/cell), cells were harvested and the levels of viral RNA (A, C, E,
672 and G) and titer (B, D, F, and H) were determined by quantitative real-time PCR and
673 $TCID_{50}$, respectively. (I-L) LLC-PK cells were transfected with siRNAs against COX-1,
674 COX-2 or scrambled-siRNA (Scram-siRNA) before inoculation with PSaV (MOI = 1
675 ffu/cell). Samples were harvested at 36 hours post-inoculation (hpi) and the levels of
676 viral RNA (I and K) and titer (J and L) were determined by quantitative real-time PCR
677 and $TCID_{50}$, respectively. Data are displayed as the mean ± standard error of the mean
678 from three independent experiments. Differences were evaluated using the One-Way
679 ANOVA. *p<0.05, **p<0.001, ***p<0.0001.
680
681 **FIG 4** Effect of COX inhibitors or siRNA in PSaV replication. (A-D) LLC-PK cells
682 were pre-treated (Pre), post-treated (Post) or pre-post-treated (Pre-Post) with non-
683 cytotoxic doses of NS-398, indomethacin, celecoxib, and SC560. At 36 hours post-
684 inoculation (hpi) with PSaV (MOI = 1 ffu/cell), cells were harvested and the level of
685 protein of viral VPg was determined by Western blot analyses. (E-F) LLC-PK cells were
686 transfected with siRNAs against COX-1, COX-2 or scrambled-siRNA (Scram-siRNA)
687 before inoculation with PSaV (MOI = 1 ffu/cell). Samples were harvested and
688 processed as mentioned above. GAPDH was used as a loading control. (G) LLC-PK

689 cells were infected with PSaV (MOI = 1 ffu/cell) and the effect of the COX-2 inhibitor
690 NS-398 on viral antigen production was determined by confocal microscopy.
691
692 **FIG 5** Addition of exogenous PGE_2 reverses the effect of COX inhibitors on PSaV
693 replication. LLC-PK cells were infected with PSaV (MOI = 1 ffu/cell), treated with
694 non-cytotoxic doses of NS-398 or indomethacin and then supplemented with exogenous
695 PGE_2 in the maintenance media. After 36 hours post-infection, cells were harvested and
696 the levels of viral RNA synthesis (A and C) and titer (B and D) were determined by
697 quantitative real-time PCR and $TCID_{50}$, respectively. Data are represented as the means
698 ± standard error of the mean from three independent experiments. Differences were
699 evaluated using the One-Way ANOVA. *p<0.05; **p<0.001; ***p<0.001.
700
701 **FIG 6** Bile acid GCDCA does not influence the expression of COX-2 during PSaV
702 infection. LLC-PK cells were either infected with PSaV (MOI = 1 ffu/cell) or
703 transfected with one microgram of *in vitro* transcribed PSaV capped RNA, and the
704 effect of the COX-2 inhibitor NS-398 or the bile acid GCDCA were examined. Infected
705 cells were harvested at 36 hours post-infection whereas transfected cells were harvested
706 after 6 days post-transfection and subjected to Western blot analysis to assess viral

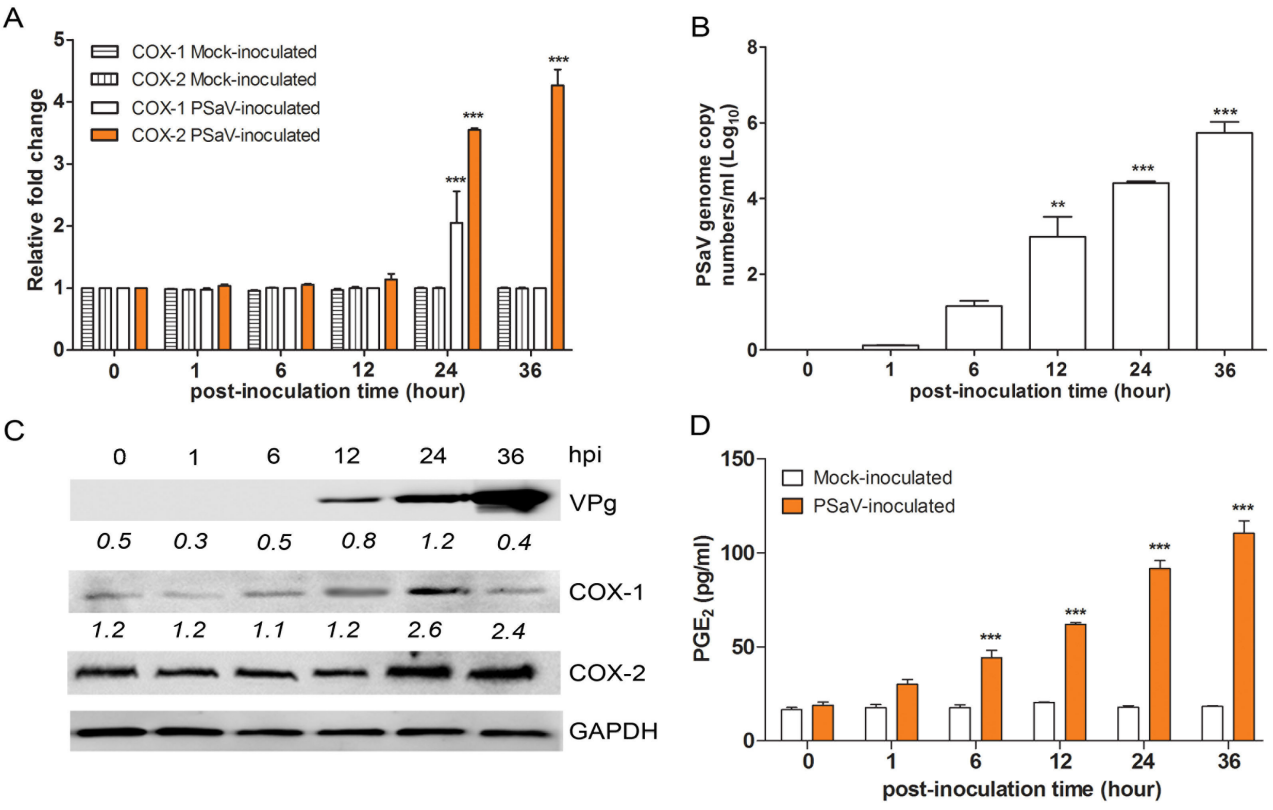
protein production (A), as well as to qPCR for viral RNA (B) and COX-2 (C). Supernatants were also collected for ELISA to quantify the levels of PGE_2 (D). Data are represented as the means ± standard error of the mean from three independent experiments. Differences were evaluated using the One-Way ANOVA. $^{**}p<0.001$; $^{***}p<0.001$.

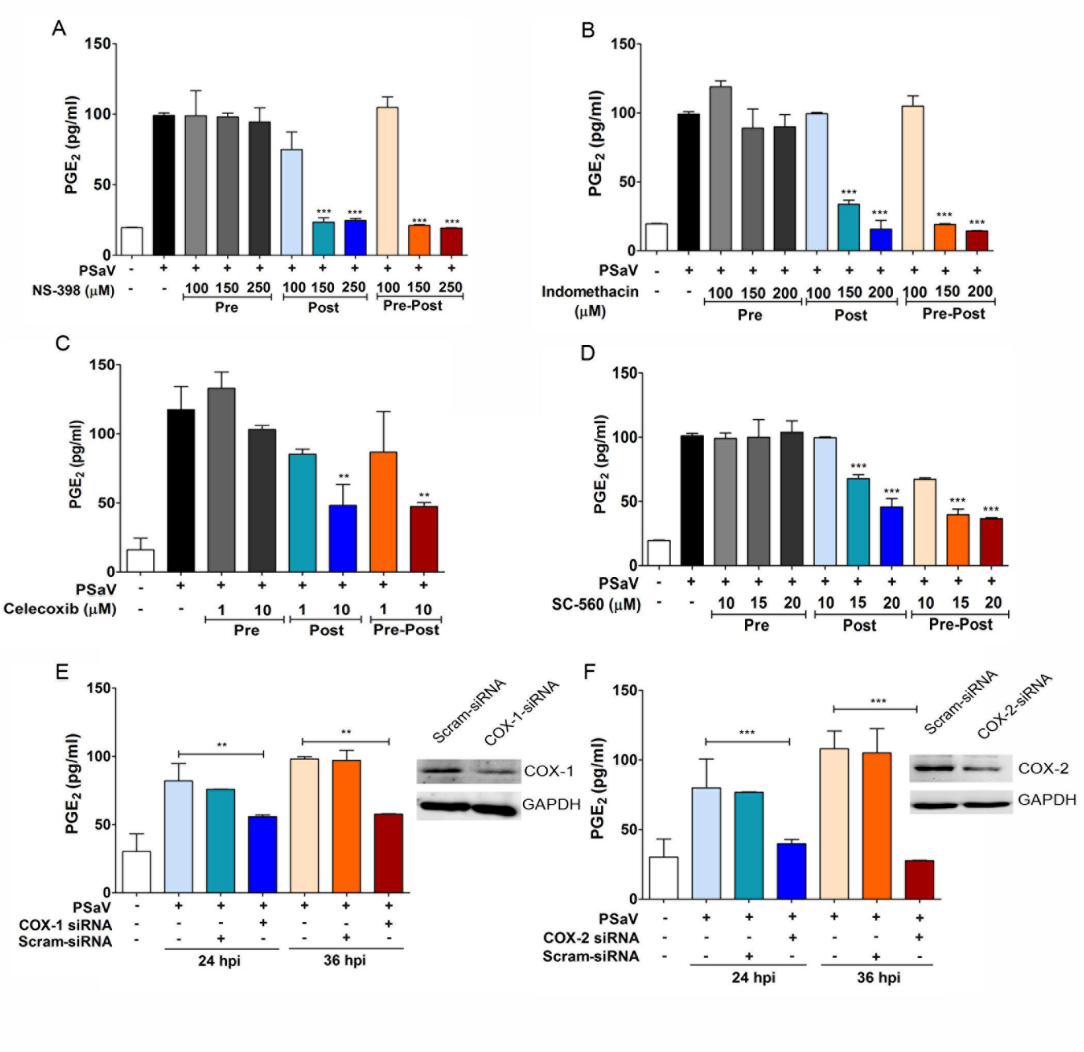
FIG 7 Role of PSaV proteins in stimulating COX-2 expression. LLC-PK cells were transfected with one microgram of pUNO plasmids containing each PSaV gene tagged with a HA epitope as indicated in the materials and methods section. As controls, pUNO empty or HA carrying (pUNO-HA) plasmids were transfected. At 36 hours post-transfection (hpt), cells were harvested and the expression levels of viral proteins (A) as well as COX-1 and COX-2 (B) proteins were determined by Western blot analysis. GAPDH was used as a loading control. In the case of COX-1 and COX-2, the expression levels were also quantified by real-time PCR, normalized to β-actin and depicted as the fold induction compared with that of the vehicle transfected cells (C). To determine the PGE_2 concentration, supernatants were collected and ELISA was conducted (D). The levels of PGE_2 in the supernatants were compared between vehicle- and PSaV gene-transfected groups. Data are presented as means ± standard error of the

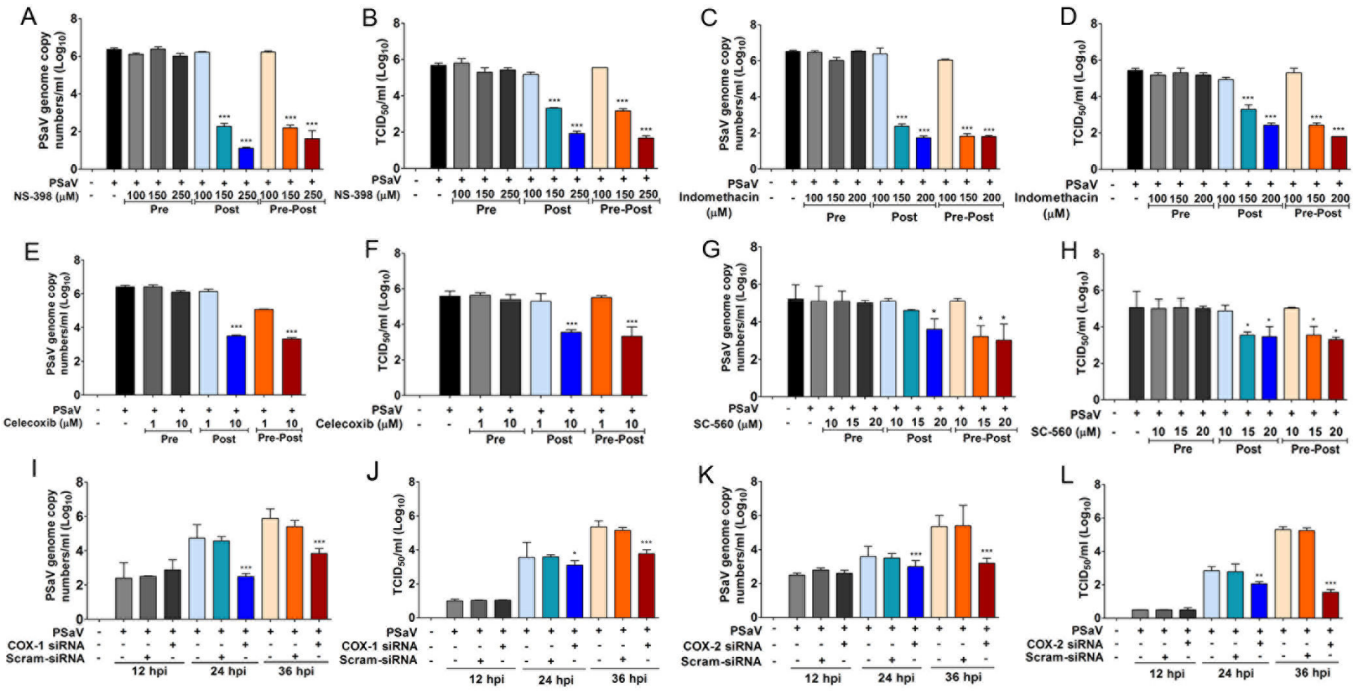
725 mean from three independent experiments. Differences were evaluated using the One-
726 Way ANOVA. ** $p<0.001$, ***$p<0.0001$.

727

728 **FIG 8** PGE_2 blocks the antiviral effect of nitric oxide (NO) for PSaV infection. (A)
729 Supernatants from mock- or PSaV-infected samples were collected and the nitrite
730 concentration was determined using the Griess reagent system as described in the
731 materials and methods section. LLC-PK cells were infected with PSaV (MOI = 1
732 ffu/cell) and subsequently treated with the COX-2 inhibitor NS-398 or the nitric oxide
733 synthase inhibitor L-NAME, either singularly or in combination. (B) The effect of
734 inhibitor treatment on nitric oxide production was then determined as described for
735 panel (A). The levels of viral titer (C), RNA (D), and protein (E) were determined by
736 $TCID_{50}$, real-time RT-PCR, and Western blot analyses, respectively. GAPDH served as
737 the loading control. Data are means ± standard error of the mean from three different
738 independent experiments. Differences were evaluated by One-Way Anova. ***$p<0.0001$.







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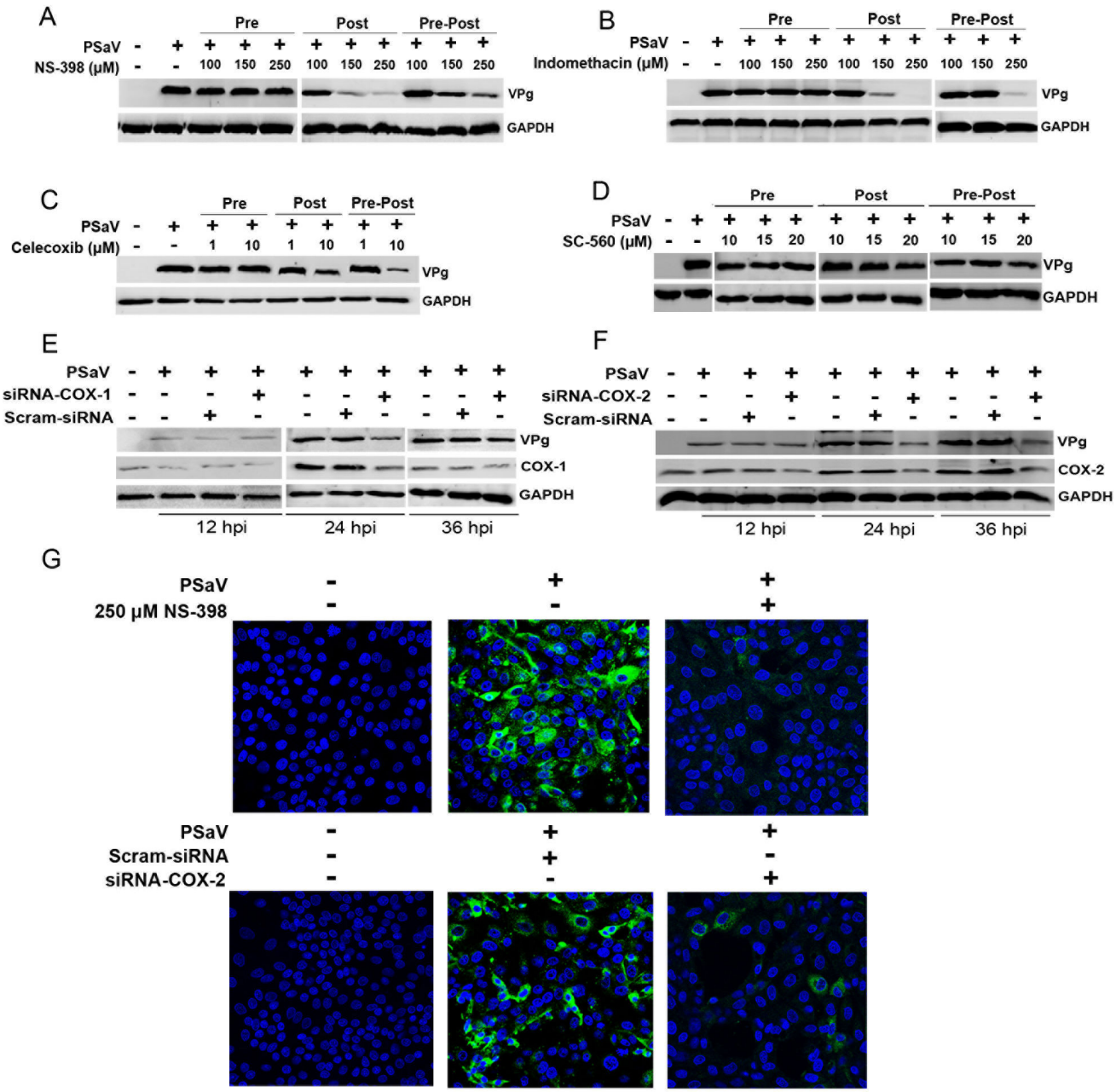

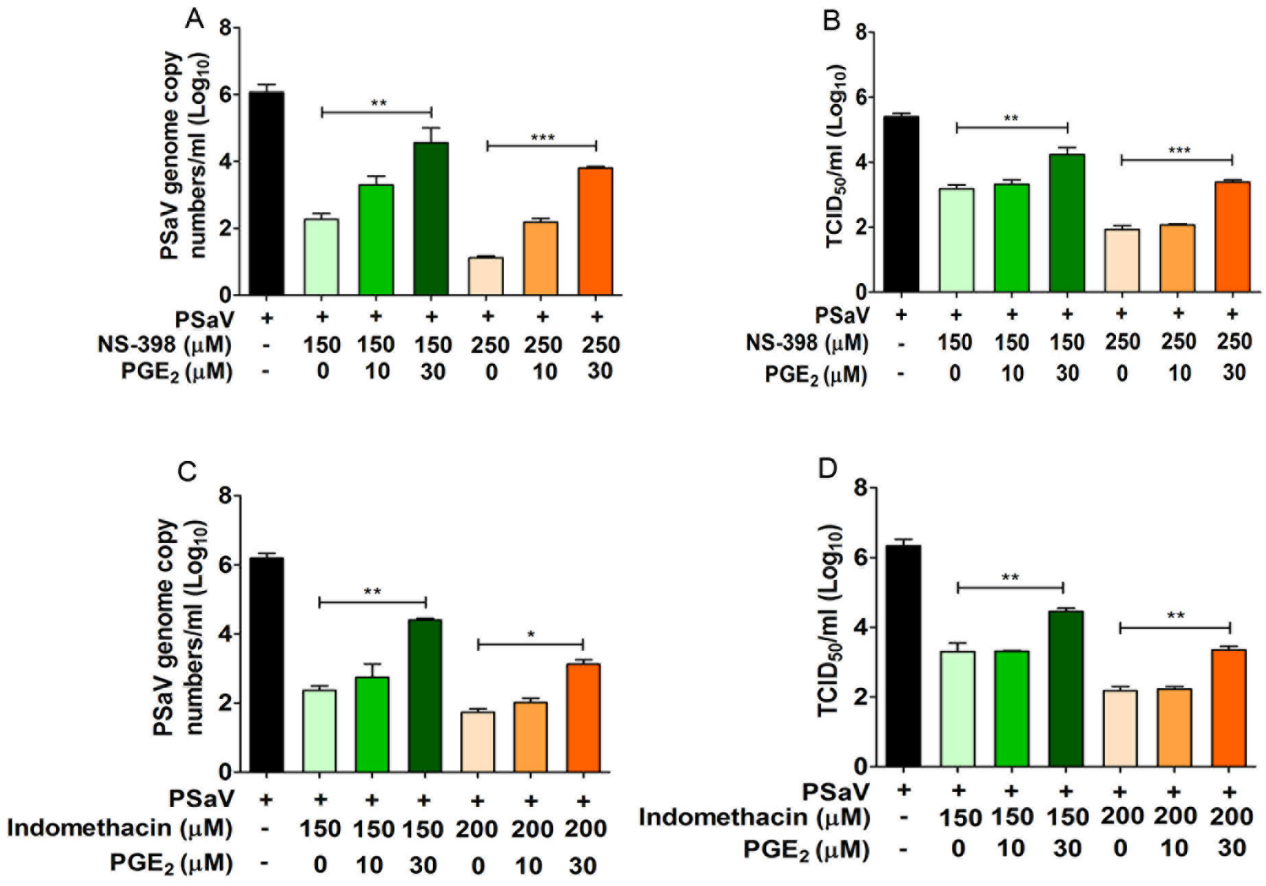

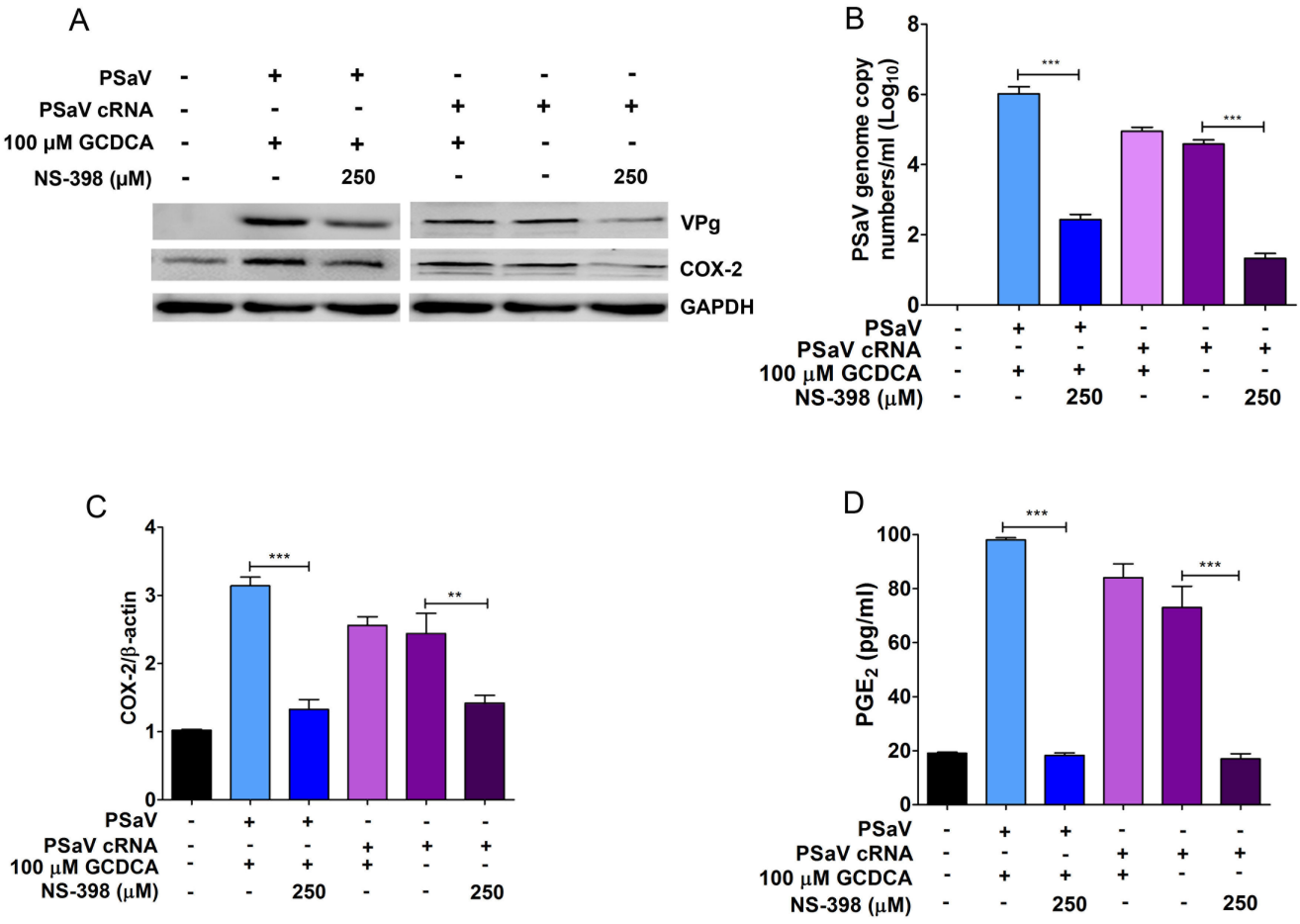

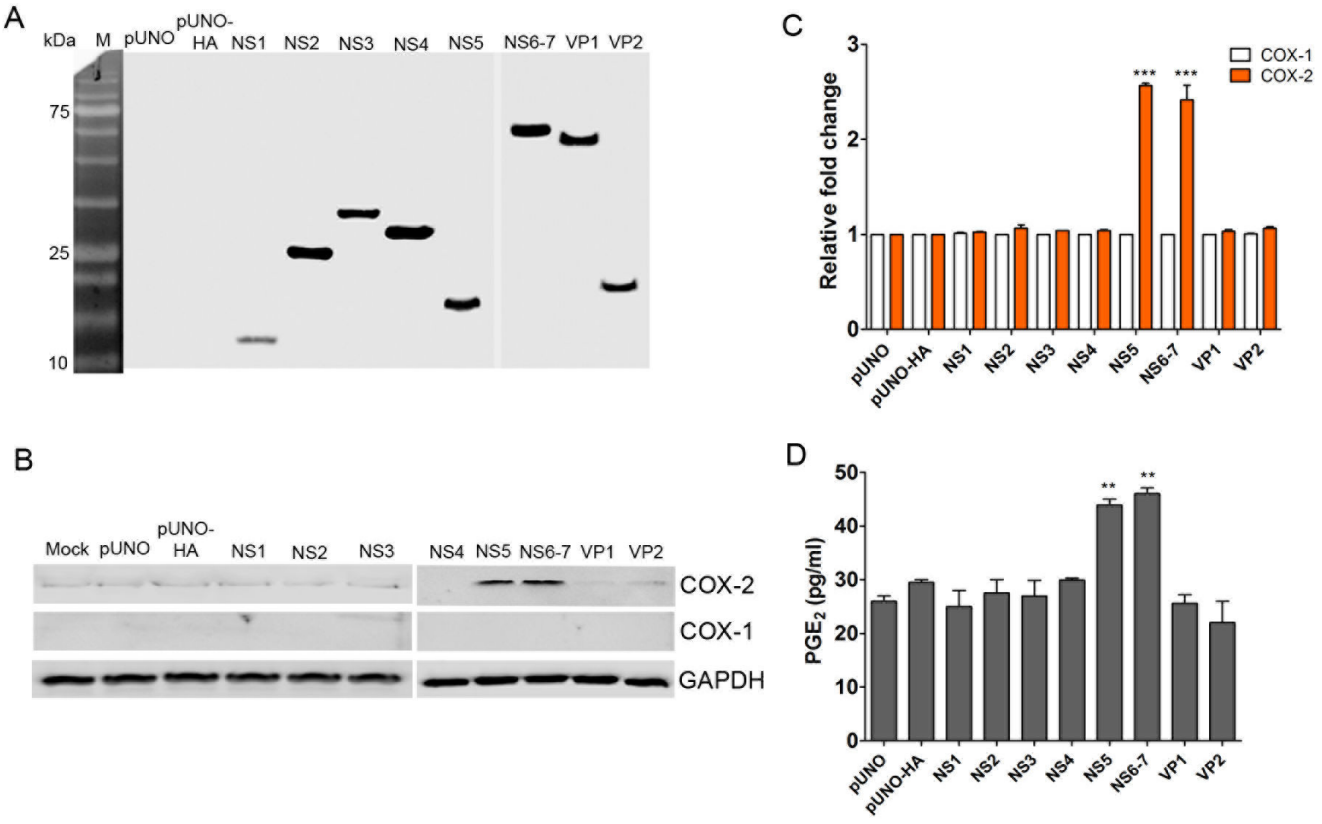

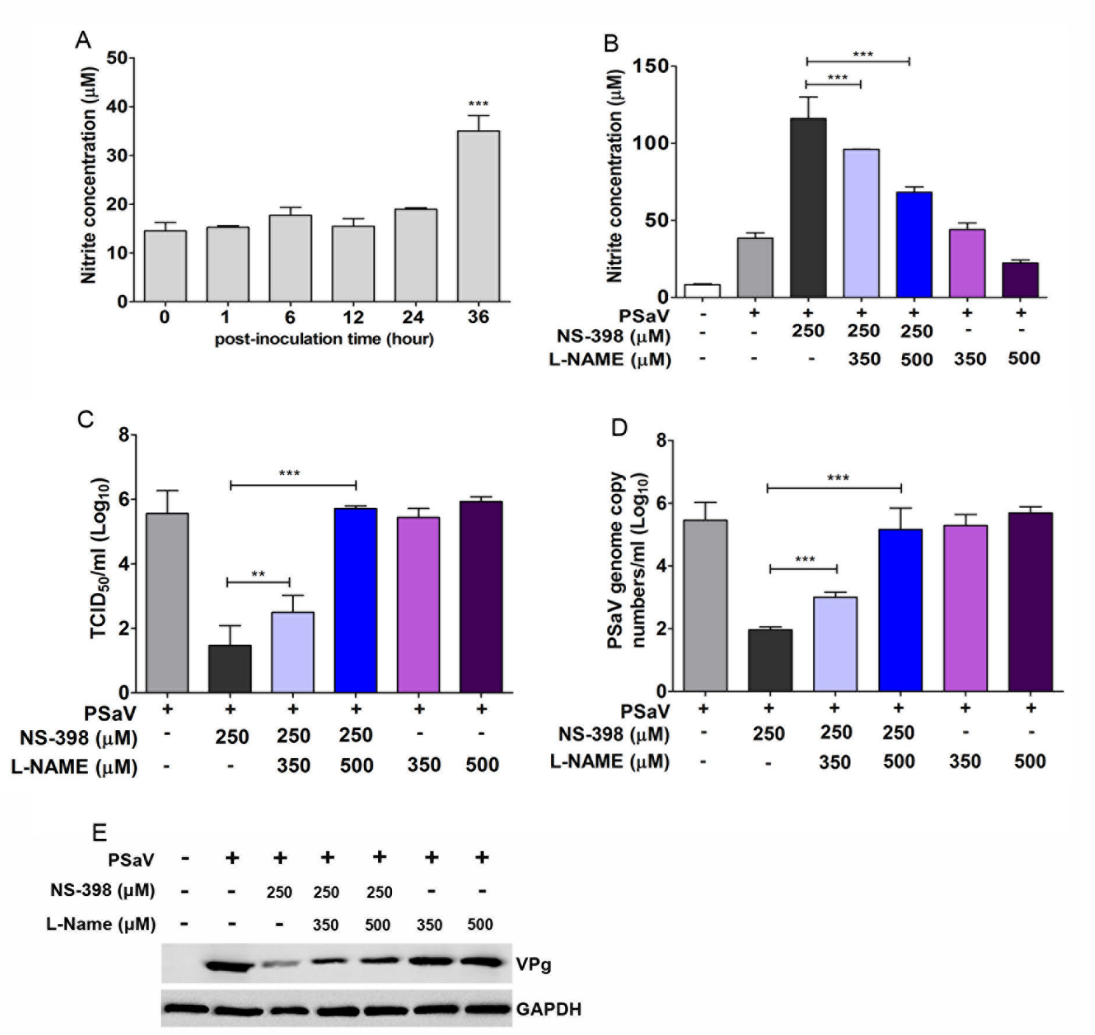

TABLE 1 Oligonucleotide primers used in this study

Target gene	Primer name	Sequence (5' -3')	Region (nt)	Size (bp)
p11	pUNO-HA-p11	F: gtg gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GCT AAT TGC CGT CCG TTG CCT ATT GGG	13-39	165
		R: atc gctagc TCA TTG CGC CAC AAA CAC GTC	177-160	
p28	pUNO-HA-p28	F: ttt gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GGG GTG GTG GAT GAT TTC TTC CGC CCC	178-204	762
		R: atc gctagc TCA CTG CGG CGT GTA GAG GC	939-923	
p35	pUNO-HA-p35	F: tac gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GCA GGC AAT GAT CTC ATC ATA TTG GGG	940-966	1017
		R: gac gctagc TCA CTC GCT GTT GTA CTT C	1956-1941	
p32	pUNO-HA-p32	F: tac gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GCC GCT GAT GTC AAA CAT CTA TGG TTC	1957-1983	855
		R: ttt gctagc TCA CTC GCT AAG CGT GTT TTC	2811-2794	
VPg	pUNO-HA-VPg	F: ac gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GCG AAA GGG AAA AAC AAA CGC GGA CGT	2812-2838	338
		R: gctagc TCA CTC ACT GTC ATA GGT GTC ACC TTT	3150-3133	
ProPol	pUNO-HA-ProPol	F: gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GGG CGT GGA TAC GTG GTA CCC ATG AC	3151-3177	1995
		R: gctagc TCACTCCATCACGAACACTTCTGGCTCTTC	5145-5128	
VP1	pUNO-HA-VP1	F: gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* GAG GCG CCT GCC CCA ACC CGT TCG GTT	5144-5169	1627
		R: gctagc TCATCGTGAGCTGTGAATGGACCTTCC	6771-6753	
VP2	pUNO-HA-VP2	F: ctc gtcgac **atg** *TAC CCA TAC GAT GTT CCA GAT TAC GCT* AGT TGG ATT GCA GGA GCA ATG CAG GGC	6774-6800	492
		R: agg gctagc TCA TCA CAC TTT GCT GTG AGT G	7265-7247	
VPg		F: CAA ACG CGG ACG TGG TGC TCG	2826-2846	145
		R: TGA TGC GCC TGA CAG TGC GCG	2970-2950	
*COX-1		F: CCG GAG GAA GTT CAT ACC TGA CCC	253-275	108
		R GCC AGG ACC CAT CTT GCC AGA	360-340	

**COX-2	F: CAC CCA TGG GTG TGA AAG GGA GG	181-203	201
	R: CCA AAG GAC AGG GCC ATG GGG	381-361	

Small underlined letters: restriction enzyme sites (Forward primer: SalI; Reverse primer: NheI).
Italic letters: polypeptide hemagglutinin sequences.
Bold letters: start codon.
*Derived from partial sequence of porcine COX-1; GenBank accession no. AF207823.1.
**Derived from partial sequence of porcine COX-2; GenBank accession no. AF207824.1.