

Gouleako and Herbert Viruses in Pigs, Republic of Korea, 2013

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Several viruses in the family *Bunyaviridae* are pathogenic to animals and cause vector-borne zoonoses. In 2013, investigation of cause of death of 9 pigs on 1 farm in the Republic of Korea found infection with Gouleako and Herbert viruses. Subsequent investigation revealed high prevalence of these viruses among pigs throughout the country.

Several viruses in the family *Bunyaviridae*, such as severe fever thrombocytopenia syndrome virus, sandfly fever Naples virus, and La Crosse virus, cause vector-borne zoonotic problems (1–7). Recently, outbreaks of severe disease caused by Rift Valley fever virus and Schmallenberg virus produced abortion storms, resulting in a high mortality rate among newborn lambs and calves (4,8). Gouleako virus (GOLV) and Herbert virus (HEBV) have been isolated from mosquitoes (*Culex* spp.) trapped in Côte d'Ivoire (9,10); however, their infectivity or virulence have not been proven. Investigation of the cause of death of pigs in the Republic of Korea identified GOLV and HEBV infection.

The Study

In March 27, 2013, a piglet, ≈8 weeks of age, on a 150-sow farm in Gyeonggi, Republic of Korea, died after onset of high fever (40°C), wasting, respiratory disease, and diarrhea. The carcass was sent to the Department of Veterinary Medicine Virology Laboratory, Seoul National University, Seoul, Republic of Korea, for diagnostics. Necropsy and microscopic examinations revealed greenish lung tissue with lymphoid depletion, consistent with severe broncho-

pneumonia. Despite the presence of multiple clinical signs, the results of routine tests for major pathogens in pigs (e.g., porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, transmissible gastroenteritis virus, porcine epidemic diarrhea virus, *Escherichia coli*, *Streptococcus* spp., and *Salmonella* spp.) were negative.

To further explore cause of the death, we used the particle-associated nucleic acid–random PCR method (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/20/12/13-1742-Techapp1.pdf>). Sequencing and BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) of the agent-specific amplicon simultaneously detected 2 viruses in lung tissue RNA samples. One partial sequence had 100% identity with 63 nt of the GOLV strain F23/CI/2004 glycoprotein gene (GenBank accession no. FJ765411). Another sequence had 97% similarity with 66 nt of the HEBV strain F23-K4 RNA-dependent RNA polymerase (*RdRp*) gene (GenBank accession no. EF423168).

Results were validated with reverse transcription PCR (RT-PCR) (online Technical Appendix). We obtained partial sequences of 235 nt of GOLV and 324 nt of HEBV. These sequences had 97.1% and 96.9% similarity with GOLV and HEBV, respectively, previously isolated from mosquitoes (9,10). The sequences were registered as GenBank accession nos. KF361520 and KF361522 and designated as GOLV/P1 and HEBV/P1, respectively.

During March–May 2013, we received a total of 9 dead pigs from the same farm; they had displayed various clinical signs. We further screened these pigs for the presence of GOLV and HEBV by using the same primer sets (online Technical Appendix) selective for their glycoprotein and *RdRp* genes, respectively. The results showed that the pigs were infected with GOLV and HEBV at a prevalence of 83.3% and 100%, respectively, mostly in lung samples (Table 1). The sequences obtained from this assay were registered as GenBank accession nos. KF361521 and KF361523 and designated GOLV/P8 and HEBV/P9, respectively.

Because of the high rate of GOLV positivity, we conducted a histopathologic RNA in situ hybridization study (online Technical Appendix). Hybridization signal was positive in lung and lymph node tissues and negative in intestine and control tissues. Hybridization was strong in the cytoplasm of mononuclear cells (deep blue color) (online Technical Appendix Figure 1).

Using the same RT-PCR method (online Technical Appendix), we investigated the prevalence of GOLV and HEBV in other swine populations in the Republic of Korea; we used the existing primer sets: (GOLV-NCF and GOLV-NCR) and (HEBV-F and HEBV-R). During March–September 2013, a total of 461 serum samples were randomly collected from 40 commercial swine farms in 9 provinces. Of these, 204 (44.3%) samples were positive for GOLV

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DOI: <http://dx.doi.org/10.3201/eid2012.131742>

Table 1. GOLV and HEBV screening results for dead pigs on 1 farm in Gyeonggi Province, Republic of Korea, 2013*

Pig no.	Clinical signs	Age group†	Month sample collected	Sample no.	Sample type	GOLV		HEBV		Other pathogens
						RT-PCR	qRT-PCR, copies/μL‡	RT-PCR	qRT-PCR, copies/μL‡	
1	None	Finisher	Mar	P0	Lung	–	NA	+	2.57 × 10 ³	
2	Wasting, cyanosis, fever, respiratory disorders, diarrhea	Weaned	Mar	P1	Lung§	+¶	2.03 × 10 ³	+‡	1.26 × 10 ²	NA
				P2	Intestine	+	1.27 × 10 ⁴	+	1.16 × 10 ²	NA
3	Diarrhea, respiratory disorders	Finisher	Mar	P3	Lung	+	1.11 × 10 ²	+	1.37 × 10 ³	PRRSV, PCV2
				P4	Intestine	–	NA	–	–	–
4	Diarrhea, respiratory disorders	Gilt	Mar	P5	Lung	+	2.53 × 10 ³	+	5.45 × 10 ⁴	PRRSV, PCV2
				P6	Intestine	–	NA	–	NA	–
5	Respiratory disorders	Weaned	Apr	P7	Lung	+	2.04 × 10 ³	+	2.78 × 10 ³	PRRSV, PCV2
				P8	Intestine	+¶	1.82 × 10 ²	–	NA	–
6	Diarrhea, respiratory disorders	Grower	Apr	P9	Lung	+	5.10 × 10 ⁵	+‡	5.46 × 10 ³	PRRSV
				P10	Intestine	–	NA	–	NA	–
7	Diarrhea	Finisher	Mar	P11	Intestine	–	NA	–	NA	<i>E. coli</i>
8	Diarrhea	Sow	Mar	P12	Intestine	+	6.52 × 10 ²	–	NA	Rotavirus, <i>E. coli</i>
				P13	Intestine	–	NA	–	NA	–

**E. coli*, *Escherichia coli*; GOLV, Gouleako virus; HEBV, Herbert virus; NA, not applicable; qRT-PCR, quantitative reverse transcription PCR; PCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus, Rota, rotavirus.

†Samples were sorted into 6 groups: female (gilt and sow), suckling (<30 d), weaned (30–60 d), grower (60–90 d); and finisher (≥90 d).

‡Amount of virus from qRT-PCR results, copies/μL (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/20/12/13-1742-Techapp1.pdf>).

§First sample in which virus was detected by particle-associated nucleic acid—random PCR.

¶GenBank, GOLV (P1, KF361520; P8, KF361521).

#GenBank, HEBV (P1, KF361522; P9, KF362523).

and 26 (5.6%) samples were positive for HEBV (Table 2). The rates of positivity for the investigated provinces are shown in Figure 1. When examined according to season, positive samples were more frequently found in the summer than in spring. For example, during July–August, rates were ≈65% (for GOLV) and 10% (for HEBV), but in March, rates for each virus were <10%. Rates for GOLV

and HEBV positivity were higher among sows (1–4 years of age) than among pigs in other age groups (Table 2).

The study was extended to include sows on other farms in the Republic of Korea because of the major

Table 2. Pig samples positive for GOLV or HEBV by RT-PCR, Republic of Korea, 2013*

Variable	No. (%) positive	
	GOLV	HEBV
Pig age group		
Gilt, n = 49	23 (46.9)	3 (6.1)
Sow, n = 76	42 (55.3)	12 (15.8)
Suckling, n = 90	20 (22.2)	5 (5.6)
Weaned, n = 90	44 (48.8)	2 (2.2)
Grower, n = 77	37 (48.1)	3 (3.9)
Finisher, n = 79	38 (48.1)	1 (1.3)
Total, n = 461	204 (44.3)	26 (5.6)
Sample collection		
Mar, n = 40	3 (7.5)	0
Apr, n = 40	5 (12.5)	1 (2.5)
May, n = 64	22 (34.4)	3 (4.7)
Jun, n = 72	27 (37.5)	3 (4.2)
Jul, n = 79	39 (49.4)	5 (6.3)
Aug, n = 82	66 (80.5)	10 (12.2)
Sep, n = 84	42 (50.0)	4 (4.8)
Total, n = 461	204 (44.3)	26 (5.6)

*GOLV, Gouleako virus; HEBV, Herbert virus; RT-PCR, reverse transcription PCR.

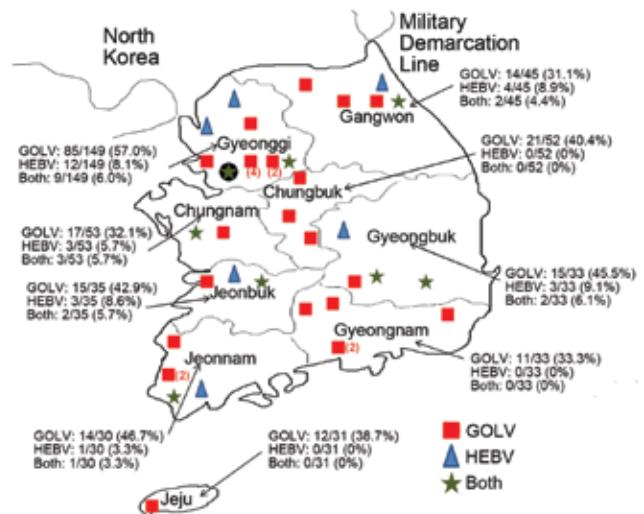


Figure 1. Distribution of swine farms investigated to determine cause of death of pigs, 9 provinces, Republic of Korea, 2013. The locations of farms are indicated, and the numbers and percentages of positive farms are shown in parentheses. Black dot indicates location of first case discovered. GOLV, Gouleako virus; HEBV, Herbert virus.

role of sows on a commercial swine farm. Pigs were divided into 3 groups and the following samples were collected: blood from healthy sows >1 year of age (n = 76), blood from abortion-problem sows (n = 13), and tissue from aborted fetuses (n = 42). Rates of virus positivity for GOLV and HEBV were higher for the 42 fetuses (33 [78.6%] and 11 [26.2%]) than for pigs in the healthy group (42 [55.3%] and 12 [15.8%]), respectively. Of the 13 abortion-problem sows, GOLV and HEBV, respectively, were found in 10 (76.9%) and 3 (23%) samples. The rates of GOLV and HEBV positivity among the healthy and abortion groups (abortion-problem sows and fetuses) were statistically compared by using the Pearson χ^2 test in SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). The only significant correlation found was for HEBV infection in the abortion group; $p \leq 0.05$.

Within the abortion group, pooled tissues from the 42 fetuses were screened for other pathogens (online Technical Appendix Table). The highest rate of positivity for the fetuses was for GOLV; 17 (40.5%) of the 42 samples were positive for GOLV only. Concurrent GOLV and HEBV infection was found in 9 (21.4%) samples and GOLV and swine influenza virus in 4 (9.5%) samples; no specific pathogens were detected in 7 (16.7%) samples (Technical Appendix Table).

The phylogenetic relationships of GOLV and HEBV isolated from swine in the Republic of Korea (online Technical Appendix) with the other members of family *Bunyaviridae* were analyzed; analyses were based on genes that encode the nucleocapsid protein (for GOLV) and *RdRp* (for HEBV). Of the GOLVs, the result showed that samples from the swine farms examined (KJ830623, KJ830624) clustered with GOLV strains from Africa (9) and showed high similarities (98.34%–98.98%) with a strain of GOLV from mosquitoes in western Africa (HQ541736) (Figure 2, panel A). Of the HEBV viruses, samples from swine farms examined (KJ830625, KJ830626) formed a branch with existing strains (KF590583, JQ659256) from mosquitoes in Côte d'Ivoire (10) (Figure 2, panel B). Similarities with each other were 94.46%–97.23%.

Two field GOLV strains (CP-1/2013 and CP-2/2013) used in this study were isolated from pig kidney (PK15) cells. Detailed information about the methods used to prove the results are shown in online Technical Appendix Figure 2.

Conclusions

We demonstrated that GOLV and HEBV are prevalent on swine farms in the Republic of Korea. Prevalence of these viruses was first suspected after particle-associated

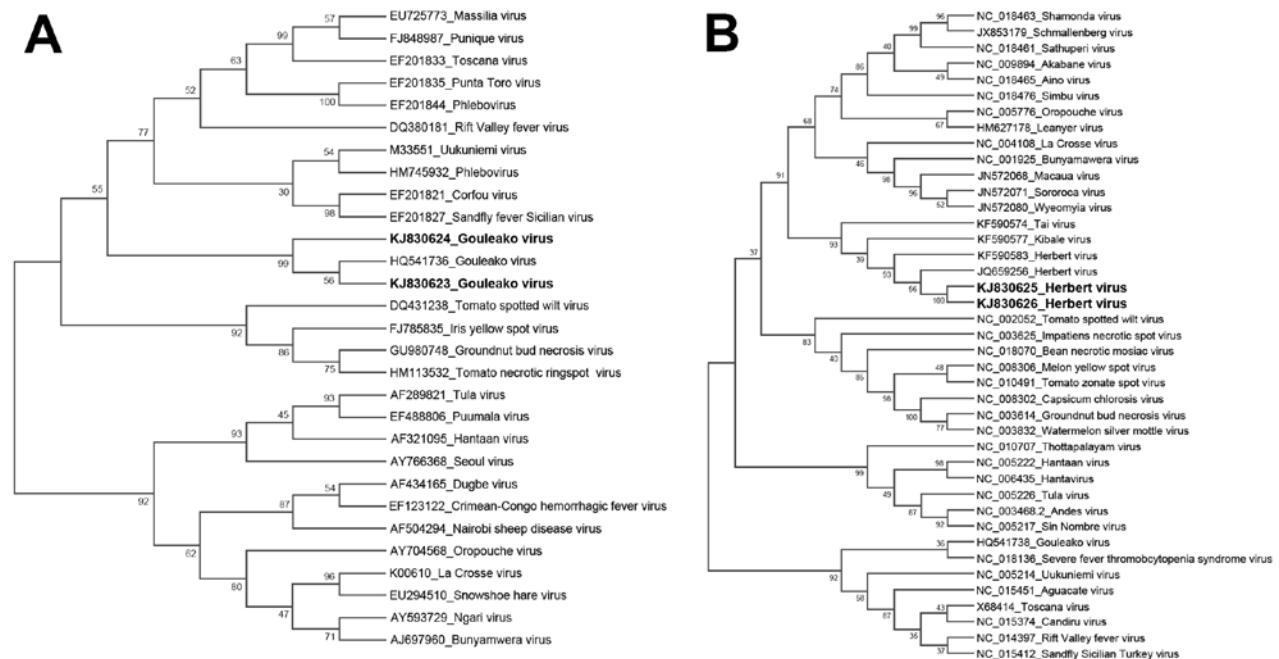


Figure 2. Phylogenetic analyses of Golueako virus (GOLV) and Herbert virus (HEBV) collected from swine in the Republic of Korea, 2013 (KJ830623–J830626, in boldface), and other family *Bunyaviridae* viruses. The bootstrap consensus trees were constructed by using the maximum-likelihood method based on the general time-reversible model, implemented in MEGA version 6.06 (<http://www.megasoftware.net>). The phylogenetic trees for GOLV (A) and HEBV (B) were inferred on the basis of nucleotide sequences of the gene encoding nucleocapsid protein (GOLV) or RNA-dependent RNA polymerase (HEBV). The bootstrap values are shown next to the branches. *Bunyaviridae* virus sequences from previous studies (9, 10) were used as reference sequences.

nucleic acid–random PCR of tissue from dead pigs, and it was proven by RT-PCR screening of a large collection of samples (serum, fetal tissue) from healthy and sick pigs throughout the country. The *in situ* hybridization method detected GOLV RNA in pig tissues and provided evidence in support of the presence of GOLV in the infected tissues of pigs. The findings of this study indicate that GOLV and HEBV may be associated with disease in pigs; investigation of the pathogenicity of the viruses in pigs, as well as their relation to other emerging viruses of swine, is needed.

Acknowledgments

We thank Su Hee Yun for expert technical assistance.

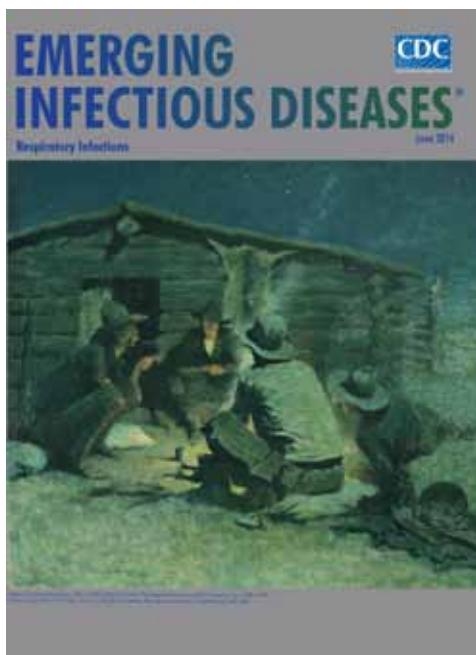
This study was supported by a grant (PJ009015) from Bio-Green 21 Program, Republic of Korea.

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Gouleako and Herbert Viruses in Pigs, Republic of Korea, 2013

Technical Appendix

Particle-Associated Nucleic Acid (PAN)–Random PCR

To identify the cause of the death, the particle-associated nucleic acid (PAN)-random PCR method was utilized (1,2). Porcine organs were separated into 3 groups as follows: lung tissue, intestinal tissue, and the rest of main organs including kidney, liver, lymph node, heart and spleen. It was performed with extracted DNA and RNA individually from these 3 kinds of tissues. The band from RNA/cDNA-based PAN-random PCR was purified by using the gel-extraction method and further processed for TA cloning and transformation (2).

RNA Extraction and RT-PCR

Total RNA was extracted by using viral RNA mini kit (QIAGEN Ltd., Manchester, UK) following the manufacturer's instructions. The RNA was then converted into cDNA with the use of random hexamers and commercial M-MLV reverse transcriptase kit (Invitrogen, USA) following the manufacturer's protocol. Finally, PCR reactions were performed with pathogen-specific primers using Takara Ex Taq PCR Mix kit (Takara Ltd., Korea).

The specific primers for detecting Gouleako virus (GOLV) were GOLV-F [5'-TCATTTTCAACCCCATTTGT-3'], GOLV-R [5'-CTAGCGACCTCCACACAACA-3'] which amplified 342-bp region of the glycoprotein coding gene. The specific primers for detecting Herbert virus (HEBV) were HEBV-F: [5'-TCCAACAATGACAAGCTCCA – 3'], HEBV-R: [5'-ACCATCTAGCGACCTCCAC -3'], which amplified 398-bp region of the gene encoded for *RdRp* gene.

Reconfirming Presence of GOLV

As many segments of genomic RNA of GOLV are available in GenBank, we designed another primer set targeting gene encodes for nucleocapsid protein of GOLV based on the sequence of the reference GOLV strain (EF423169). The primer pairs were GOLV-NCF [5'-TCTTGCCAGTGTGAGTTTGC-3'] and GOLV-NCR [5'-TTGGATCCAGTTGGTCTTCC-3'], which amplified 300-bp fragment of the nucleocapsid coding gene. The RT-PCR targeting nucleocapsid coding gene results confirmed the existence of this virus in the porcine samples by demonstrating the same positive results compared to the existing primer set (GOLV-F and GOLV-R).

Real-Time Quantitative Reverse Transcription PCR

The real-time quantitative reverse transcription PCR (RT-qPCR) assay using Maxima SYBR Green kit (Thermo Fisher Scientific Inc., Lithuania) was performed (3) to estimate viral load in tissue samples (8 positive for GOLV and 7 for HEBV). The same primer set was used (GOLV NCF and GOLV NCR) to detect GOLV. To enhance the sensitivity and accuracy, the specific primer set was designed for HEBV as follows: HEBV-QF [5'-TCAGTGGCAAATTTCCAAAA-3'] and HEBV-QR [5'-TTAAAAAGGGGGCTTCAACC-3']. The quantity of GOLV ranged from 1.11×10^2 to 5.10×10^5 (copies/ μ L) and HEBV ranged from 1.16×10^2 to 5.45×10^4 (copies/ μ L) (Technical Appendix Table 1).

Histopathology for RNA in situ Hybridization

Dead pig no. 2 is presented in Table 1; the following 3 tissue types were collected in 10% neutral buffered formalin: lung, lymph node, and intestine. And after 1 day of fixation, samples underwent dehydration through graded alcohols and a toluene step and embedded in paraffin wax. Sections were then cut at a thickness of 5 μ m, mounted on "Superfrost/plus" slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at room temperature. Probe sets were designed with a reference GOLV nucleocapsid protein gene (GenBank accession no. HQ541736.1, region 112–780 covered by probeset) from Affymetrix Ltd., genomics Asia teams. The in situ hybridization was utilized to QuantiGene® ViewRNA ISH tissue kit (Affymetrix Ltd., USA), according to the manufacturer's instructions.

GOLV Isolation, Purification, and Sequencing

This study also attempted to isolate GOLV from RT-PCR positive 25 serum samples (5 pools). Both PK15, and HEK293 cells inoculated grown in Dulbecco's minimum essential medium (DMEM) containing 5% fetal bovine serum (FBS) with cultured the cells at 37°C in a 5% carbon dioxide atmosphere. The PK15 cells were detected after 42 h incubation with cytopathic effect (CPE). After observation of CPEs, the supernatant of isolates was used for further blind passage, identified by RT-PCR, real time RT-PCR, and using virus titrated using Reed and Muench method (4) (Technical Appendix Figure 2). After 5th passages, PK15 cell monolayers were inoculated with 10-fold diluted virus inoculums in 6-well culture plates, and incubated for 1 h. The inoculums were then removed, and the cells were overlaid with 48°C DMEM containing 1.8% agarose, 10% FBS, 1% neutral red, and incubated at 37°C in a 5% CO₂ incubator for 5 days. The formation of plaques with GOLV could be seen 100 h after infection; they appeared as round uncolored areas contrasting with the red color, were aspirated by using tips, and were suspended in DMEM. For the 5th passage, infectious virus was demonstrated by plaque assay 1.1×10^6 plaque-forming units (pfu/mL). For the clear characterization of virus isolates, the suspension of pure plaque was inoculated to PK15 cells with maintained in DMEM supplemented with 5% fetal bovine serum (FBS). After observation of CPE, the supernatant of isolates used for characterization, was identified by RT-PCR and sequencing by primer walking techniques (5) for reference (GenBank accession no. HQ541736.1) from Macrogen Ltd., genomics teams, Korea. We obtained the complete sequences of the GOLV's S segment (1,087nt) from the 2 field GOLV strains CP-1/2013 (KJ830623) and CP-2/2013 (KJ830624).

HEBV Sequencing

Attempts were also made but failed to get HEBV isolates from 26 HEBV positive serum mentioned above. However, by another HEBV specific primers (HEBV-PF [5'-TTGAATCAGGGTTACAGGCTT-3'], and HEBV-PR [5'-GCATCGATGTACCTTTTAGG-3']), which amplified 1,081-bp fragment of the *RdRp* gene, we obtained partial sequences of 832bp from samples CP-3/2013 (KJ830625), and CP-4/2013 (KJ830626).

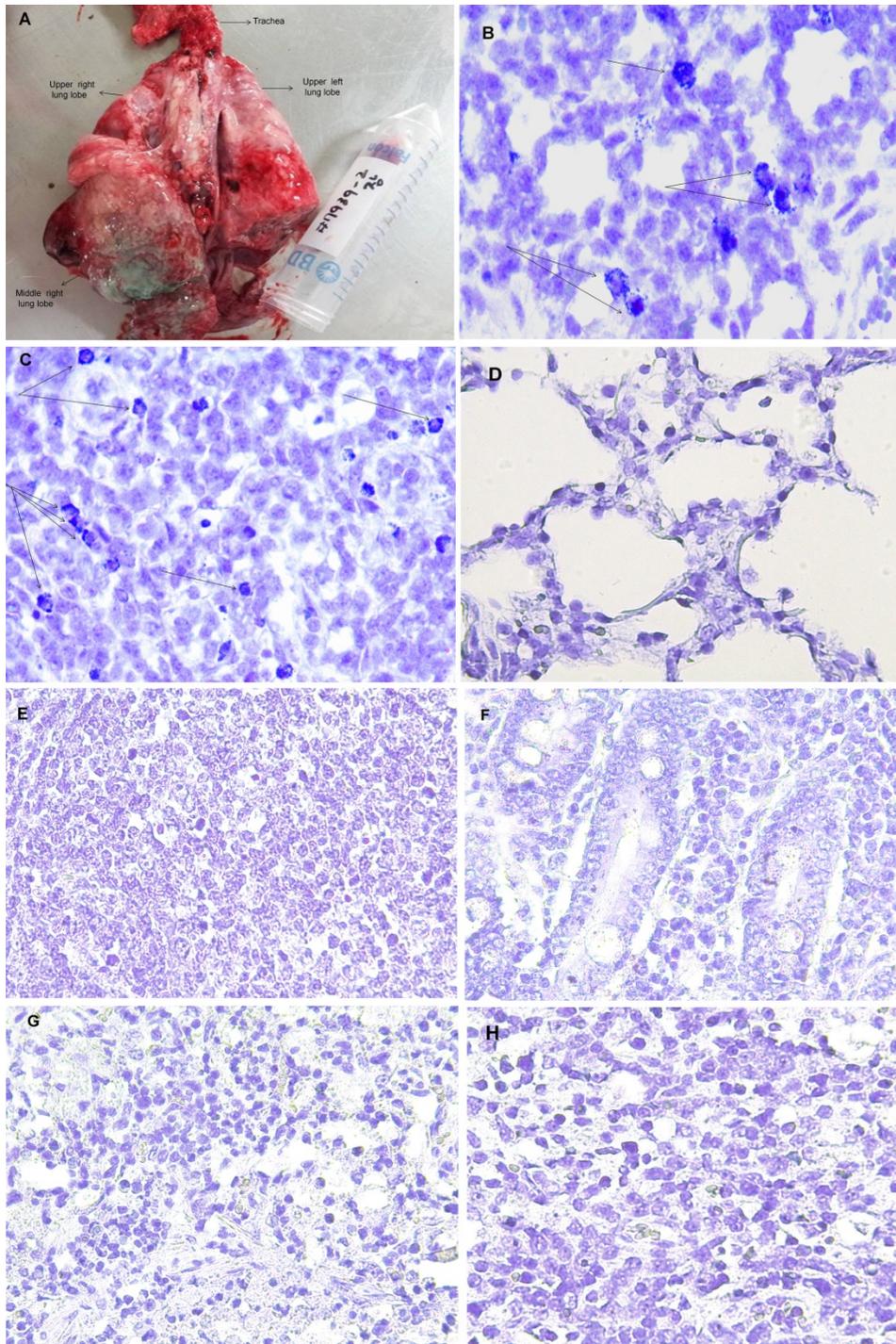
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Technical Appendix Table . GOLV infections associated with abortion in pigs, South Korea, 2013

Pathogen	No. (%) samples positive
GOLV alone	17 (40.5)
GOLV + 1 pathogen	
HEBV	9 (21.4)
SIV	4 (9.5)
PCV2	2 (4.8)
PRRSV	1 (2.4)
GOLV + 2 pathogens	
HEBV, SIV	1 (2.4)
PCV2, SIV	1 (2.4)
No pathogens detected	7 (16.7)
Total	42 (100)

*GOLV, Gouleako virus; HEBV, Herbert virus; PCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus; SIV, swine influenza virus.



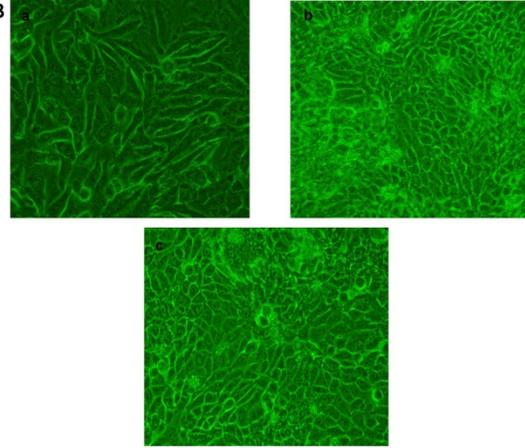
Technical Appendix Figure 1. RNA in situ hybridization assay targeting the nucleocapsid protein coding gene of Gouleako virus (GOLV) in porcine tissues. A) Gross appearance of greenish lesion in right middle lung. B) Positive signal of lung tissue and C) lymph node tissue. Negative control; tissue sections from a healthy pig (8 weeks old), which was RT-PCR negative for GOLV: D) lung, E) lymph node, F) Intestine, and omitted target probe set: G) lung, H) lymph node. The specific signal was visualized by using a standard brightfield microscope using 40× objective lens.

2.A

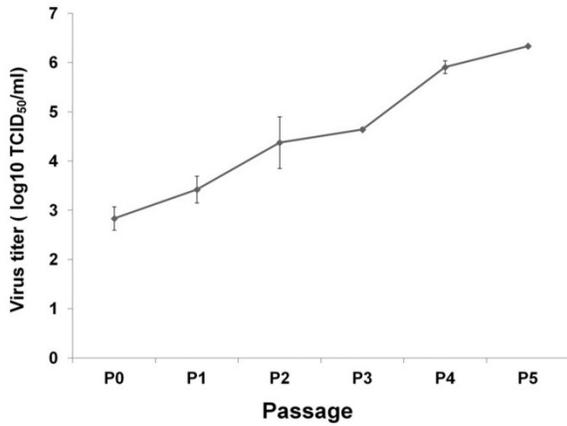
Cell \ Passage	P0	P1	P2	P3	P4	P5
PK15	+ /CPE					
HEK293	NT/-	NT/-	NT/-	NT/-	NT/-	NT/-

* RT-PCR/CPE

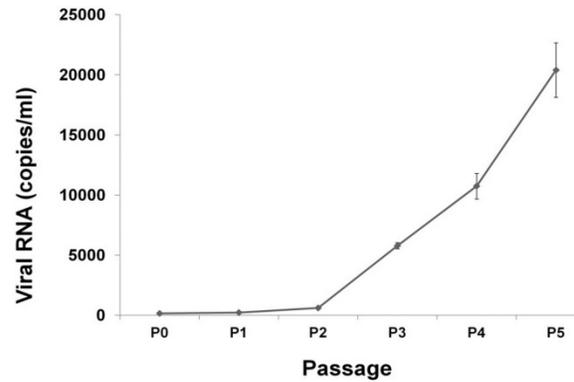
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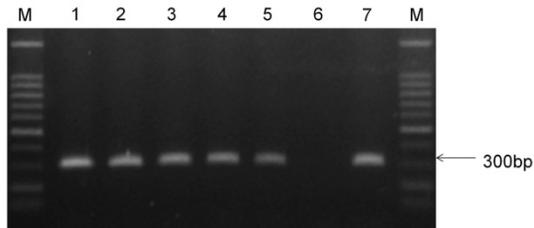
2.C



2.D



2E



Technical Appendix Figure 2. The growth of Gouleako virus (GOLV) on pig kidney (PK15) cells. A) Isolation results of GOLV on PK15 and HEK293 cell lines. B) CPE of PK15 cells induced upon GOLV infection; a) is negative control, b) and c) show the observed CPE at the viral titer of $10^{3.615}$ TCID₅₀/mL and $10^{4.166}$ TCID₅₀/mL, respectively. C) The titers of GOLV between passages. D) The quantitative real-time RT-PCR results of GOLV between passages (reported as number of copies/mL). E) Agarose gel electrophoresis of PCR product on the nucleocapsid protein gene of GOLV. From left to right: lane M, 100-bp DNA ladder; lanes 1 to 5, passage levels 1,2,3,4, and 5, respectively ; lane 6; negative control ; lane 7; positive control.