

Virus isolation and epidemiological study of a novel porcine morbillivirus as a putative cause of fetal death and encephalitis

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Industry Summary

Paramyxoviruses that are known to naturally infect swine include porcine rubulavirus, Menangle virus, Nipah virus, and porcine parainfluenza virus. There are reports of less well-characterized paramyxoviruses associated with central nervous and respiratory disease in pigs. However, none of these viruses are classified in the genus *Morbillivirus*. In the spring of 2020, the Veterinary Diagnostic Laboratory at Iowa State University received twenty-two porcine fetuses (neonatal mortality, stillbirths, mummified fetuses, and fetuses with moderate autolysis) from six litters submitted from Mexico for diagnostic investigation. PCV2, PCV3, PRRSV, PPV1, and *Leptospira sp.* were not detected by qPCR or RT-qPCR in any litter. Metagenomics sequencing identified a new virus in the genus of *Morbillivirus*: Porcine Morbillivirus (PoMV). Other currently known members in the genus *Morbillivirus*, including measles virus (MeV), rinderpest virus (RPV), peste des petits ruminants virus (PPRV), canine distemper virus (CDV), phocine distemper virus (PDV), cetacean morbillivirus (CMV), and feline morbillivirus (FeMV), are highly contagious pathogens and can cause serious human and animal diseases. Therefore, there is an urgent need to determine if this new virus associated with swine fetal death is present and, if yes, its prevalence in the U.S. swine population. A total of 450 clinical samples (brains, lungs, and spleens from neonatal mortalities, stillbirths, and mummified fetuses) were collected from all over the United States and

subjected to PoMV rRT-PCR assay. Our current assessment has not found PoMV in the U.S. swine populations tested thus far. In addition, virus isolation would be valuable for future characterization of PoMV (diagnostics, prevention, pathogenicity, and pathogenesis investigations). Isolation of PoMV in various cell lines (MARC-145 (a clone of the MA-104 cell line, ATCC CRL-2378), MDCK (ATCC NBL-2), PK-15 (ATCC CCL-33), ST (CRL-1746), Vero (ATCC CCL-81), BHK-21 (CCL-10), LLC-MK2 (CCL-7), ZMAC (ATCC PTA-8764), and primary porcine kidney cells using available PoMV PCR-positive tissue homogenates was attempted in this study. Unfortunately, a PoMV isolate that could efficiently replicate in cell culture has not yet been obtained.

Scientific Abstract

A novel porcine morbillivirus (PoMV), as a putative cause of fetal death, encephalitis, and placentitis, was detected in fetus samples from Mexico. However, PoMV was not yet isolated, the pathogenicity was not yet determined, and its presence and prevalence in the U.S. swine population was unknown. In this study, 450 clinical samples (brains, lungs, and spleens from neonatal mortalities, stillbirths, and mummified fetuses) were collected from all across the United States and subjected to PoMV rRT-PCR assay. Our current assessment has not found PoMV in the U.S. swine populations tested thus far. In addition, isolation of PoMV in various cell lines (MARC-145 (a clone of the MA-104 cell line, ATCC CRL-2378), MDCK (ATCC NBL-2), PK-15 (ATCC CCL-33), ST (CRL-1746), Vero (ATCC CCL-81), BHK-21 (CCL-10), LLC-MK2 (CCL-7), ZMAC (ATCC PTA-8764), and primary porcine kidney cells) using available PoMV PCR-positive tissue homogenates was attempted in this study. Unfortunately, a PoMV isolate that could efficiently replicate in cell culture has not yet been obtained. Virus isolation would be valuable for future characterization of PoMV. Unfortunately, no additional PoMV PCR-positive clinical

samples from Mexico could be obtained and the pig samples collected from various U.S. states were all PoMV PCR-negative. This limits our efforts to optimize conditions for additional VI attempts. In the future, if more PoMV PCR-positive samples become available, VI can be attempted again.

Keywords

Porcine morbillivirus, virus isolation, real-time-RT-PCR, virus isolation, epidemiology

Introduction

The family *Paramyxoviridae* encompasses a group of large, enveloped, pleomorphic (mostly spherical) viruses with RNA genomes of 14.6 to 20.1 kb. The family is currently composed of four subfamilies and seventeen genera with more than 70 species and contains globally significant viral pathogens. Currently, the genus *Morbillivirus* within subfamily *Orthomyxovirinae* includes highly contagious human and animal viruses such as measles virus (MeV), rinderpest virus (RPV), peste des petits ruminants virus (PPRV), canine distemper virus (CDV), phocine distemper virus (PDV), cetacean morbillivirus (CMV), and feline morbillivirus (FeMV). Paramyxoviruses that are known to naturally infect swine include porcine rubulavirus, Menangle virus, Nipah virus, and porcine parainfluenza virus. There are reports of less well-characterized paramyxoviruses associated with central nervous and respiratory disease in pigs. However, none of these viruses are classified in the genus *Morbillivirus*.

In the spring of 2020, the Veterinary Diagnostic Laboratory at Iowa State University received twenty-two porcine fetuses from six litters submitted from Mexico for diagnostic investigation. These included one neonatal mortality, three stillbirths, fourteen mummified fetuses, and four

fetuses with moderate autolysis. PCV2, PCV3, PRRSV, PPV1, and *Leptospira sp.* were not detected by qPCR or RT-qPCR in any litter. Metagenomics sequencing identified a new virus in the genus *Morbillivirus* and we named it Porcine Morbillivirus (PoMV). Phylogenetic analyses determined PoMV to be most closely related to canine distemper virus (62.9% nt identity) and phocine distemper virus (62.8% nt identity) in the *Morbillivirus* genus. Intranuclear inclusions were commonly observed in neurons and glial cells of fetuses with encephalitis. Furthermore, fluorescence in situ hybridization assay detected PoMV RNA within neurons, respiratory epithelium, and lymphocytes in the brains, lungs, and spleens of neonatal mortalities, stillbirths, and mummified fetuses (the article has been accepted by *Emerging Infectious Diseases* and is in press). However, PoMV was not yet isolated, the pathogenicity was not yet determined, and its presence and prevalence in the U.S. swine population was unknown.

Objectives

- 1) Attempts to isolate porcine morbillivirus (PoMV) in cell culture from clinical samples
- 2) Determine the presence/incidence of PoMV in swine populations of the United States

Materials & Methods

Clinical samples. More than 500 clinical samples including brain, lung, and spleen samples were collected from neonatal mortalities, stillbirths, and mummified fetuses that were submitted to Veterinary Diagnostic Laboratory at Iowa State University and were stored at -80°C for future investigation.

Four PoMV PCR-positive tissue homogenates from a stillborn fetus A, stillborn fetus B, litter D with mummified fetuses, and litter E with mummified fetuses collected from a farm in Mexico in January 2020 were subjected to virus isolation attempts. Fetal thoracic tissues from Litters A and

E had a higher viral load (Cq values were 19.7 and 19.4, respectively) and fetal thoracic tissues from Litters B and D had a lower viral load (Cq values were 23.4 and 20.2, respectively).

Cell lines. Cell lines MARC-145 (a clone of the MA-104 cell line, ATCC CRL-2378), MDCK (ATCC NBL-2), PK-15 (ATCC CCL-33), ST (CRL-1746), Vero (ATCC CCL-81), BHK-21 (CCL-10), LLC-MK2 (CCL-7), ZMAC (ATCC PTA-8764), and primary porcine kidney cells prepared at the ISU VDL were used in this study to attempt isolation of porcine morbillivirus. MARC-145 cells were cultured in regular RPMI-1640 medium supplemented with final concentrations of 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mg/ml gentamicin, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. MDCK, PK-15, ST, Vero, BHK-21, and LLC-MK2 cells were cultured in regular MEM medium supplemented with final concentrations of 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mg/ml gentamicin, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. ZMAC cells were cultured in RPMI-1640 medium with L-glutamine & 25 mM HEPES supplemented with 1× MEM non-essential amino acids, 4 mM sodium pyruvate, 2 mM L-glutamine, 0.81% glucose, 10% fetal bovine serum, 0.01 µg/ml mouse macrophage colony stimulating factor (mouse M-CSF, Shenandoah Biotechnology, Inc., Warwick, PA), 0.05 mg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. All cells were maintained in a 37°C incubator with 5% CO₂.

Virus isolation. Tissue homogenates were filtered through 0.45 µm size syringe filter and then inoculated into each cell line cultured in 24-well plates (0.2 mL per well) in the presence of trypsin. The plates were incubated at 37°C with 5% CO₂ for 1-2 h. The inoculum was removed and fresh culture medium was added. The cells were incubated for 4-5 days at 37°C with 5% CO₂ and the development of cytopathic effect (CPE) was monitored. After the first passage, the cells were

subjected to freeze-thaw once. The mixtures were centrifuged at 3,000× g for 10 min. The supernatants were harvested for further propagation or saved at -80°C for PCR testing. Following similar procedures, the cell lysates were passed in the respective cell lines for 2-3 additional passages. The cell culture lysates at different passages were tested by PoMV real-time RT-PCR following the previously described procedures (Arruda et al., 2021) to confirm the outcomes of virus isolation.

Real-time reverse transcription PCR. Porcine morbillivirus (PoMV) primers (MBLV-900F, 5'-GGGCTAGCAAGCTTTATCCTCAC-3'; MBLV-988R, 5'-GTAACTCACCAGCAAACATCATGC-3') and probe (MBL-959P, 5'-6-FAM-CAGTGCCGGGTACATAGTTTCTATCCCAA-TAMSp-3') were synthesized by Integrated DNA Technologies (<https://www.idtdna.com>). Samples were processed to obtain 20% tissue homogenate for RNA/DNA extraction with the Applied Biosystem 5X MagMax Pathogen RNA/DNA Kit and Thermo Fisher Scientific KingFisher Flex automated extraction instrument. The real-time PCR reaction was performed in a 25 µL PCR mixture that contained 12.5 µL 2× AgPath-ID RT-PCR Buffer (Applied Biosystems), 1 µL 25X RT PCR Enzyme Mix, 1 µL (0.4 µmol) of each of the primers, 0.5 µL (0.2 µmol) of probe, 4 µL nuclease-free water, and 5 µL extracted RNA. The amplification was performed at 48°C for 10 min, 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 45 s. Samples with a Cq value <40 were considered positive. Fetal thoracic tissues from 2 litters that originated from a 3,000-head sow farm in the United States experiencing increased mummified fetuses and stillborn fetuses were used for real-time RT-PCR negative controls. Samples from Litter A that originated from Mexico where PoMV was detected were used for positive control.

Results

Virus isolation

In the beginning, all four tissue homogenates (A, B, C and D) were inoculated into each of the cell lines MARC-145, MDCK, PK-15, ST, Vero, BHK-21, and LLC-MK2 and passed in each line for two passages with the exception of the LLC-MK2 cells, which were no longer in good condition at that time. No CPE was observed in any of the inoculated cells. The Passage 0 (P0) and P1 cell culture lysates harvested from these cells were tested by PoMV real-time RT-PCR. The results are summarized in Table 1.

The P1 cell culture lysates in the MARC-145, MDCK, PK-15, Vero, and BHK-21 cells had increasing PCR Ct values compared to the respective P0 cell culture lysates, suggesting that there was no efficient virus replication in these cell lines; thus, no further passages were conducted in these cell lines.

In ST cells, the P1 cell culture lysates from inocula D and E had slightly lower Ct values compared to the respective P0 cell culture lysates. Hence, two more passages were conducted in ST cells. At the P2 and P3, all cell culture lysates derived from samples A, B, D, and E were negative by porcine morbillivirus PCR, indicating that VI attempts in ST cells did not result in isolation of a virus that can continuously and efficiently replicate.

In LLC-MK2 cells, the P0 cell culture lysates had promising PCR results especially for sample E whose P0 cell culture lysate had Ct value of 25.9. When LLC-MK2 cells were in good condition later on, the sample E P0 cell culture lysate underwent three more passages (P1, P2, and P3). Unfortunately, Ct values increased when passage number increased and the P3 cell culture lysate was PCR negative. The data suggest that VI attempt in LLC-MK2 cells was unsuccessful either.

Then, VI was attempted in ZMAC cells and primary porcine kidney (PPK) cells. Due to the limited volume of the tissue homogenates, VI was only attempted in ZMAC and PPK cells for samples D and E. With continuous passages in ZMAC and PPK cells, the cell culture lysates from the higher passages had increasing Ct values, suggesting no efficient virus replication. Thus, the VI in ZMAC and PPK cells was considered unsuccessful.

Table 1. Summary of porcine morbillivirus PCR results (Ct values) on virus isolation materials harvested at different passages in a variety of cell lines

Cell line	Passage 0				Passage 1				Passage 2				Passage 3			
	A	B	D	E	A	B	D	E	A	B	D	E	A	B	D	E
MARC-145	31	34.7	29.4	27.5	35.1	≥40	35.5	31.9								
MDCK	30.2	33.8	31.1	27.4	34.8	≥40	36.2	32.8								
PK-15	31.3	34.3	30.4	27.3	37	≥40	36.9	34.4								
ST	31.7	34.5	32.2	28.3	31.5	33.4	29.4	27.1	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Vero	31.6	34.5	31.2	28.3	34.7	≥40	36.4	34.8								
BHK-21	31.1	33.3	30.4	28.7	37.4	≥40	36	34.9								
LLC-MK2	29.6	33.2	29.1	25.9				33.2				36.9				≥40
ZMAC			29.7	28.9			31.7	29.4			≥40	34.2				
PPK			29.3	26.3			34.6	33.7								

Determine the presence/incidence of PoMV in swine populations of the United States

A total of 150 lung tissue samples, 150 brain samples, and 150 spleen tissue samples from neonatal mortality and mummified fetus were examined by using PoMV rRT-PCR assay. The Ct values of all tested samples were >40, thus were negative for PoMV. Our current assessment has not found PoMV in the U. S. swine populations tested thus far.

Discussion

Porcine morbillivirus was identified for the first time in 2020 from pig fetuses collected from Mexico (Arruda et al., 2021). Although PoMV was identified from cases with fetal death,

encephalitis, and placentitis, the etiological role of PoMV had not been definitively confirmed. Obtaining a PoMV cell culture isolate would be very valuable to further characterize PoMV. Isolation of PoMV in various cell lines using four available PoMV PCR-positive tissue homogenates was attempted in this study. Since no CPE was observed and there was no PoMV-specific antibody for immunofluorescence staining, we tested the cell culture lysates at different passages by PoMV real-time RT-PCR to confirm the VI outcomes. Based on the PCR Ct values, it appears that a PoMV isolate that can efficiently replicate in cell culture has not been obtained. Unfortunately, no additional PoMV PCR-positive clinical samples from Mexico could be obtained and the pig samples collected from various U.S. states were all PoMV PCR-negative. This limits our efforts to optimize conditions for additional VI attempts. In the future, if more PoMV PCR-positive samples become available, VI can be attempted again.

Since PoMV was associated with fetal death and encephalitis in swine, it is very urgent to determine if this novel virus is also present in U.S. swine population. Various tissues from neonatal mortality and mummified fetus samples submitted from all over the country were subjected to PoMV rRT-PCR assay. Our current assessment has not detected PoMV in the U. S. swine populations tested thus far.

Reference

Arruda B, Shen H, Zheng Y, Li G. Novel morbillivirus as a putative cause of fetal death and encephalitis among swine. *Emerg Infect Dis.* 2021, 27(7): 1858-1866.