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Acute neurologic disease in *Porcine rubulavirus* experimentally infected piglets

Jenifer Herrera[a], Luis Gómez-Núñez[a], Rocío Lara-Romero[a], Fernando Diosdado[a], Atalo Martínez-Lara[a], Miguel Jasso[b], Humberto Ramírez-Mendoza[b], Armando Pérez-Torres[c], José Francisco Rivera-Benítez[a,*]

[a] *Centro Nacional de Investigación Disciplinaria en Microbiología Animal, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Mexico City, Mexico*
[b] *Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, UNAM, Mexico City, Mexico*
[c] *Facultad de Medicina, UNAM, Mexico City, Mexico*

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ABSTRACT

The objective of this study was to evaluate the clinical disease, humoral response and viral distribution of recent *Porcine rubulavirus* (PorPV) isolates in experimentally infected pigs. Four, 6-piglet (5-days old) groups were employed (G1-84, G2-93, G3-147, and G4-T). Three viral strains were used for the experimental infection: the reference strain LPMV-1984 (Michoacán 1984) and two other strains isolated in 2013, one in Queretaro (Qro/93/2013) and the other in Michoacán (Mich/147/2013). Each strain was genetically characterized by amplification and sequencing of the gene encoding hemagglutinin-neuroamidase (HN). The inoculation was performed through the oronasal and ocular routes, at a dose of 1×10^6 $TCID_{50}$/ml. Subsequently, the signs were evaluated daily and necropsies were performed on 3 different days post infection (dpi). We recorded all micro- and macroscopic lesions. Organs from the nervous, lymphatic, and respiratory system were analyzed by quantifying the viral RNA load and the presence of the infectious virus. The presence of the viral antigen in organs was evidenced through immunohistochemistry. Seroconversion was evaluated through the use of a hemagglutination inhibition test. In the characterization of gene HN, only three substitutions were identified in strain Mich/147/2013, two in strain LPMV/1984 (fourth passage) and one in strain Qro/93/2013, with respect to reference strain LPMV-84, these changes had not been identified as virulence factors in previously reported strains. Neurological alterations associated with the infection were found in all three experimental groups starting from 3 dpi. Groups G1-84 and G3-147 presented the most exacerbated nervous signs. Group G2-93 only presented milder signs including slight motor incoordination, and an increased rectal temperature starting from day 5 post infection (PI). The main histopathological findings were the presence of a mononuclear inflammatory infiltrate (lymphocytic/monocytic) surrounding the ventricles in the brain and focal interstitial pneumonitis with distention of the alveolar sacs in the lungs. PorPV and RNA distribution were identified in the organs of the nervous, lymphatic, and respiratory systems of the piglets analyzed at different times (days 5, 10, and 15 PI). The viral antigen was detected in the brain and lungs in most of the assessed groups. Seroconversion was evident in groups G1-84 and G2-93. Groups G1-84 and G3-147 were the most clinically affected by the experimental infection. Both strains were isolated in the state of Michoacán. The virulence of the new isolates maintains similar characteristics to those reported more than 30 years ago.

1. Introduction

Blue eye disease (BED) is caused by *Porcine rubulavirus* (PorPV) (Moreno-López et al., 1986). PorPV is an enveloped, single-strained, negative-sense RNA virus that belongs to the *Paramyxoviridae* family (Wang et al., 2011). Blue eye disease was first identified in farms in La Piedad, Michoacán, Mexico in the early 1980′s (Stephano et al., 1988). The BED has only been identified in Mexico and there are no

* Corresponding author. Present address: Laboratorio de Virología. Centro Nacional de Investigación Disciplinaria en Microbiología Animal, INIFAP, Km. 15.5 Carretera México–Toluca, Colonia Palo Alto, C.P. 05110, Mexico City, Mexico.
E-mail address: rivera.francisco@inifap.gob.mx (J.F. Rivera-Benítez).

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reports on its presence in other countries (Kirkland et al., 2012). PorPV infection can provoke nervous and respiratory disease in piglets and growing pigs (Reyes-Leyva et al., 2004; Reyes-Leyva et al., 2002; Rivera-Benitez et al., 2013a; Stephano et al., 1988). In pregnant sows, it may cause abortions or mummified fetuses; infertility, epididymitis, and orchitis has been observed in boars (Ramírez-Mendoza et al., 1997 Hernández-Jauregui et al., 2004; Rivera-Benitez et al., 2013b). This infection may cause corneal opacity in 1–10% of the cases (Stephano et al., 1988). Blue eye disease is enzootic to the central and mid-western areas of Mexico (Escobar-Lopez et al., 2012). There are three genetic groups characterized based on the hemagglutinin-neuraminase (HN) gene sequence and the clinical effect they produce (Reyes-Leyva et al., 2002; Sánchez-Betancourt et al., 2008). New PorPV isolates have been genetically characterized recently (Cuevas-Romero et al., 2016). However, to this day, the clinical characteristics of the disease under experimental conditions have not been evaluated yet. The first studied strain, named La Piedad Michoacán Virus (LPMV) has been used in experimental infections of piglets (Moreno-López et al., 1986; Allan et al., 1996). Other virus strains (PAC-3) have been employed in experimental infections of growing and adult pigs (Ramírez-Mendoza et al., 1997; Hernández-Jauregui et al., 2004; Rivera-Benitez et al., 2013b). The strains that currently circulate in the field in different geographic regions have not been used for studies on virulence. For this reason, it is relevant to describe the virulence of the viral strains collected in the enzootic zone of BED. The objective of this study was to evaluate the clinical characteristics, viral load, and sero-conversion that occur after experimental infection using recently-isolated PorPV strains.

2. Materials and methods

2.1. Virus and cells

Three viral strains were used for the experimental infection: LPMV/1984, Qro/93/2013 and Mich/147/2013. Strain LPMV/1984 was isolated during a BED outbreak in La Piedad, Michoacán, where piglets experienced neurological problems and a high mortality rate. Strain Qro/93/2013 was isolated in 2013, in a farrow-to-finish farm where the signs only included stunted growth, respiratory signs in growing and fattening pigs. Strain Mich/147/2013 was isolated in La Piedad, Michoacán, where growing pigs presented respiratory problems. The virus strains were replicated in PK-15 cells (pig kidney), maintained in Dulbecco's minimal essential medium (D-MEM), and supplemented with 5% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) (Reyes-Leyva et al., 1997). The Reed and Muench method was used to obtain viral titers by calculating the infective dose in a 50% cell culture ($TCID_{50}$). All viral stocks used were harvested in their fourth passage of cell culture.

2.2. Genetic and phylogenetic analysis

To verify the genetic identity of the strains to be used, the hemagglutinin-neuroamidase (HN) gene was amplified and sequenced. One aliquot of strains Qro/93/2013, Mich/147/2013, and reference strain LPMV/1984 were used for the extraction of the RNA by means of columns (RNeasy Mini kit, QIAGEN). The obtained RNA was quantified and was used in the amplification of the HN gene of PorPV. Briefly, to 5 μl of total RNA (containing a miminum of 500 ng), the components of the reaction from a comercial kit (RT-PCR One Step, QIAGEN) were added, as well as two pairs of primers to amplify the complete ORF HN, Fw1-LGN-5′- GCAATGTCTCAATTAGGGACTGATC-3′, Rv1-LGN-5′-CCACCAAGAACTACTGCGTTGATAAT-3′ (1262 bp), and Fw2-LGN-5′-CTACTCAATGATCCACGATATATGAGTGGT-3′, Rv2-LGN-5′-TCTCGTAAGAAGGGTACAATCTGAAATTCAC-3′ (1327 bp). Amplification products were visualized in a 1% agarose gel. Gel bands were excised and purified in columns (QIAquick Gel Extraction Kit, QIAGEN). Purified products were sequenced in both senses using the Applied Biosystems 3130xl Genetic Analyzer at the Synthesis and Sequencing Unit of the Biotechnology Institute, UNAM. The obtained sequences were compared to the database of the GenBank using the NCBI's BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were edited with the BioEdit v 7.1.11 software (Hall, 1999) and aligned with ClustalW contained in the same program. To determine the phylogenetic relation with the reported PorPV strains, a phylogenetic tree was constructed using neighbor-joining (JTT model) with 1000 bootstrap replicates using the MEGA v. 6.0 package (Tamura et al., 2013). For the analysis of genogroups, the three sequences obtained in this study as well as the 18 sequences reported in the GenBank were included: (PAC1/1990: KP229773.1; PAC2/1990: EF413172.1; PAC3/1992: EF413173.1; PAC4/1993: EF413174.1; PAC6/2001: EF413175.1; PAC7/2002: EF413176.1; PAC9/2003: EF413178.1; LPMV-CI/1991: AY463798.1; LMPV-CII/1999: AY487249.1; LPMV-CIII/1999: AY487251.1; LPMV-CIV/2002: AY487250.1; PorPV/Mx/1/Jalisco/2007: KT037087.1; PorPV/Mx/1/ Guanajuato/2008: KT037088.1; PorPV/Mx/1/Michoacan/2008: KT037089.1; PorPV/Mx/2/Jalisco/2009: KT037090.1; PorPV/Mx/2/Michoacan/2009: KT037091.1; PorPV/INIFAP/01/clone/Mx/2010: KC928078.1; PorPV/Mx/3/Michoacan/2013: KT037092.1). We performed a mapping of epitopes, glycosylation sites, and active HN site to identify possible changes in the amino acids sequence of the used strains.

2.3. Animals and experimental infection

Twenty-four 5-day old crossbred (Yorkshire × Landrace) conventional piglets were obtained from a commercial farm. The piglets came from a PorPV-free farm, which had previously been evaluated through serologic tests and real time *q*RT-PCR. Four experimental groups were employed with six piglets each. The piglets were housed in the isolation units of the *Centro Nacional de Investigación Disciplinaria en Microbiología Animal* (CENID-MA), *Instituto Nacional de Investigación Pecuaria, Agrícola y Forestal* (INIFAP). All pigs were fed with milk substitute every four hours, mini-starter feed, and free access to water. The experimental protocol was approved by the Internal Technical and Scientific Collegiate Group (INIFAP) (Experimental project number: Date 03/2013-19144832016). The experimental procedures were carried out similarly for each group. The groups were identified in accordance to the strain they were experimentally infected with. These identifications were: G1-84, six piglet group inoculated with strain LMPV/1984 (reference strain); G2-93, six piglet group inoculated with strain Qro/93/2013 (strain isolated in the state of Queretaro in 2013); G3-147, six piglet group inoculated with strain Mich/147/2013 (strain isolated in the state of Michoacán in 2013) and G4-T, six piglet control group which was not infected. The experimental inoculation was performed through routes: 2.5 ml orally, 4 ml nasally, and 1 ml through the eye. The viral dose was adjusted to a titer of 1×10^6 $TCID_{50}$/ml.

2.4. Clinical observations

From their arrival an up to the end of the experiment, the pigs were inspected to identify any clinical signs associated to the experimental infection. The following data were registered: rectal temperature, body weight, presence of nervous signs (excitement, tremor, hyper salivation, incoordination, ataxia, opisthotonos, depression, or death), presence of respiratory signs (sneezing,