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Evaluation of the pathogenicity of mammalian orthoreovirus type 3 (MRV3) in germ-free gnotobiotic pigs and of the efficacy of an inactivated vaccine against MRV3 infection in neonatal conventional piglets

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ABSTRACT

A novel U.S. strain of mammalian orthoreovirus type 3 (MRV3) isolated from diarrheic pigs in 2015 was reportedly highly pathogenic in pigs. In this study, we first developed an inactivated MRV3 vaccine and determined its protective efficacy against MRV3 infection in conventional neonatal piglets. A pathogenicity study was also conducted in gnotobiotic pigs to further assess the pathogenicity of MRV3. To evaluate if piglets could be protected against MRV3 infection after immunization of pregnant sows with an inactivated MRV3 vaccine, pregnant sows were vaccinated with 2 or 3 doses of the vaccine or with PBS buffer. Four-day-old piglets born to vaccinated and unvaccinated sows were subsequently challenged with MRV3. The results showed that piglets born from vaccinated sows had lower levels of fecal viral RNA shedding at 1, 3, and 4 days post-challenge, suggesting that the inactivated MRV3 vaccine can reduce MRV3 replication. Surprisingly, although the conventional piglets were infected, they did not develop severe enteric disease as reported previously. Therefore, in an effort to further definitively assess the pathogenicity of MRV3, we experimentally infected gnotobiotic pigs, a more sensitive model for pathogenicity study, with the wild-type MRV3 virus. The infected gnotobiotic piglets all survived and exhibited only very mild diarrhea in some pigs. Taken together, the results indicate that the novel strain of MRV3 recently isolated in the United States infected but caused only very mild diarrhea in pigs, and that maternal immunity acquired from sows vaccinated with an inactivated vaccine can reduce MRV3 replication in neonatal pigs.

1. Introduction

The *mammalian orthoreovirus* (MRV) belongs to the *Orthoreovirus* genus of the family *Reoviridae*, and consists of three serotypes: MRV1, MRV2, and MRV3 (Day, 2009; Dermody et al., 2013). MRV is a non-enveloped double-stranded RNA virus, and its dsRNA genome contains 10 segments, which is divided into three size classes: three large segments (L1, L2 and L3), three medium segments (M1, M2 and M3), and four small segments (S1, S2, S3 and S4), encoding three λ, three μ, and four σ proteins, respectively (Dermody et al., 2013). MRV infects a wide range of species including swine (Cox et al., 2005; Thimmasandra Narayanappa et al., 2015; Zhang et al., 2011), cattle (Cox et al., 2005), horses (Conner et al., 1984), dogs (Decaro et al., 2005), cats (Mochizuki et al., 1992), bats (Lelli et al., 2013, 2015; Li et al., 2016), and humans (Tyler et al., 2004). In general, MRV was considered to be non-pathogenic, although occasionally mild respiratory and/or enteric diseases have been linked to MRV infections (Dermody et al., 2013; Tyler et al., 2004). However, severe diseases have also reportedly been associated with MRV infection (Lelli et al., 2016; Ouattara et al., 2011; Steyer et al., 2013).

Recently, MRV3 was isolated from diseased pigs in China (Dai et al., 2012; Qin et al., 2017; Zhang et al., 2011), Korea (Kwon et al., 2012),

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the United States (Thimmasandra Narayanappa et al., 2015), and Italy (Lelli et al., 2016). Particularly, the MRV3 isolated from feces and blood meal of pigs in the United States was reported to be highly pathogenic in neonatal pigs (Thimmasandra Narayanappa et al., 2015). Whether the porcine MRV3 is a primary or secondary agent in piglet diarrhea is unclear, however, the discovery of the novel strain of MRV3 in fecal samples of piglets from the outbreaks of epidemic diarrhea and in swine blood meal suggested that the MRV3 may contribute to the severity of diseases, if not the outbreaks of the disease.

Therefore, the initial objective of this present study was to develop an inactivated MRV3 vaccine and determine its protective efficacy against MRV3 infection in piglets born to vaccinated sows, since the previous study reported severe disease in pigs experimentally-infected with the U.S. MRV3. The unexpected results from the vaccine study showing that the piglets born to unvaccinated sows did not develop severe disease at all after challenge with MRV3 led us to further definitively evaluate the pathogenicity of the U.S. MRV3 using gnotobiotic pigs, which are more sensitive for pathogenicity studies.

2. Materials and methods

2.1. MRV3 viruses

The MRV3 isolates, FS03 and BM100, used in the study were isolated in 2015 from the feces and blood meal of pigs, respectively (Thimmasandra Narayanappa et al., 2015), but these two isolates are genetically identical. To avoid potential mutations during serial cell culture passages, we produced two virus stocks through minimal numbers of passages in cell culture. The virus inoculum used for animal infection in this study was the third passage of the plaque-purified MRV3 FS03 isolate. The inactivated MRV3 vaccine was prepared from the fourth passage of the plaque-purified MRV3 BM100 isolate.

2.2. Infectivity titration of MRV3

MRV3 infectivity titration was performed on confluent cell monolayers of Vero cells grown in 96-well plates (CoStar™, Corning®). The virus stock was serially diluted 10-fold with medium, and 100 µL of each dilution was inoculated onto each of 5 wells of Vero cells. The cell culture plates were incubated at 37 °C with 5% CO_2 for 1 h, and subsequently 100 µL medium was added to each well. Plates were continuously incubated at 37 °C with 5% CO_2 for 5 days, after which the wells were evaluated for the presence of cytopathic effect (CPE) induced by MRV3 infection. The 50% endpoint was calculated as $TCID_{50}$/ml using the Reed-Muench method.

2.3. MRV3-specific ELISA

To establish the MRV3 ELISA for antibody detection, we first cloned the full-length MRV3 S1 gene into pTri-Ex 1.1 vector, expressed and purified the recombinant MRV3 σ1 protein using the *E. coli* expression system. The purified recombinant MRV3 σ1 protein was used as the coating antigen in the MRV3-specific ELISA. Following titration and optimal dilution of the purified recombinant MRV3 σ1 antigen, polystyrene 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were coated (100 µL/well) with the purified antigen and incubated at 4 °C overnight. After washing 3 times, and the plate was first blocked with 300 µL per well of a solution containing 1% bovine serum albumin, followed by incubation with serially-diluted serum samples. The bound antibodies were detected by goat-anti-pig secondary antibody-HRP conjugates (MP Biomedicals, Inc.).

2.4. Reverse transcription PCR (RT-PCR) amplification of MRV3 S1 fragment

Total RNAs from fecal or serum samples were isolated using TRIzol™ LS Reagent (Invitrogen) according to the manufacturer's instruction. A one-step RT-PCR was carried out to amplify the MRV3 S1 fragment in a 200 µL PCR tube using SuperScript™ III One-Step RT-PCR System (Invitrogen, CA). The primer set includes: FS03S1:366F22 (5′ GGATT ACGCAATGACTACAGCA 3′) and FS03S1:959R21 (5′ CCTATCCACATA CTTCGCCTA 3′). Briefly, 5 µL of the extracted RNA and 0.5 µL of MRV3 S1-specific primers were mixed with 2 × reaction mix, SSIII/Taq enzymes mix in a 25 µL reaction. The thermal cycling conditions included a reverse transcription at 55 °C for 15 min; initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 15 s, annealing at 55.4 °C for 30 s, extension at 68 °C for 30 s, and one final extension at 68 °C for 5 min. The amplified RT-PCR products were examined by agarose electrophoresis or subject to a second round nested PCR amplification. For the nested PCR, 5 µL of the first-round RT-PCR product was used as the template for the second round nested PCR in 50 µL reaction using GoTaq® Green Master Mix (Promega, WI). The prime set of the second round nested PCR was: FS03S1:418U23 (5′ GCGACACT GGATCATTAAC GACT 3′) and FS03S1:924L22 (5′ GGCTCATCCCAAT ACTACCACT 3′)

2.5. Quantification of porcine MRV3 RNA by quantitative RT-PCR (RT-qPCR)

Viral RNAs were quantified in pig fecal samples by RT-qPCR using MRV3-specific primers and probe targeting the MRV3 S1 segment. Briefly, the fecal samples from pigs were suspended in sterile PBS at 10% (w/v). The fecal suspensions were centrifuged at 8000 × g at 4 °C for 15 min, and the supernatants were transferred to fresh tubes for RNA extraction. Total RNAs were extracted from 250 µL of 10% fecal suspensions or diluted serum samples with TRIzol™ LS Reagent (Invitrogen). MRV3 RNAs were quantified using the SensiFAST™ Probe No-ROX One-Step kit (BIOLINE USA Inc. USA) with the forward primer (FS03S1:306F22 5′ CTTGATTCGAGT GTTACCCAGT 3′), reverse primer (FS03S1:414R21 5′ TAATGATCCAGTGTCGCGTTC 3′), and a hybridization probe (FS03S1:345L23 5′ CCTGCAAATCCTGTCTCAAGCTG 3′, which contains a 5′ 6-carboxy fluorescein fluorophore and 3′ black hole quencher (BHQ) by following a protocol described previously (Jothikumar et al., 2006). The RT-qPCR assay was performed in a CFX96 real-time PCR system (Bio-Rad Laboratories). *In vitro* transcribed and purified MRV3 S1 segment RNAs were used to produce a standard curve in RT-qPCR assay. The thermal cycling conditions of the RT-qPCR assay are as follows: 45 °C for 10 min (reverse transcription); 95 °C for 2 min (initial denaturation); and 95 °C for 5 s followed by 55 °C for 20 s (PCR amplification) for 40 cycles. The detection limit of the RT-qPCR assay is 10 viral genomic copies as previously reported (Jothikumar et al., 2006; Mokhtari et al., 2013).

2.6. Preparation of an inactivated MRV3 vaccine

The MRV3 BM100 virus, which was isolated from blood meals of pigs, was used as the seed virus for vaccine preparation. Briefly, the BM100 virus was propagated in BHK-21 cells, and the infected cells were frozen and thawed 3 times to release the intracellular virions. The cell debris was removed by centrifugation at 4000 × *g* for 20 min at 4 °C. The infectious titer of the virus in the supernatant was determined using the $TCID_{50}$ method in 96-well plates. Subsequently, the MRV3 BM100 virus stock was inactivated by binary ethyleneimine (BEI) at 37 °C. To determine the inactivation kinetics of MRV3, serial samples (0.5 mL) with different inactivation time points were collected at 6, 12, 24, 48 and 72 h post-inactivation (hpi). BEI was neutralized with 10% 1 M sodium thiosulfate (STS) to a final concentration of 2%. The tissue culture supernatant of serial samples was serially diluted 10-fold and inoculated onto Vero cells in 96-well culture plates to determine the kinetics of BEI inactivation of MRV3. The time point of the sample that showed no obvious CPE after three blind passages was set as the cut-off for the MRV3 inactivation point. To prepare the inactivated vaccine for