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Genetic and pathogenic characterization of a novel reassortant mammalian orthoreovirus 3 (MRV3) from a diarrheic piglet and seroepidemiological survey of MRV3 in diarrheic pigs from east China

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ABSTRACT

Mammalian orthoreoviruses (MRVs), which cause gastrointestinal and respiratory illness, have been isolated from a wide variety of mammalian species including bats, minks, pigs and humans. Here we report the isolation and genetic and pathogenic characterization of a novel MRV type 3 (MRV3), named MRV-ZJ2013, from the diarrheic feces of piglets in Zhejiang province, China. Genomic and phylogenetic analysis shows that MRV-ZJ2013 may have originated from reassortments among mink, bat, and pig MRVs, suggesting the hypothesis that interspecies transmission has occurred in pig herds. Neonatal piglets infected with MRV-ZJ2013 displayed mild clinical signs such as poor appetite and soft feces, but vomiting and diarrhea were not observed. Fecal virus shedding was detected only in three out of six piglets, each for one- or two-day post-infection. In contrast, piglets inoculated with a virulent porcine epidemic diarrhea virus (PEDV) strain as the control group had severe signs characterized by acute vomiting and watery diarrhea. These findings suggest that the virulence of MRV-ZJ2013, if any, was likely not significant compared to that of PEDV. A seroepidemiological survey of MRV by means of an indirect enzyme-linked immune-sorbent assay (ELISA) based on a recombinant MRV3 capsid protein sigma1 as antigen revealed a high seroprevalence (77%) in 1037 samples from diarrheic pigs of different ages from 24 herds in seven provinces of east China between 2015 and 2016, indicating that MRV3 is endemic in pig herds in China, and may contribute collectively to enteric disease along with other porcine pathogens.

1. Introduction

Diarrhea is a common disorder in pigs, and the associated dehydration is a leading cause of mortality among piglets, leading to substantial economic losses in China, South Korea and the United States. The etiology of diarrhea is varied, including multiple viral, bacterial, and parasitic pathogens, but viruses such as porcine epidemic diarrhea virus (PEDV) (Huang et al., 2013), porcine delta coronavirus (PDCoV) (Jung et al., 2015; Wang et al., 2015b), transmissible gastroenteritis virus (TGEV) (Kim et al., 2000), rotavirus (RV) (Santos and Hoshino, 2005) and other enteroviruses (EV) (Saif, 1999), are the predominant factors in most cases. Repeated outbreaks have also been reported on farms in which piglets had been immunized by vaccines targeting these viruses. Recently, mammalian orthoreovirus (MRV), which can cause diarrhea alone or in co-infections with other known pathogens, has been of great concern in Asia, especially in China and South Korea (Dai et al., 2012a; Kwon et al., 2012a; Zhang et al., 2011b).

Orthoreoviruses belong to the genus *Orthoreovirus* in the family *Reoviridae*, and are divided into two subgroups, fusogenic and non-fusogenic, based on their ability to induce cell–cell fusion and syncytium formation (Day, 2009). Members of the species MRV are nonfusogenic, and are classified into four major serotypes (type 1 Lang, type 2 Jones, type 3 Dearing, and type 4 Ndelle) (Day, 2009). The MRV dsRNA genome contains 10 segments divided into three size classes based upon their characteristic mobility in gel electrophoresis: three large segments (L1, L2 and L3), three medium segments (M1, M2 and M3), and four small segments (S1, S2, S3 and S4). The total genome size is approximately 23,500 base pairs (Mertens, 2004).

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MRVs were traditionally believed to be the causative agents of mild respiratory and enteric infections, without significant clinical impact. However, in the last decade, increasing numbers of studies in humans and other mammals have shown that they can cause severe illness in humans and other mammals, including upper respiratory tract infections, encephalitis, and diarrhea (Chua et al., 2008; Ouattara et al., 2011a; Steyer et al., 2013a). MRVs have been isolated from a broad range of mammalian species, including bats (Hu et al., 2014; Kohl et al., 2012; Lelli et al., 2013; Lorusso et al., 2014; Thalmann et al., 2010; Wang et al., 2015a; Yang et al., 2015), civet cats (Li et al., 2015), cows (Anbalagan et al., 2014), mink (Lian et al., 2013), pigs (Dai et al., 2012b; Kwon et al., 2012b; Thimmasandra Narayanappa et al., 2015; Zhang et al., 2011a), dogs (Decaro et al., 2005) and humans (Chua et al., 2008; Ouattara et al., 2011b). However, porcine MRV failed to arouse the concern of researchers until 2007, when it was first reported as the cause of diarrhea in newborn piglets in China (Zhang et al., 2011a). Serotypes 1 and 3 of porcine MRV were subsequently isolated in China (Dai et al., 2012b; Zhang et al., 2011a) and the prevalence and genetic diversity of porcine MRVs circulating in South Korea was assessed in 2012 (Kwon et al., 2012b). They found that 19.0% of diarrheic fecal samples collected from 78 pig farms around the country tested positive for porcine MRV (Kwon et al., 2012b). In 2015, a novel type 3 MRV (MRV3) was isolated and characterized in the USA, having caused severe diarrhea and acute gastroenteritis in neonatal piglets with 100% mortality by 3 days post-infection (Thimmasandra Narayanappa et al., 2015). Therefore, porcine MRV may contribute to enteric disease alone or in combination with other swine pathogens.

Type 2 MRVs (MRV2) have recently been isolated, possibly originating from reassortment between bat, pig, and/or human MRV strains which have been associated with diarrhea, acute gastroenteritis and necrotizing encephalopathy (Wang et al., 2015a). Six bat MRV strains have been isolated which share high S1 segment sequence similarity with MRVs recovered from diseased mink, piglets, or humans. These studies suggest that interspecies transmission can occur between bats and pigs (Yang et al., 2015), with alteration of viral characteristics upon intragenic rearrangement and reassortment of the reovirus genomes. Here, we describe a porcine MRV3 strain with evidence of reassortment between mink, bat and pig viruses isolated from the diarrheic feces of piglets in Zhejiang province, China.

2. Materials and methods

2.1. Virus isolation

Vero (African green monkey kidney) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin, w/v). Fecal specimens, collected from 5 day-old diarrheic pigs in Zhejiang province in 2013, were homogenized in DMEM containing antibiotics, centrifuged at 4000 × *g* for 15 min, and the supernatants were collected. Samples were used to inoculate confluent monolayers of Vero cells with 0.5% (w/v) trypsin at 37 °C and 5% CO_2 and observed daily for 7 days to track development of cytopathic effect (CPE). The unknown virus was adapted and passaged 5 times serially using the culture supernatant in Vero cells then subjected to the next generation sequencing on an Illumina MiSeq platform by a commercial company (Huada Gene Technology Co., Ltd.). Briefly, random RT-PCR was performed using first reverse transcription and then primer extension using Klenow DNA polymerase and a primer with degenerate 3′-end (GCCGACTAATGCGTAGTCNNNNNNNNN). The double stranded DNA was further amplified and the PCR product was then used as input to generate a library for Illumina MiSeq (2 × 250 bases) using Nextera™ XT Sample Preparation Kit with dual barcoding. The isolated virus was named MRV-ZJ2013 after determination of the genome.

2.2. Plaque assay and generation of MRV-ZJ2013 virus stock

Monolayers of Vero cells grown to 90% confluency in 6-well plates were inoculated with 10-fold serial dilutions of MRV-ZJ2013 suspended in modified Eagle's medium (MEM) supplemented with 0.5% (w/v) trypsin. The virus was allowed to adsorb to the cell monolayer by incubating 2 h at 37 °C followed by removal of the inoculum then 2 ml of agar overlay (1% agar in MEM supplemented with 1% penicillin/streptomycin and 0.5% trypsin) was added to each well and allowed to solidify at room temperature for 10 min. After incubation at 37 °C for 2 days, cells were fixed by 2% formaldehyde solution and stained with crystal violet for visualization of plaques. The plaque-purified MRV-ZJ2013 was propagated in Vero cells as described above, with virus particles harvested from cells by three freeze-thaw cycles and the resulting suspension purified from cell debris by low-speed centrifugation (4000 × *g* for 15 min) used as the virus stocks for the subsequent study. The titer of the virus stock was determined by the plaque assay.

2.3. Electron microscopy

Vero cells infected by the MRV-ZJ2013 (at 6, 12, and 24 h post-inoculation, hpi) were fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) and 1% OsO4 in phosphate. Specimens were dehydrated in a graded series of ethanol dilutions (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 15–20 min at each step, then transferred to absolute acetone for 20 min. Subsequently, the specimens were placed in one of three mixtures of absolute acetone and Spurr resin (1:1, 1:3, and pure Spurr resin) for 1 h, 3 h, and overnight, respectively. Finally, ultrathin sections were stained by uranyl acetate and alkaline lead citrate for 5–10 min and observed using a Hitachi Model H-7650 TEM.

2.4. Characterization of in vitro growth and physicochemical properties of MRV-ZJ2013

Viral growth kinetics were examined by infecting Vero cells with MRV-ZJ2013 at an MOI (multiplicity of infection) of 0.01 for 2 h at 37 °C, after which the inoculum was replaced by maintenance medium. Supernatants of infected cells after freeze-thaw cycles were collected at 0, 6, 12, 24, 36, 48, 60, and 72 hpi, and virus titers ($TCID_{50}$) at each time point were determined in triplicate on Vero cells. UV-inactivated MRV-ZJ2013 was used as a negative control. Temperature sensitivity was assayed; MRV-ZJ2013 was heated to 37, 55, 65, 80, and 100 °C for 1 h, and infectious titers ($TCID_{50}$) were determined in triplicate on Vero cells.

2.5. Sequence and phylogenetic analysis

Total RNA was extracted from the isolated virus with TRIzol reagent according to the manufacturer's instructions (Invitrogen), and cDNAs were amplified with specific primers for each of the 10 segments according to the manufacturer's instructions (SuperScript II, Invitrogen). A total of 14 primer pairs were designed to amplify the complete genome of MRV-ZJ2013 (the primer sequences will be made available upon request). The RT-PCR products were individually subcloned into a pCR-Blunt vector (Invitrogen) followed by Sanger sequencing to determine the consensus sequences. The nucleotide and deduced amino acid (aa) sequences of the L class (L1, L2 and L3), M class (M1, M2 and M3) and S class (S1, S2, S3, and S4) segments were compared with the sequences available in GenBank using Mega5.2 program. Evolutionary analysis was performed using the maximum likelihood method and the Jukes-Cantor evolutionary model, with bootstrap consensus trees inferred from 1000 replicates taken to represent the evolutionary history of the taxa analyzed.