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Identification of novel and diverse rotaviruses in rodents and insectivores, and evidence of cross-species transmission into humans

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ABSTRACT

Rotaviruses are an important cause of severe diarrheal illness in children globally. We characterized rotaviruses sampled in humans, insectivores (shrews) and rodents from urban and rural regions of Zhejiang province, China. Phylogenetic analyses revealed seven genotypic constellations of human rotaviruses with six different combinations of G and P genotypes – G3P[8] (50.06%), G9P[8] (36.16%), G1P[8] (8.92%), G2P[4] (4.63%), G3P[3] (0.12%), and G3P[9] (0.12%). In rodents and shrews sampled from the same locality we identified a novel genotype constellation (G32-P[46]-I24-R18-C17-M17-A28-N17-T19-E24-H19), a novel P genotype (P[45]), and two different AU-1-like rotaviruses associated with a G3P[3] genotype combination. Of particular note was a novel rotavirus from a human patient that was closely related to viruses sampled from rodents in the same region, indicative of a local species jump. In sum, these data are suggestive of the cross-species transmission of rodent rotaviruses into humans and for reassortment among human and animal rotaviruses.

1. Introduction

Rotaviruses are an important cause of severe diarrheal illness in infants and young children globally, causing 453,000 deaths per year in those <5 years of age (Estes and Greenberg, 2013, Tate et al., 2008). Rotaviruses are members of the genus *Rotavirus* of the family *Reoviridae* and possess a genome comprising 11 double-stranded RNA segments that encodes 6 structural (VP1-VP4, VP6, VP7) and 6 non-structural (NSP1-NSP6) proteins. Of the documented species (rotavirus A-H) (Matthijnssens et al., 2012) and the tentative species (rotavirus I) (Mihalov-Kovács et al., 2015), rotavirus A (RVA) is responsible for the majority of seasonal endemic diarrheal disease in young children. Based on sequences of the VP7 and VP4 genes RVAs have been further classified into G and P genotypes, respectively. To date, at least 31 G and 44 P genotypes have been identified worldwide (Matthijnssens et al., 2011, Trojnar et al., 2013; Rotavirus Classification Working Group (RCWG), 2015). Recently, a uniform system for rotavirus nomenclature was established for RVA based on nucleotide identities of the 11 rotavirus genome segments and phylogenetic analysis (Matthijnssens et al., 2008). Globally, the most common combinations in humans are G1, G2, G3, G4 or G9 and the P[4] or P[8] genotypes, especially G1P[8] which accounts for approximately 31% of human strains globally (Bányai et al., 2012, Dóró et al., 2014). Notably, genotype distributions vary among geographic regions, and several unusual strain combinations have recently been identified (Dóró et al., 2014, Nordgren et al., 2012).

As well as humans, rotaviruses infect a wide range of vertebrates including domestic and wild mammals and birds (Estes and Greenberg, 2013). Rodentia (rodents) is the largest order of

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mammals, with approximately 2277 species distributed globally (Wilson and Reeder, 2005). It is therefore no surprise that rodents are also the largest zoonotic source of human infectious diseases (Luis et al., 2013, Meerburg et al., 2009). In addition to known pathogens, rodents are also an obvious source for the discovery of novel viruses, and new rodent arenaviruses, coronaviruses, and hantaviruses have been described recently (Gonzalez et al., 2007, Guo et al., 2013, Holmes and Zhang, 2015, Li et al., 2015, Wang et al., 2015). To date, however, relatively little attention has been directed toward the rotaviruses that might circulate in rodent populations (Everest et al., 2011, Firth et al., 2014, Greenwood & Sanchez, 2002, Linhares et al., 1986, Sachsenröder et al., 2014). However, as rodents (especially rats) often live in close proximity to humans and domestic animals, and at high densities, they may play an important role in the cross-species transmission of rotaviruses, including to human populations.

Zoonotic rotavirus infections in humans are not uncommon, and there are a growing number of reports describing the interspecies transmission of rotavirus among animals and from animals to humans (Ben Hadj Fredj et al., 2013, Bonica et al., 2015., Gautam et al., 2015, Martella et al., 2010; Medici et al., 2015, Zhou et al., 2015). Like influenza viruses, zoonotic rotaviruses can become increasingly "humanized" by reassortment with co-infecting human viruses (Cowley et al., 2013, Jeong et al., 2014, Matthijnssens et al., 2009, Matthijnssens et al., 2010, Theuns et al., 2015). Clearly, to better understand the evolution and emergence of rotaviruses it is important to determine the diversity, evolution and origins of rotaviruses in those animals that live in close proximity to humans, including rodents.

Wenzhou is located on the southwestern coast of Zhejiang province, China, and is a prefecture-level city (Figure S1). Wenzhou incorporates both urban and rural areas with a total population of 9.12 million, of which 1.69 million can be classed as pediatric. Wenzhou experiences a high level of rotavirus infections, totaling $>65{,}000$ cases each year. Longquan, also in Zhejiang province, is located 250 km west of Wenzhou, and is a county-level city with a population of approximately 280,000. More than 90% of the Longquan's total area is mountainous. Compared with Wenzhou, Longquan experiences a relatively low level of rotavirus infection. In this study we investigated the diversity of rotaviruses in humans and small mammals (rodents and shrews) in Wenzhou and Longquan, as well as the evolutionary relationships between these viruses and those circulating in the local human population.

2. Method and materials

2.1. Patient sampling

Between October 2013-December 2014 a total of 1099 stool specimens were collected from diarrheal patients visiting the Diarrheal Department of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou city, Zhejiang province, China. Patient demographic data and clinical symptoms, including any complications, were collected and evaluated. Signed individual written informed consent for fecal sample collection was obtained from the guardian of each patient.

2.2. Trapping of small animals and sample collection

Between March 2013–October 2014, 1865 small mammals (rodents and insectivores) were captured in Longwan, Lucheng, Ruian, and Wencheng counties/districts of Wenzhou city, as well as from Longquan city, as described previously (Guo et al., 2013) (Fig. S1). All animals were treated according to the "Rules for Implementation of Laboratory Animal Medicine" from the Ministry of Health, China. Trapped animals were identified by morphological examination and further verified by sequence analysis of the cytochrome b (Cyt-b) gene (Guo et al., 2013). All animals were anesthetized before they were sacrificed, and every effort was made to minimize suffering. Stool samples were collected from each animal and used for the detection of rotaviruses.

2.3. RT-PCR and sequencing

Total RNA was extracted from stool samples using the Bioteke fecal RNA isolation kit (Bioteke, Beijing, China) according to the manufacturer's instructions. All samples were screened for the presence of rotaviruses using nested RT-PCR. Two pairs of primers based on conserved regions of sequences of the VP7 segment of known group A rotaviruses were used (Table S1). RT-PCR was performed under the following conditions: incubation at 50 °C for 30 min and 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 40 s, annealing at 46 °C for 40 s, and extending at 72 °C for 60 s. The second amplification was performed as follows: incubation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 40 s, annealing at 46 °C for 40 s, and extending at 72 °C for 50 s. Both the VP7 and VP4 genes were recovered from each of the rotavirus positive samples. Additionally, at least one complete genome sequence of each genotype was recovered by RT-PCR using primers based on conserved regions of known viruses. All primer sequences are described in Table S2.

PCR products purified using the QIAquick Gel Extraction kit (Qiagen) were sent to Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) for sequencing. They were sequenced with the PCR primers in both directions on an ABI Prism 3130 automated capillary sequencer (Applied Biosystems, Foster City, CA). Additionally, primer walking was performed to obtain the complete sequence of large genomic segments. Sequencing results were compared with the non-redundant nucleotide database at GenBank using Blastn to confirm they were of rotavirus origin. All rotavirus sequences obtained here from humans and animals have been deposited in GenBank and assigned accession numbers KU243375-KU243694 (Table S3).

2.4. Phylogenetic analysis

In addition to the sequences recovered here, reference sequences that cover the phylogenetic diversity of rotaviruses were compiled for evolutionary analyses. The following rotavirus data set sizes were used: VP7=84 sequences; VP4=70 sequences; VP1=72 sequences; VP2=73 sequences; VP3=72 sequences; VP6=76 sequences; NSP1=77 sequences; NSP2=76 sequences; NSP3=75 sequences; NSP4=73 sequences; NSP5=74 sequences. The 5′ and 3′ terminal sequences of each segment complementary to the primers were excluded.

All viral genome sequences obtained in this study were manually edited using the Seqman program implemented in the DNAStar v7.1 software package (DNASTAR, Inc., USA). Complete coding regions were aligned using the ClustalW method implemented in MEGA v5.05 (Tamura et al., 2011). DNAStar was also used to calculate nucleotide and amino acid identities.

Phylogenetic trees of these data were inferred using the maximum likelihood (ML) method implemented in the PhyML v3.0 package (Guindon et al., 2009). The best-fit nucleotide substitution model was determined using jModeltest (Posada 2008). Accordingly, the VP1, VP2, VP3, NSP1 and NSP4 segments were analyzed using the HKY+I+Γ model; the VP4, VP6, NSP2 and NSP3 segments were analyzed using the GTR+Γ model; VP7 was analyzed using the HKY+Γ model; while NSP5 was analyzed using the GTR+I+ Γ model. In addition, 1000 bootstrap replicates were used to assess the support for individual nodes on each tree.