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Research Article

Isolation and Molecular Characterization of a Porcine Rotavirus from Henan Province of China

Erxin Wang[1], Lei He[1], Hewei Zhang[2], Xiangchao Cheng[1] and Chunjie Zhang[1]*

[1]Animal Disease and Public Security Academician Workstation of Henan Province/The Key Lab of Animal Disease and Public Health, Henan University of Science and Technology, China

[2]State Key Laboratory for Molecular Biology of Special Economic Animals, Institute of Special Economic Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, P.R. China

***Corresponding author:** Chunjie Zhang, Animal Disease and Public Security Academician Workstation of Henan Province/The Key Lab of Animal Disease and Public Health, Henan University of Science and Technology, China

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Abstract

A porcine rotavirus, strain HN-001, was isolated from fecal sample of a diarrhea piglet in Henan province, China. The virus emerged specific CPEs after 6 blind passages on MA-104 cells. The isolate has been successfully adapted on MA-104 cell line and virus titer reached to 105.0 TCID50/ml. Sequencing and phylogenetic analysis showed that the VP4 gene of new isolate had a high homology with that of YM strain (genotype P[7]). The VP7 gene of the isolate belonged to genotype G[5]. An experimental infection was conducted in 3-day old piglets with an oral inoculation of cell culture material of the new porcine rotavirus isolate. The infected piglets showed severe diarrhea at 24h after inoculation. The piglets died or were euthanized 50 hours post inoculation. At necropsy, large amount of watery cheese-like material was found in stomach and watery fluid in small intestines with thinned wall. The jejunum and ileum were partly erated with some yellow contents. The cecum was filled with yellow liquid and inflated. The results indicated that the new porcine rotavirus isolate is pathogenic strain and had caused a severe disease in piglets.

Keywords: Porcine rotavirus; Isolation; Characterization; Pathogenicity

Introduction

Porcine rotaviruses belong to the genus rotavirus in the family Reoviridae [1]. Porcine rotaviruses have so far been divided into four serogroups, named as A, B, C, and E [2]. Rotavirus appears as a wheel-like particle under negative staining electronic microscopy (EM) [3]. The rotavirus genome is composed of 11 double-stranded RNA segments, and the sizes range from 0.6 to 3.3 kb [4]. VP4, VP6, and VP7 have recently become the focuses of research due to their unique biological functionalities. Based on the antigenicity of VP4 and VP7, group A rotavirus is divided into genotypes P and G. Group A porcine rotavirus is consist of 37P genotypes and 27 G genotypes [5].

The incubation period for acute porcine rotavirus infections in piglets usually ranges from 16 to 24h, the clinical symptoms for infections include depression, diarrhea, and a large amount of mucosal feces [6]. Porcine rotaviruses mainly exist in the intestines of piglets at beginning of infection excrete into feces and spread throughout to other piglets by a fecal/oral route [7]. Porcine rotavirus is very resistant to surrounding environment and disinfectants and viral infectivity in feces can last for 7-9 months at room temperature [8].

In last two years, pig herds with serious diarrhea have been reported in a large number of areas of China. During disease survey and diagnosis, a rotavirus, HN-001, was isolated from fecal samples of young piglet with acute diarrhea in Henan province of China. The virus isolation, molecular characterization, and its pathogenic evaluation in piglets will be described.

Materials and Methods

Virus isolation

A total of 50 fecal samples were collected from piglets of ten day old in traditional small-scale pig farm in Henan province of China. The positive fecal samples were collected and mixed with phosphate-buffered saline (PBS, 0.1 M, pH 7.2). The samples were further processed by freezing and thawing twice followed by a centrifugation at 3000 rpm for 12 min at 4°C. The collected supernatant was filtered with a 0.22 μ filter (Life science, USA) and aliquoted and stored at -80°C in Laboratory for analysis. All the fecal samples were screened using a Rapid Rota Ag Test kit (BioNote.Inc, Korea) before performing virus isolation in tissue culture and positive samples were subject to further virus isolation .

The samples were pretreated with trypsin solution (Hyclone, USA) at a final concentration of 20 μg/mL at 37°C for 1h before inoculation onto the monolayer of MA-104 cells [9] in 24-well plates (Costar, USA). The negative control group was treated in the same method instead of maintenance medium (MEM, Hyclone, USA). Each well of the plate was inoculated with 200ul treated samples, 4 wells per sample. The plates were slightly shaked every 20 minutes for an hour in the incubator. One milliliter of MEM was added to each well of the plates. The plates were placed into the incubator at 37°C and observed daily for appearance of potential CPEs. The cell culture supernatant were collected and inoculated into fresh cells after 3 days of incubation. Cell culture supernatant, with or without CPEs, was collected, and stored at -8°C for further characterization.

Virus Titration and Immunofluorescence assay

MA-104 cell monolayers were propagated and prepared in 96-well cell culture plates two days before virus titration. The cell monolayers were washed with PBS twice before virus inoculation. Testing virus samples were 10-fold serially diluted in MEM in a separate set of test tubes. Samples at dilutions of 10-1, 10-2, 10-3, 10-4, 10-5, and 10-6 were inoculated to the cell monolayers at 100μl per well. Each sample dilution was added to 8 replicated wells on the plates. Cell monolayer

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with same amount of medium were served as negative controls. The CPEs were observed and recorded daily upto 5 days. The TCID50 of each testing samples was determined using the Karber method [10]. Monolayers of MA-104 cells were inoculated with rotavirus at a MOI of 0.01 and incubated for 12 h at 37°C. The cells were fixed with 80% ice-cold ethanol for 0.5 h at -20°C. 100× diluted monoclonal antibody specially against PoRV was added to the cells as primary antibodies for 40 min at 37°C followed by a 2000× diluted FITC-conjugated donkey anti-mouse IgG antibody (Life Technologies, Carlsbad, CA, USA). The cells were analyzed by fluorescence microscope.

RT-PCR

Total RNA was extracted from cell culture supernatant using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. A reverse transcription was performed using the M-MLV Reverse Tran scriptase (Taraka, Japan) to synthesize cDNA for further gene amplification. A pair of primers were designed for a 762bp portion of VP4 gene published in GenBank (Accession number: AY523636.1), with a forward primer 5'-GCC TTC TTA CAT TTA TGA CAC-3'and a reverse primer 5'-GTT CCA TTG CAT TTC TAT GC-3'. PCR reactions were carried out in PCR buffer containing 3 µl of cDNA, 2 µl of each dNTPs (2.5mmol), 0.5 µl of each primer, 0. 25 µl of rTaq in a 25 µL reaction volume. The PCR program included an initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 45s, 54°C for 45s and 72°C for 1min, followed by a 10 min extension at 72°C.

A complete VP7 gene of 1041 bp was amplified with a pair of primers, VP7-F 5'-GGC TTT ATT AGA GAC AAT TTC CGT-3' and VP7-R 5'-GCT CAC ACC ATA CAC TTC TAA CCT AAG-3'. The same PCR amplification reagents for VP4 were used VP7 gene amplification. The PCR program included an initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 45s, 57°C for 45s and 72°C for 1min, followed by a 10 min extension at 72°C.

Sequencing of the VP4 and VP7 gene

For each amplicons of VP4 and VP7, PCR was run at least twice and purified using the QIA quick Gel Extraction Kit (Qiagen, Germany) according to the instructions. The purified products of the VP4 and VP7 genes were cloned using the PMD-18T vector (Takara, Japan) and transformed into DH5α competent cells. The plasmid DNAs sequenced using an ABI3730 Prism DNA analyzer.

Pathogenicity evaluation

An experimental infection was carried out in order to determine whether the isolated virus could cause clinical disease in newborn piglets or not. Six 3-day-old piglets, free of rotavirus antibodies and antigens, were involved in the experimental infection. Piglets were transferred into isolators at Laboratory immediately, with three piglets in each isolator. The temperature in the isolators was maintained at 32°C or higher using heat lamps. Piglets were fed pasteurized whole milk supplemented with probiotic once a day in the morning and regular milk three times a day and approximately 70mL milk each time.

The 6 piglets, either males or females, were randomly assigned into two groups of 3 piglets. One group of piglets were orally received 5.0 ml of cell culture material of porcine rotavirus HN-001strain, equal to 105.0 TCID50/ml. The other group was orally received 5.0 ml phosphate buffered saline (PBS) per piglet as control. After inoculation, the piglets were closely observed every two hours a day for diarrhea and other gastrointestinal clinical signs. Piglets were humanely euthanized after the appearance of severe diarrhea or becoming moribund. The piglets were necropsied and intestinal material from each piglet was collected and stored at -80°C for further analysis. At end of the study, all the piglets were euthanized humanely.

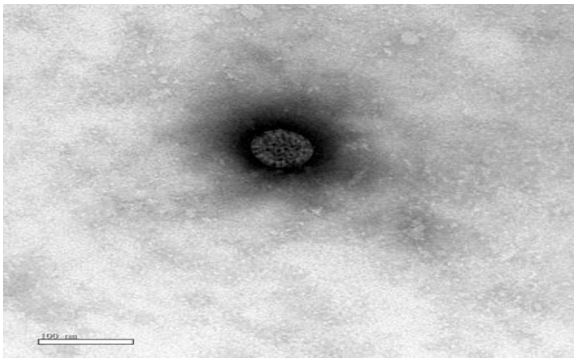

Figure 1: Electron microscopy images of rotavirus-like particles of strain HN001 infecting the MA-104 cells in cell culture media.

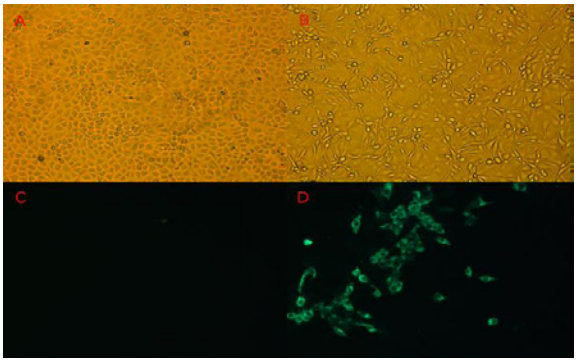

Figure 2: Cytopathic effects and Immunofluorescence assay of PoRV isolate
A and C were set as control.
B: At 24 h postinfection, the cytopathic effects were recorded as clustering, detachment.
B2: Cells were examined by IFA using PoRV-specific monoclonal antibody.

Results

Virus isolation

To successfully isolate the virus, a blind passage strategy was applied. Structural analysis of the infected cells by electron microscopy confirmed that the rotavirus virion was round with wheel-shape appearance. (Figure 1) Specific CPEs for rotavirus became visible in the MA-104 cells after continuously eight blind passages. Significant CPEs were detected in cell culture at 24 hours after 6 passages. The characteristic CPE for rotavirus, such as cell round up, clustering in grape bunches, seining were observed (Figure 2). As the virus adapting to the MA-104 cell, the virus propagated rapidly and virus titer has reached to 105.0TCID50/ml on day 5. Results from the immunofluorescence assay revealed that the attached fluorophore could be detected in infected MA-104 cells, whereas none were detected in the control group.

Identification of the VP4 and VP7 genes

The VP4 and VP7 genes were successfully amplified by the RT-

Table 1: Amino acid comparison (% aa identity) of the VP4 of the porcine strain HN-001 with other P genotypes.

Strain	GenBank accession No.	Species	P genotype	% Identity of VP4 gene
SA11	X14204	Simian	1	27.60%
SA11-N5	JQ688676.1	Simian	2	24.10%
K9	EU708926.1	Canine	3	24.90%
DS-1	AB848005.1	Human	4	54.80%
P343	U35851.1	Porcine	5	53.50%
Gottfried	M33516.1	Porcine	6	65%
YM	M63231.1	Porcine	7	92.10%
DRC88	DQ005111.1	Human	8	63.80%
T152	AB077766.1	Human	9	56.60%
69M	EF672556.1	Human	10	72.30%
I321	L07657.1	Bovine	11	58.40%
FI-14	D13398.1	Equine	12	73.20%
MDR-13	L07886.1	Porcine	13	65.60%
B4106	AY740738.1	Human	14	73.10%
Lp14	L11599.1	Lamb	15	55.10%
EW	U08429.1	Murine	16	62.60%
PO-13	AB009632.2	Avian	17	57.40%
GBR	JF712558.1	Horse	18	55.30%
Mc323	D38052.1	Human	19	63.50%
EHP	U08424.1	Murine	20	65.20%
Hg18	AF237665.1	Bovine	21	48.80%
160-01	AF526374.1	Rabbit	22	51.30%
A34	AY174094.1	Porcine	23	41.40%
TUCH	FJ816611.1	Rhesus	24	68.40%
Dhaka	GU199520.1	Human	25	57.30%
134-04	DQ061053.1	Porcine	26	62.30%
344-04	DQ242615.1	Porcine	27	68%
Ecu534	EU805773.1	Human	28	52.80%
Azuk-1	AB454420.1	Bovine	29	37.20%
DEU 03	NC-021581.1	Chicken	30	2.80%
03V0567	JQ920006	Chicken	31	3.60%
61-07-ire	FJ492835.1	Porcine	32	69.70%
Dai-10	AB513836.1	Bovine	33	72.30%
FGP51	AB571047.1	Porcine	34	75.10%
DEU	NC-021631.1	Turkey	35	2.70%
SG3	AB823215.1	Glider	36	68.60%
GER	JX204814.1	Pheasant	37	66.30%

PCR programs and revealed on agarose gel electrophoresis as the expected fragments, 762bp for VP4 gene and 1041bp for VP7 gene, respectively.

Sequencing and Phylogenetic analysis of the VP4 gene

The amplified VP4 gene of HN-001 strain was sequenced and its deducted amino acid sequence were compared with known amino acid sequences in the Genebank (Table 1) to further determine the P genotype of the isolate [11]. The similarity of amino acid sequence of VP4 gene of HN-001 strain to existing rotavirus strains ranges from 2.7% to 92.1%, with the highest degree of amino acid identity found in strain YM [12], belonged to genotype P[7]. The lowest identify was found in the strain DEU, of genotype P[35] [13]. Blast analysis revealed that the strain HN-001 had a high homology with porcine rotavirus strain CRW-8 which belonged to P[7].

Phylogenetic analysis of the deduced VP4 amino acid sequences indicated that strain HN-001 is closely related to strain YM and the two strains are grouped to form a distinct cluster (Figure 3A). JS-

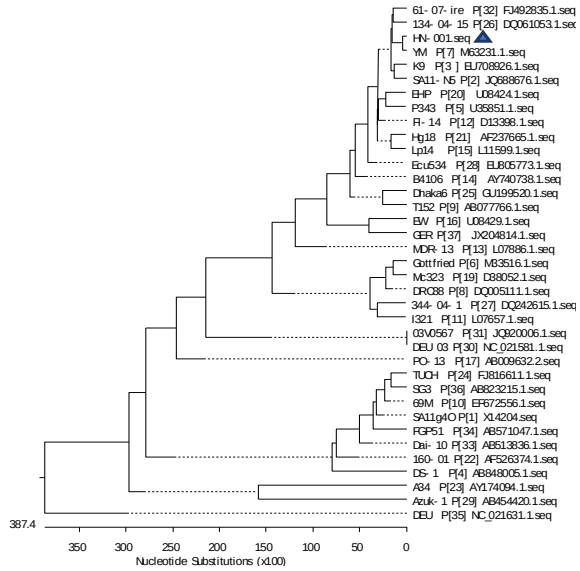

Figure 3A: Phylogenetic tree based on the partial VP4 sequence of Porcine rotavirus detected in Henan province.

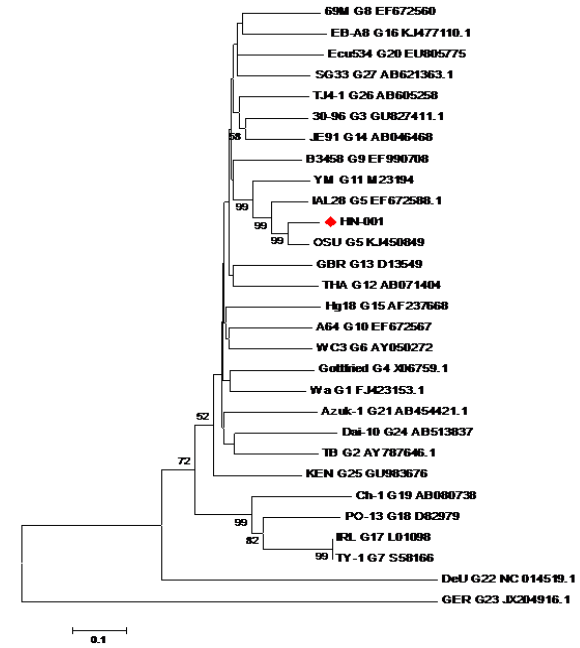

Figure 3B: Phylogenetic tree based on the partial VP7 sequence of Porcine rotavirus detected in Henan province.

Table 2: Amino acid comparison (% aa identity) of the VP7 of the porcine strain HN-001 with other G genotypes.

Strain	GenBank accession No.	Species	G genotype	% Identity of VP7 gene
Wa	FJ423153.1	Human	1	65.60%
TB	AY787646.1	Human	2	54.20%
30-96	GU827411.1	Rabbit	3	73.10%
Gottfried	X06759.1	Porcine	4	17.40%
IAL-28	EF672588.1	Porcine	5	88.60%
WC3	AY050272	Bovine	6	55.70%
Ty-1	S58166	Turkey	7	36.80%
69M	EF672560	Human	8	69.10%
B3458	EF990708	Porcine	9	77.20%
A64	EF672567	Human	10	80.40%
YM	M23194	Porcine	11	73.20%
THA	AB071404	Human	12	22.10%
GBR	D13549	Horse	13	18.30%
JE91	AB046468	Horse	14	44.70%
Hg18	AF237668	Bovine	15	77.20%
EB-A8	KJ477110.1	Murine	16	75.20%
IRL	L01098	Turkey	17	2.50%
PO-13	D82979	Pigeon	18	61.10%
Ch-1	AB080738	Avian	19	9.70%
Ecu534	EU805775	Human	20	68.20%
Azuk-1	AB454421.1	Bovine	21	73.20%
Deu	NC-014519.1	Chicken	22	47.10%
GER	JX204916.1	Pheasant	23	56.20%
Dai-10	AB513837	Bovine	24	60.20%
KEN	GU983676	Porcine	25	70.50%
TJ4-1	AB605258	Porcine	26	75.10%
SG33	AB621363.1	Porcine	27	73.10%

01-2014 is closer to strain 61-07-ire [14], 134-04-15[15] in terms of genetic distance, indicating a possible genetic relationship. The DEU which isolated from the chicken showed the furthest distance with the isolate strain HN-001.

Sequencing and Phylogenetic analysis of the VP7 Gene

A full length of VP7 gene of strain HN-001 was amplified using RT-PCR and resulting a full-length of open reading frames (ORFs) of 981 nt codes for the VP7 protein of 327 amino acids. Blast analysis has shown that the strain HN-001 has a high degree of homology with US strain A2, belonging to genotype G[5]. The amino acid similarity of strain HN-001 to other existing strains ranges from 2.5% to 88.6%. (Table 2) The lowest amino acid similarity was found with strain IRL [16] a turkey isolate, belonging to genotype G[17]. The isolate IAL-28 have the highest amino acid similarity to the strain HN-001.

Phylogenetic analyses of the VP7 genes suggested various degrees of sequence identity to other genotypes. (Figure 3B) Strain HN-001, IAL-28 and A64 were grouped together to form a cluster. Although the strain CH-1[17] belonged to the group A rotavirus, as with other strains described above, it was genetically distant from other genotypes and formed a separate genetic branch, indicating that avian rotavirus is genetically distant from other species.

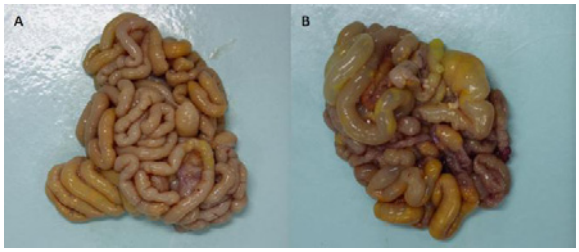

Figure 4: The tissues of the infected and control groups were grossly examined, the analyze was as follows.
A was set as control
B The transparent intestine walls with accumulation of yellowish fluids were observed in the infected piglets.

Pathogenicity Results

The piglets showed clinical signs 16- 24 hours after orally inoculation with rotavirus strain HN-001. The clinical signs include diarrhea with yellow and watery feces. The piglets showed serious depression, loss of appetite, poor health condition, serious dehydration. The infected piglets either died or became moribund at Day 4 after inoculation and were euthanized. All the piglets in control group showed no abnormal clinical signs. At necropsy, watery white cheese-like material was seen in stomach and a large quantity of fluid in the small intestines with thinned wall. The jejunum and ileum were partly aerated with some yellow contents. The cecum together with the colon was filled with yellow liquid and was inflated (Figure 4).

Discussions

Porcine rotavirus infection is a very common infection in pig herds and usually co-infected with other diarrhea viruses, such as PEDV, TGEV, Kobu and E.coli. Porcine rotaviruses were first adapted to grow in porcine primary kidney cells with pretreated trypsin in 1984 [18]. In this study, the samples were pretreated with equal volumes of trypsin in the same manner and followed blind passages on MA104. After 6 passages, the virus was successfully adapted to the cells and produced CPEs and virus titer could reach to 105.0 TCID50/ml. A preliminary experimental challenge model has been evaluated with pure porcine rotavirus culture in young piglets and the virus isolate alone could induce serious clinical diseases such as diarrhea. The model can provide the tools for the research of pathogenic mechanism.

Porcine rotaviruses have a strong viability in the environment and are insensitive to many chemical disinfectants and preservatives [19]. This is perhaps one reason why the prevalence of rotavirus is very often reported. Due to the numerous genotypes of VP4 and VP7 genes, no effective vaccine available were shown on the market. Although live vaccine was reported in the market, the results were far from the expected [20]. VP4, VP6, VP7 were known as the antigens of the virus and had been focused. A subunit vaccine representing dominant porcine rotavirus genotype could provide a solution for farms to prevent and control the diseases directly or indirectly caused by porcine rotaviruses. The research data from current study will provide valuable information for researching the disease and a future

vaccine design.

Conoclusion

This study indicated that the VP4 gene of the isolate belonged to genoty pe P[7], while the VP7 gene belonged to the genotype G[5]. The experimental infection model showed that the new isolate is pathogenic str ain. This experimental infection model has established a foundation for further studies on vaccine development.

Acknowledgements

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