

Basic Study

Development of a human rotavirus induced diarrhea model in Chinese mini-pigs

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Abstract

AIM: To establish a new animal model for the research of human rotavirus (HRV) infection, its pathogenesis and immunity and evaluation of potential vaccines.

METHODS: 5-d, 30-d and 60-d-old Chinese mini-pigs, Guizhou and Bamma, were inoculated with a single oral dose of attenuated strain Wa, G1, G3 of HRV, and PBS (control), respectively, and fecal samples of pigs from 0 to 7 d post infection (DPI) were collected individually. Enzyme linked immunosorbent assay was used to detect HRV antigen in feces. The HRV was tested by real-time PCR (RT-PCR). The sections of the intestinal tissue were stained with hematoxylin and eosin to observe the morphologic variation by microscopy. Immunofluorescence was used to determine the HRV in intestinal tissue. HRV particles in cells of the ileum were observed by electron micrography.

RESULTS: When inoculated with HRV, mini-pigs younger than 30 d developed diarrhea in an age-dependent manner and shed HRV antigen of the same inoculum, as demonstrated by RT-PCR.

Histopathological changes were observed in HRV inoculated mini-pigs including small intestinal cell tumefaction and necrosis. HRV that was distributed in the small intestine was restricted to the top part of the villi on the internal wall of the ileum, which was observed by immunofluorescence and transmission electron microscopy. Virus particles were observed in Golgi like follicles in HRV-infected neonatal mini-pigs. Guizhou mini-pigs were more sensitive to HRV than Bamma with respect to RV antigen shedding and clinical diarrhea.

CONCLUSION: These results indicate that we have established a mini-pig model of HRV induced diarrhea. Our findings are useful for the understanding of the pathogenic mechanisms of HRV infection.

Key words: Human rotavirus; Animal model; Chinese mini-pigs; Diarrhea; Intestine

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Core tip: Rotavirus (RV) is the leading cause of serious dehydrating diarrhea in infants and young children worldwide. Animal models for RV infection research have provided key insights into the RV infection, its pathogenesis and immunity, and have offered opportunities for design and evaluation of potential vaccines. Our study indicated that human RV (HRV) could effectively replicate in the intestinal villi of the ileum of Chinese mini-pigs and lead to histopathological alterations and diarrhea. This research offers a new animal model for studying the pathological changes and immunogenicity of HRV infection and a useful tool in the design and evaluation of RV vaccines.

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INTRODUCTION

Rotavirus (RV) is well known as the major cause of severe gastroenteritis in infants under five years of age, responsible for an estimated 480000 to 640000 deaths per year worldwide^[1]. Currently, there is no specific medicine for treating RV diarrhea; thus, a safe and effective vaccine would be the best way to control RV^[2]. To date, all approved RV vaccines delivered through oral imitation to mimic natural infection have been in attenuated form. Although natural RV infection has shown substantial protection against RV infection, potential virulence recovery of attenuated RV remains a safety concern. Moreover, the immune response

mechanism to RV infection is elusive, which hampers the development of RV vaccines. Therefore, an ideal animal model of human RV (HRV) infection is urgently needed to improve the understanding of the immune response to RV infection in humans. Previously tested animal models include mouse, rabbit, rat, lamb, calf and gnotobiotic pig^[3-8]. However, our understanding of the immune protection mechanism against HRV infection has been limited to these models. Due to host restriction, small animals such as rodents and rabbits are prone to refractory HRV infection, limiting their potential for studying the pathogenesis and immunity of HRV infection. On the other hand, while larger animals, such as Large White cross-bred pigs could be infected with HRV, genetic consistency and hereditary stability may affect the HRV infection^[9-24].

Chinese mini-pigs have been used in many medical experiments as animal models^[25]. In this study, two breeds of Chinese mini-pigs, Guizhou and Bamma, were selected to develop an HRV induced diarrhea model because of the following advantages: (1) higher genetic consistency and hereditary stability compared to conventional pigs, with the group similarity coefficient close to 0.928 after inbreeding for nearly 20 generations; (2) early sexual maturation with high reproduction rate; and (3) small size (the neonate Guizhou mini-pig weighs 0.3-0.5 kg and the one-month-old weighs 1.0-1.5 kg), comparable to rabbits^[26,27]. We examined whether HRV can replicate efficiently in Chinese mini-pigs due to the different gene background. Furthermore, we were interested in elucidating whether different G serotypes of human group A RV can replicate, spread, and induce disease. Our study findings indicated that HRV could effectively replicate in the intestinal villi of the ileum of Chinese mini-pigs and lead to histopathological alterations and diarrhea. This research offers a new animal model for studying the pathological change and immunogenicity of HRV infection and a useful tool in the design and/or evaluation of RV vaccines.

MATERIALS AND METHODS

Experimental mini-pigs

Guizhou and Bamma mini-pigs from the Experimental Animal Center of the Third Military Medical University (Chongqing, China) were placed in germ-free isolator units immediately after birth. Prior to inoculation with HRV, all mini-pigs were seronegative for RV antibodies for a dilution ratio of 1:10, as detected by enzyme linked immunosorbent assay (ELISA).

Virus stocks

Wa strain of HRV was originally obtained from Dr. Elschner and MA104 cells were used to grow the virus in the presence of 0.5 µg/mL trypsin. The dose and median infection dose (PID50) of Wa strain HRV were determined as previously described^[13]. The PID50 of the Wa HRV stock inoculum for inoculation in mini-pigs

was almost 10^6 focus forming units (FFUs).

A total of 42 stool samples collected from children with acute RV induced diarrhea in two consecutive winter seasons from October 2003 to April 2005 were used in this study. Viral RNA was extracted and used as templates for real-time PCR (RT-PCR). G and P typing was realized by nested amplification of various gene sequences of the proteins of VP7 and VP4, which respectively consist of six G- and five P-type-specific primers (multiplex PCR)^[28]. Two of the samples typed as serotype G1 or G3 were selected for use as animal inocula. After multiple centrifugations to remove the fecal debris from the selected samples, the supernatant was used to inoculate mini-pigs orally for 3-5 d. The intestinal contents were collected and three passages were subsequently conducted. The intestinal content obtained from the 4th mini-pig passage was used as the virulent Wa HRV stock inoculum. The titers of infectious virus from the virulent Wa HRV stock inoculum were detected by fluorescent-focus assay (FFA) and confirmed by FFU^[29]. The stock inoculum was identified and analyzed by dilution of serially diluted (10-fold, in duplicate) single layer cells of MA104. Infectivity titers were indicated *via* FFU. On the basis of the toxicity, 1:10 was the minimum dilution for fecal samples identified for infectivity titers. If the fluorescent foci with the dilution ratio of 1:10 were not visualized by fluorescence microscopy, the sample was labeled as negative.

Inoculation of mini-pigs with HRV

In this study phase, 5-, 30- and 60-d-old mini-pigs were inoculated with a single oral dose of attenuated strain Wa, G1, G3 of HRV, and PBS (control), respectively. Prior to oral inoculation, mini-pigs received 5 mL NaHCO_3 (100 mmol/L) to reduce gastric acidity and, after 5 to 10 min, 5 mL of virus were slowly instilled into the mouth at the back of the throat using a needleless syringe.

Sample collection and processing

The fecal samples from the studied pigs were collected from 0 to 7 d post infection (DPI) individually. In order to avoid cross-contamination, cages and bedding were changed every day during the sample collection period. Presence of diarrhea was identified in the individual fecal samples by gently pressing the abdomen of neonatal pigs every day. As described earlier, cold (4 °C) PBS containing gentamicin (2 mg/mL, Sangon, Shanghai, China), streptomycin (200 U/mL), and penicillin (200 U/mL) was used to process fecal samples as 10% solution for the subsequent test^[30]. Based on the assessment of color, consistency, and the amount of feces, diarrhea was scored from 1 to 4^[31]. A score > 2 was considered indicative of diarrhea. Three 1 cm long sections, consisting of the duodenum (which was proximal to the stomach), ileum (located at the distal part of the intestine), and jejunum (situated

in the middle section of the intestine between the duodenum and ileum) of each pig, were obtained and homogenized.

Detection of HRV antigen in feces by ELISA

As described previously, the expression of HRV antigen in samples of fecal or intestinal contents was detected using aforementioned mono-antibodies by ELISA^[32]. A reaction was considered positive if: (1) the difference between the optical density (OD) values of the well to which the sample containing virus was added and the well lacking antigen was > 0.1; and (2) the difference between the OD values of the well to which the sample containing virus and the negative control well was equal to or exceeded two standard deviations.

To further ensure that pigs only shed the impregnated virus, fecal suspensions obtained from the pigs that were virus-infected as well as positive for RV (as established by ELISA) were examined by RT-PCR. Nucleic acids were extracted from virus that was recovered from fecal materials, and the results were determined by RT-PCR. The PCR serotype-specific primers were as previously described^[33]. All experiments were performed in triplicate at the minimum, yielding reproducible results.

Preparation of small intestine tissue sections and histopathological observation

To evaluate the distribution of neutral and acid mucopolysaccharides and the histopathology, 10% zinc-formalin was used to fix sections obtained from the small intestine 24 h prior to transferring to 70% graded ethanol series for dehydration. Next, intestinal tissue samples were embedded in paraffin wax before being sectioned at the 4 μm thickness, as described previously^[27,32].

Histopathological findings in sections obtained from the small intestine of newborn pigs sacrificed at 48 h post-inoculation (hpi) were evaluated. The sections were stained with hematoxylin and eosin^[34]. The stained villi were observed under a light microscope in order to identify any changes in vacuolization, presence of enterocyte injury or inflammation.

Detection of RV in tissues

Frozen sections from pigs sacrificed at 24, 48, and 72 hpi were allowed to air dry completely before being fixed for 10 min with 95% alcohol. PBS containing 0.05% Tween 20 (PBS-T) was used to wash the slides twice before placing them in distilled water and blocking them with 2% normal bovine serum (Jingmei, China) for 0.5 h at room temperature (RT). After decanting the excess bovine serum, and at room temperature, the slides were incubated with either sheep anti-VP6 or anti-VP7 serum (Dako Diagnostics, Inc.) at a dilution of 1:100 for 2 h at RT. Next, slides were washed twice with PBS-T and incubated for 2 h at RT with donkey anti-sheep Ig antibody conjugated

with fluorescein isothiocyanate, which was mixed with PBS at a dilution of 1:200 (Sigma Chemical Co., St. Louis, MO, United States). After incubation, slides were flushed gently with PBS-T and sealed with moderate aqueous mounting medium (Dako Diagnostics, Inc.) and covered with coverslips. The Leica TCS-SP5 microscope was used to detect fluorescence (Leica, Inc., Germany).

Additional samples of mid-duodenum, mid-jejunum, and mid-ileum collected from newborn pigs sacrificed at 24, 48 and 72 hpi were fixed with buffer containing 2.5% glutaraldehyde and 0.1 mol/L cacodylate (pH 7.4) before adding buffer containing 1% osmium tetroxide. Plastic was used to embed the fixed samples, to ensure that they could be sectioned in thin slices and analyzed by transmission electron microscopy (TEM). Sections (400 Å) were stained with uranyl acetate and were observed under a HITACHI H600 TEM.

Statistical analysis

SPSS Version 11 for MS windows (SPSS, Inc., Chicago, IL, United States) was used to perform statistical analyses. In brief, one-way analysis of variance (ANOVA) was used for the comparison of distinct data for the virus shedding (in mean days), diarrhea rate and severity scores of disease for groups of different ages. In addition, the gradients of the cumulative weight data obtained each day were analyzed by linear regression and were compared for equality of means by using the *t*-test. Pearson's correlation coefficient was used to calculate correlation coefficients. Difference was deemed statistically significant at $P < 0.05$.

RESULTS

Clinical and serological responses of HRV-inoculated mini-pigs

We inoculated 5-d-old mini-pigs with G1 HRV field strain, G3 HRV field strain, G1 HRV attenuated Wa strain, or PBS. Mini-pigs were randomly divided into four groups. All mini-pigs received oral inoculations of G1 HRV field strain and G3 HRV field strain and developed obvious clinical signs of infection within the 7-d observation period. In brief, mini-pigs infected with human RV produced feces that were profuse, watery and often floccular; thus, they became anorexic within 12–72 h of the inoculation. In contrast, none of the mini-pigs that were treated with oral inoculations of G1 HRV attenuated Wa strain or PBS developed clinical signs of infection.

Antigen of HRV (Figure 1A) or infectious virus (Figure 2A) was not detected by ELISA or FFA in 5-d-old mini-pigs that were inoculated with G1 HRV attenuated Wa strain over the 7-d study period. The pigs that were inoculated with G1 HRV attenuated Wa strain shed virus antigen, starting from as early as 1 DPI (Figure

1A), while in those inoculated with G1 HRV field strain (Figure 1B) and G3 HRV field strain (Figure 1C) these effects were noted during a period of 1 to 3 d. The virus antigen quantities shed by the mini-pigs were correlated with each of the infectious viruses ($r = 0.852$, $P = 0.019$; Pearson's correlation coefficient). Neonatal pigs that were impregnated with G1 HRV attenuated Wa strain shed virus antigen for 2 d, while they shed infectious virus for 1 d (Figures 1A and 2A). The quantity of the infectious virus shed by pigs impregnated with G3 HRV field strain was higher compared to those inoculated by G1 HRV field strain, for both Guizhou pigs and Bamma pigs (Figure 1B and C, Figure 2B and C, respectively). Neonatal pigs inoculated with G1 HRV field strain and G3 HRV field strain shed virus antigen or inoculated virus during the entire 7-d period (Figure 1B and C, Figure 2B and C, respectively). The control group, mini-pigs impregnated with PBS only shed neither antigen nor infective virus (Figures 1D and Figure 2D, respectively).

We found that the excreted virus and human virus with which pigs were inoculated were the same, rather than porcine RV, by detecting the RNA extracted from the virus collected from selected fecal samples by RT-PCR. The virus from newborn mini-pigs inoculated with G1 HRV field strain and G3 HRV field strain was the same to the respective virus inoculum. No evidence of virus was found in samples from neonatal mini-pigs inoculated with G1 HRV attenuated Wa strain, most likely because of the low quantity of virus excreted by these pigs.

All 5-d-old virus-inoculated Guizhou pigs that were not PBS-inoculated, as well as Bamma pigs, developed diarrhea (Table 1). However, because of efficient horizontal transmission, the severity of diarrhea developed by PBS-inoculated littermates of G1 HRV field strain and G3 HRV field strain inoculated group and their respective virus-inoculated littermates was similar, while the PBS-inoculated littermates of G1 HRV attenuated Wa strain inoculated group developed mild diarrhea (score of 4 in the Guizhou group and 6 in the Bamma group, respectively). The mean severity of disease was based on the scores obtained by dividing the total score by the total number of samples every day.

Histopathological lesions of the intestine from 5-d-old mini-pigs

In order to confirm whether human RV infection caused histopathological lesions in the intestine of the mini-pigs that were inoculated with only 0.5 mL PBS or 0.5×10^7 FFUs of G1 HRV field strain and G3 HRV field strain, these individuals were euthanatized from 0 to 168 hpi. Postmortem examination revealed no obvious changes in the intestine of the control mini-pigs that were euthanatized at each time point. In G1 HRV field strain and G3 HRV field strain infected mini-pigs, fluid accumulation in the small intestine was first viewed

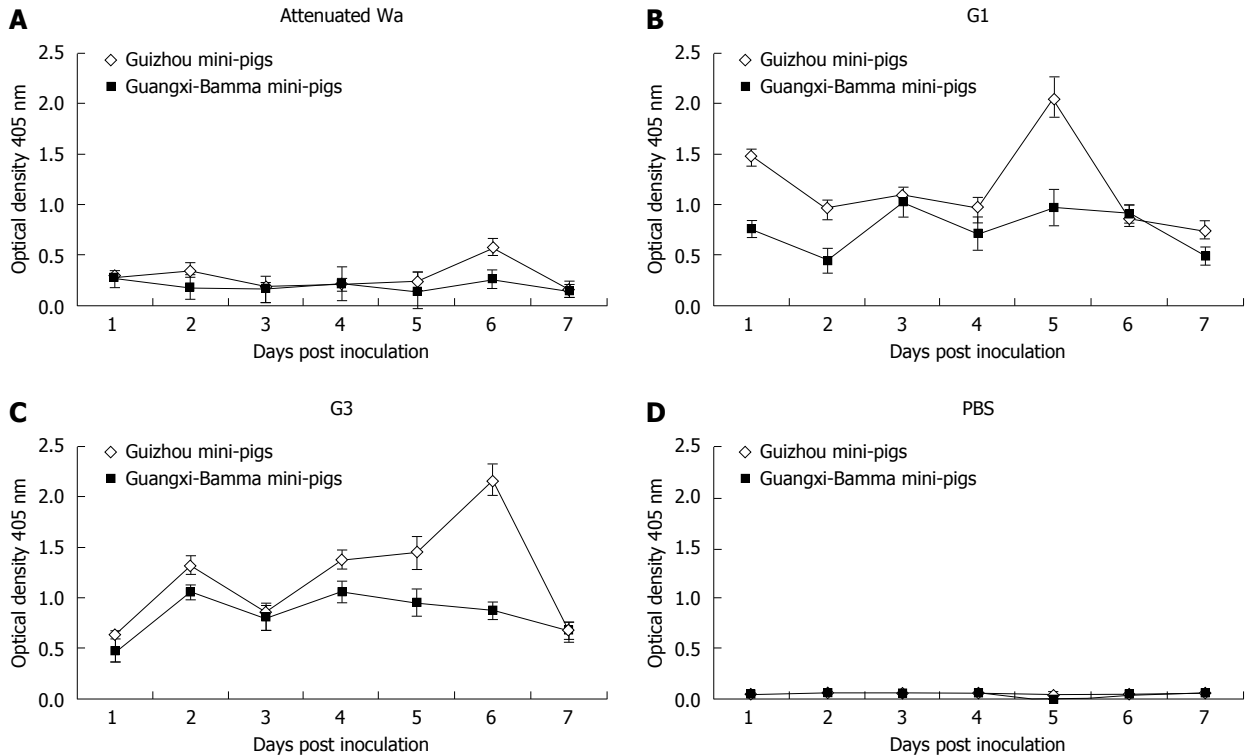


Figure 1 The curves of viral antigen-shedding in fecal samples from 3- to 5-d-old PBS or human rotavirus-inoculated mini-pigs. The amount of viral antigen in fecal samples from the mini-pigs impregnated with 1 mL of 0.5×10^7 FFUs of human attenuated RV Wa (A), 0.5×10^7 FFUs of human RV G1 (B), 0.5×10^7 FFUs of human RV G3 (C), and 1 mL of PBS (D). Viral antigen shedding from 0 to 7 DPI was assessed by ELISA and the OD, as net readings, was identified at 450 nm. Only values exceeding 0.1 were considered positive. Fresh fecal samples from mini-pigs were collected every day and were stored at -80°C . Every virus-inoculated group consisted of three virus-impregnated and one PBS-impregnated mini-pig to supervise transmission among mock-impregnated littermates. All the PBS inoculations were performed before any inoculation with the virus. FFUs: Focus forming units; DPI: Days post infection.

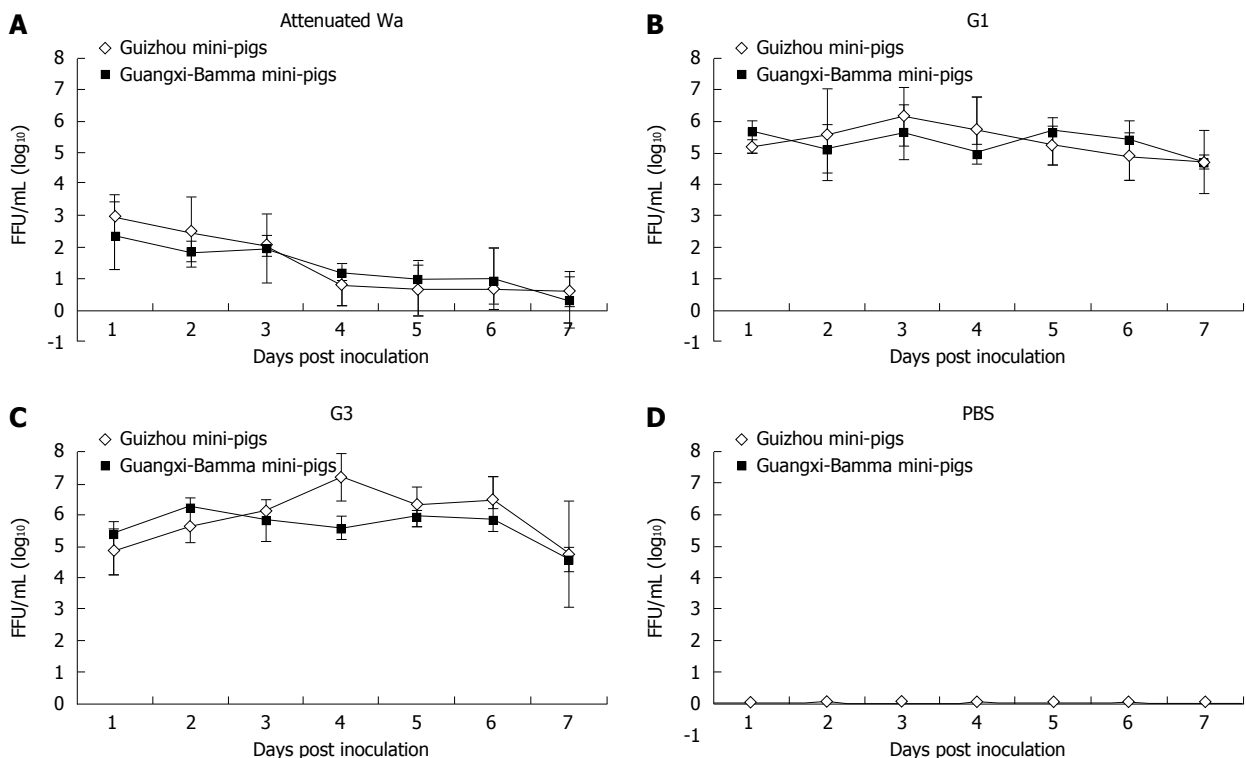


Figure 2 The curves of infectious virus-shedding of 3- to 5-d-old mini-pigs impregnated with 1 mL of human rotavirus or PBS. The curves of infectious virus-shedding from 3- to 5-d-old mini-pigs impregnated with 1 mL of 0.5×10^7 FFUs of human attenuated rotavirus Wa (A), 0.5×10^7 FFUs of human G1 (B), 0.5×10^7 FFUs of human RV G3 (C), and 1 mL of PBS (D). Infectious RV shedding in individual fecal samples from 0 to 7 DPI was assessed by FFA and was presented by FFU. FFUs: Focus forming units; DPI: Days post infection.

Table 1 Infectivity (ID50) of the different G serotypes of human rotavirus inoculum for mini-pigs (3-5 d old)

Virus shedding ¹		Diarrhea ³							
Inoculum RV serotype (G)	Animal species	Animal number	Percentage shedding	Mean onset (d)	Mean duration (d)	Peak titer shed (FFUs/mL)	Percentage with diarrhea	Mean onset (d)	Mean cumulative faecal score
Wa (attenuate)	Guizhou	3	33% ²	2	2	1 × 10 ³	0	0	4
	Bamma	3	33% ²	2	1	5 × 10 ²	0	0	6
G1	Guizhou	3	100%	1	6	2 × 10 ⁶	100% (3/3)	1.2	14
	Bamma	3	100%	1	6	1 × 10 ⁶	100% (3/3)	1	12
G3	Guizhou	3	100%	1	7	2 × 10 ⁷	100% (3/3)	1.5	16
	Bamma	3	100%	1	7	5 × 10 ⁶	100% (3/3)	1	12
PBS	Guizhou	2	0	0	0	0	0	0	0
	Bamma	2	0	0	0	0	0	0	0

¹Determined by FFA assay; ²The virus shedding animal is randomly selected; ³Diarrhea present if daily fecal consistency score ≥ 2, mean cumulative fecal score = \sum (Daily fecal scores from 1-7 DPI)/n. FFUs: Focus forming units; DPI: Days post infection.

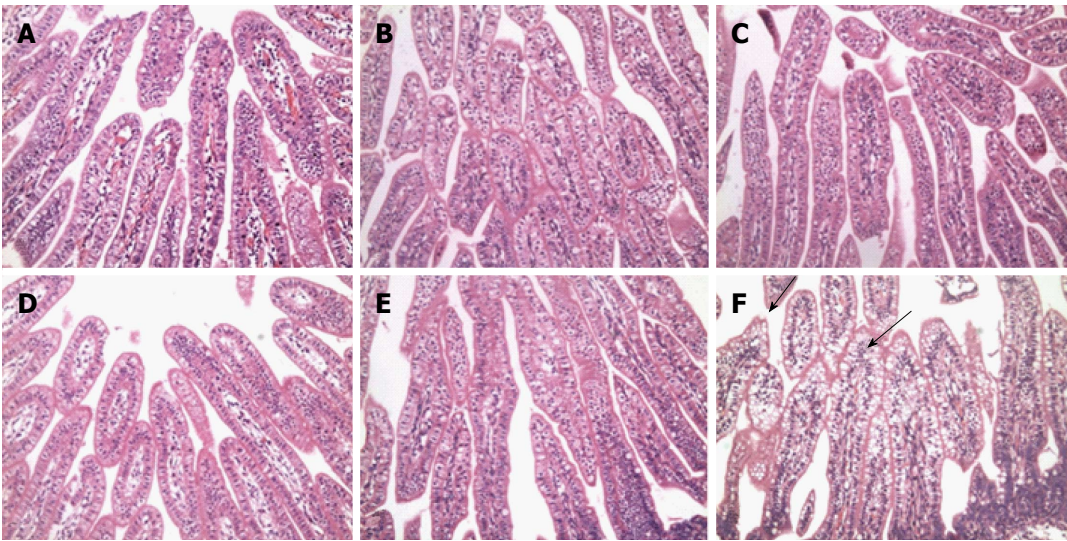


Figure 3 Histopathological lesions in the small intestine during rotavirus infection at 72 hpi. Sections of the small intestine were stained with hematoxylin and eosin. Duodenal (A), jejunal (B), and ileal (C) mucosae collected from mock-infected control newborn mini-pigs or duodenal (D), jejunal (E), and ileal (F) mucosae that were collected from 5-d-old Group A virulent G1-impregnated newborn mini-pigs. Magnification, × 200.

at 12 hpi, and again from 96 to 168 hpi, and was accompanied by distention that extended throughout the entire gut, including the small and large intestine.

No histological lesions were observed in all of the 5-d-old control mini-pigs at any time point (Figure 3A-C). While obvious signs of lymphocyte inflammation could be noted, villus height and width in the G1 HRV field strain and G3 HRV field strain inoculated 5-d-old mini-pigs changed. In addition, obvious histopathological lesions in the ileum were observed, including large vacuoles in the enterocyte lining, which were mainly distributed on the surface of the villi (Figure 3D-F). Vacuolization at the tops of the villi in the ileum was rarely observed, and was identified only in some of the control mini-pigs inoculated with PBS. Vacuolization in the ileum reached the maximum from 48 to 72 hpi and declined at 96 hpi. Histopathological changes of vacuoles declined at 168 hpi, in terms of both size and number.

Using TEM analysis, we determined that supra nuclear cytoplasmic vacuoles were present in the

ileum of RV-inoculated mini-pigs (Figure 4A and B). The vacuoles in the virus-exposed mini-pigs, however, were significantly distensible (Figure 4B). The markedly enlarged enterocytes indicated that intracellular organelles were pushed aside, and nuclei were squeezed. There were small, ill-defined fragments in the vacuoles, and cytoplasmic debris was noted in the background of general amorphous or finely granular structure. Therefore, normal transport vesicles appeared to be expanding. Viral particles in the intestine or near the vacuoles of the RV-inoculated mini-pigs at 24 hpi were detected by TEM (Figure 4C).

Detection of RV antigen in sections of the small intestine from 5-d-old mini-pigs

To confirm the distribution of structural protein VP6 in RV, frozen sections of intestinal tissue (including the duodenum, jejunum and ileum) of HRV-impregnated mini-pigs were stained by immunofluorescence (Figure 5). No RV antigen was tested in any section of the

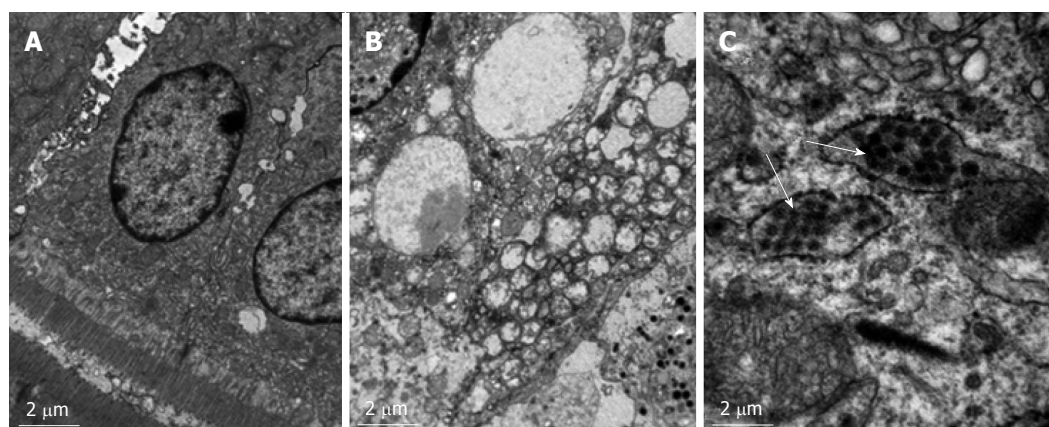


Figure 4 Ultrastructural visualization of the ileum from 5-d-old mini-pigs by electron micrograph. PBS-inoculated (A) and HRV-inoculated (B and C) at 48 hpi. Vacuoles devoid of virus particles (B) and virus particles encapsulated in Golgi-like follicle (white arrow) were observed in HRV-infected neonatal mini-pigs (C). Original magnification, $\times 40$. HRV: Human rotavirus.

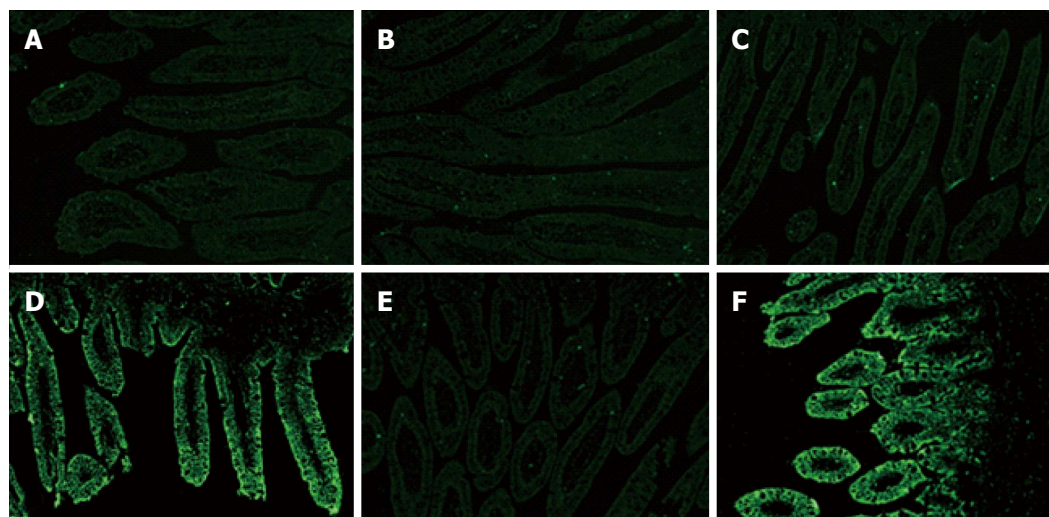


Figure 5 Distribution of immunofluorescence specific to VP6 antigen of rotavirus in the intestine. The duodenum (A), jejunum (B) or ileum (C) from 5-d-old mock-impregnated control newborn mini-pigs and the duodenum (D), jejunum (E) or ileum (F) from HRV-incubated newborn mini-pigs at 24 hpi. No rotavirus antigen was detected at 24 hpi in any part of the small intestinal epithelium in mock-impregnated control newborn mini-pigs. The expression of rotavirus antigen in the ileum was significantly higher than in the jejunum in HRV-infected neonatal mini-pigs at 24 hpi. HRV: Human rotavirus.

small intestine collected from 5-d-old control mini-pigs (Figure 5A-C). VP6 protein of RV was tested in epithelial cells which were located at the top part of the villi in samples of the jejunum and ileum, but not in the duodenum of HRV-infected 5-d-old mini-pigs at 24 hpi (Figure 5D-F). At any time point, RV antigen was not detected in the lower part of the villi of the whole small intestine collected from HRV-inoculated 5-d-old mini-pigs, and the same finding was noted for crypts. The distribution of RV antigen at 24, 48 and 72 hpi was almost identical. Little or no RV antigen was found over 168 hpi in any region of the intestine.

Infection of mini-pigs at different ages inoculated with human Group A rotavirus

To observe the age difference in mini-pigs inoculated with human Group A RV, we examined the mini-pigs at 30 d, 60 d and 6 mo of age. The time points were

chosen because mini-pigs were weaned at age of 2 mo and sexually mature at 6 mo of age. Six mini-pigs ($n = 6$) were inoculated with 0.5×10^7 FFUs of HRV. Similar to previous reports pertaining to rats, mice and conventional pigs, RV disease was age restricted to neonatal and 30 d old mini-pigs^[13,32,35,36]. None of the 60-d-old or 6-mo-old mini-pigs inoculated with RV developed disease (Table 2). All the RV-inoculated mini-pigs started to shed virus antigen at 1 to 2 DPI and continued to do so for 1 or 2 d.

Compared to the neonatal mini-pigs or 30-d-old mini-pigs inoculated with the corresponding RV strains, the timing and duration of virus antigen shedding in mini-pigs aged 60 d and 6 mo significantly decreased ($P < 0.001$). These findings indicate that RV infection in mini-pigs is also age limited (data not shown), in line with the observations pertaining to rabbits, mice and rats. To confirm that virus antigen test was not simply

Table 2 Inoculated mini-pigs with human rotavirus at different points in maturity

Age (d)	Number	Inoculum	Diarrhea ¹			Viral antigen shedding ²		
			Percentage (%)	Average (days happened)	Persistent (d)	Percentage (%)	Average (d)	Persistent (d)
3-5	3	G1 HRV	100 (3/3)	1	3-5	100 (3/3)	1	5-7
	3	G3 HRV	100 (3/3)	1	3-6	100 (3/3)	1	5-7
	2	PBS	0	0	0	0	0	0
30-35	3	G1 HRV	100 (3/3)	3	3-5	100 (3/3)	1	4-7
	2	PBS	0	0	0	0	0	0
56-60	3	G1 HRV	0	0	0	0	2	2-3
	2	PBS	0	0	0	0	0	0

¹Diarrhea present if daily fecal consistency score ≥ 2 , mean cumulative fecal score = \sum (Daily fecal scores from 1-7 DPI)/*n*; ²Determined by FFA assay. HRV: Human rotavirus; DPI: Days post infection.

the result of residual virus inoculums, we evaluated the fecal samples shed by the mini-pigs inoculated with virus to establish the infectious virus quantity. As the quantity of the virus antigen shed was negligible, the infectivity with rotavirus was not detectable.

DISCUSSION

In this study, two breeds of Chinese mini-pigs, namely, Guizhou and Bamma, were used to develop Group A HRV induced diarrhea models. Their smaller size and susceptibility to infection with HRV allowed us to conveniently study HRV induced diarrhea disease. To our knowledge, this is the first study to demonstrate that Chinese mini-pigs can be infected with HRV and subsequently develop clinically typical diarrhea. Mini-pigs inoculated with either G1 or G3 HRV shed RV and had clinical diarrhea for an average of 3 d. Pigs inoculated with G3 HRV shed RV at slightly higher rates than those inoculated with G1 HRV. Moreover, compared to Bamma pigs, Guizhou pigs seem more sensitive to HRV, with respect to shedding RV and developing clinical diarrhea.

The animal models for RV infection have offered key insights for understanding of RV infection, its pathology and immunity, and testing of prospective vaccines in children^[12-21,23,24,27,29,32,36-56]. To date, five models - including two small (mouse and rabbit) and three large laboratory (sheep, pig and cow) animal species - had been used to research the pathogenesis of both human and animal RV infection. Large animal models have been utilized to study the pathogenesis of RV infection because they are sensitive not only to homologous but also to heterologous RV, and develop severe disease symptoms. The limitations of the large animal models include high cost, limited availability, the need of specialized equipment and staff, and difficulty in handling multiple animals, which restrict their use in large-scale studies. Conversely, models of small animals have some merit, such as cost-effectiveness, short gestation and multiple births, the feasibility of including a large number of animals in experimental studies, the practicability of segregating sufficient number of infected animals, and other

merits. However, using adult mouse and rabbit models of RV infection for vaccine testing is affected by several restrictions, in particular: (1) HRV strains in either animal fail to replicate efficiently^[26]; (2) clinical disease was not observed in extant studies^[27]; and (3) only isogeny virus strains that were isolated from the same species replicate effectively and transmit horizontally to the control animals that had not been inoculated. Therefore, development of an ideal small animal model combines the efficient intestinal replication of HRV strains with small size and convenience for large-scale studies.

Both Guizhou mini-pigs and Guangxi mini-pigs examined in this study have been used widely as animal models in medical research^[25]. We investigated whether G1 HRV field strain and G3 HRV field strain could efficiently replicate in mini-pigs. To our knowledge, HRV in mini-pigs has not been reported to date. The present study was designed to develop a mini-pig model of human G1 or G3 serotype RV infection that is useful for defining basic parameters of active immunity, immunogenicity, and protective efficacy of vaccines^[57,58].

In this study, mini-pigs inoculated with HRV (including serotype 1 and serotype 3) developed clinical symptoms and pathological features of homologous virulent RV infections in children, lambs, calves, rodents and rabbits. Our results have successfully established virological and clinical parameters of RV infection in mini-pigs. More specifically, we have demonstrated that (1) viral replication, transmission, and disease of attenuated and virulent RV of human origin can persist from birth to 30 d, as detected by viral antigen or infectious virus shed by the virus-incubated pigs; (2) mini-pigs that were inoculated with human RV can develop diarrhea before reaching 30-d maturity, indicating that the disease has age-related restrictions; (3) pathological changes that do appear in newborn mini-pigs include small intestinal cell tumefaction, necrosis and lymph node inflammation^[30]; (4) the distribution of RV antigen is restricted to the top part of the villi in the ileum; and (5) RV infection of mini-pigs (including neonatal and those that are 30 d old) resulted in weight reduction during

the early stages of infection.

On average, the mini-pigs included in the study developed diarrhea within 3 d from inoculation, with the earliest onset noted at 1 d after impregnation. The onset of diarrhea coincides with the early stages of viral shedding despite different RV serotypes (either G1 or G3) and different mini-pig breeds (either Guizhou or Bamma). Viral antigen in small intestinal tissue was tested from 24 to 72 hpi, showing that diarrhea is connected with the presence of replicating virus in the intestinal tissue. Fluorescence intensity of structural RV protein VP6 in the ileum was higher than in the jejunum and less pronounced fluorescence staining was detected in the duodenum in mini-pigs inoculated with RV.

In this study, for the first time, we have identified human RV particles in the ileum of mini-pigs by electron microscopy. No previous study has reported HRV particles in this region of the small intestine. As only porcine RV particles have been previously reported in the pig intestine^[31], our novel findings indicate that mini-pigs are as sensitive to human RV as conventional pigs and other animals.

It has been three decades since RV has been determined as the most common cause of severe dehydrating diarrhea^[59]. However, children's morbidity and mortality remain high. Yet, making progress in developing successful vaccine strategies is hindered by our limited understanding of pathogenic mechanism of RV. Knowledge of the molecular and cellular basis of host responsiveness to RV infection remains crucial, especially in the context of malnutrition, which exacerbates severity of the disease and will most likely affect the efficacy of RV vaccines. Our results suggest that mini-pigs provide important advantages over normal pigs, mice and rabbits as animal models in the study of pathogenic mechanisms of RV infections, particularly in studies designed to evaluate RV vaccines and protection against infection and disease.

In conclusion, two breeds of Chinese mini-pigs, namely, Guizhou and Bamma, were used to develop Group A HRV induced diarrhea models. Their small size and susceptibility to infection with HRV allowed us to conveniently study HRV induced diarrhea. To our knowledge, this is the first study to demonstrate that Chinese mini-pigs can be infected with HRV and subsequently develop clinically typical diarrhea. Mini-pigs inoculated with either G1 or G3 HRV shed RV and had clinical diarrhea for an average of 3 d. Pigs inoculated with G3 HRV shed RV at slightly higher levels compared to those inoculated with G1 HRV. Moreover, Guizhou pigs seem more sensitive to HRV than Bamma pigs with respect to shedding RV and developing clinical diarrhea. Our results suggest that mini-pigs provide important advantages over normal pigs, mice and rabbits as animal models in investigations of pathogenic mechanisms of RV infections, particularly in studies designed to evaluate RV vaccines and protection against infection and disease.

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COMMENTS

Background

Rotavirus (RV) can cause severe gastroenteritis in infants in the world, and there is no specific medicine for treating RV diarrhea. Therefore, a safe and efficacious vaccine is very necessary. The unclearness of immune response mechanism of RV infection limits the development of vaccines. An ideal animal model of human RV (HRV) infection is urgently needed to improve the understanding of the immune response to RV infection in humans.

Research frontiers

It was reported that Chinese mini-pigs, Guizhou and Bamma, have many advantages as animal models in medical experiments.

Innovations and breakthroughs

This is the first to use Chinese mini-pigs to establish an infection model of RV.

Applications

This research offers a new animal model for studying the pathological changes and immunogenicity of HRV infection and a useful tool in the design and evaluation of RV vaccines.

Terminology

Mini-pigs inoculated with either G1 or G3 HRV shed RV and had clinical diarrhea for an average of 3 d. Pigs inoculated with G3 HRV shed RV at slightly higher levels compared to those inoculated with G1 HRV and Guizhou pigs seem more sensitive to HRV than Bamma pigs with respect to shedding RV and developing clinical diarrhea.

Peer-review

The manuscript has reported an original research performed in two kinds of Chinese mini-pig. Both can be infected efficiently with HRV. Obvious histopathological changes and rotavirus particles can be observed in the intestinal villi of the ileum by TEM. The small size of Chinese mini-pigs gave convenience for understanding pathogenic mechanisms of human rotavirus. The idea is new and the findings provided potential use for testing of vaccine efficacy for preclinical test. The manuscript is in general well written.

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