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Original Article

Comparison of IgA Antibody Titer Induced by Human-Bovine Rotavirus Candidate Vaccine with Bovine Rotavirus and Rotarix

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Abstract

Rotavirus (RV) is the most common cause of acute gastroenteritis in early childhood worldwide. Gastroenteritis is a preventable disease by the vaccine, and vigorous efforts were made to produce attenuated oral rotavirus vaccines. In recent years, despite the existence of three types of live attenuated rotavirus vaccines, several countries, such as China and Vietnam, have intended to produce indigenous vaccines based on rotavirus serotypes circulating among their population. In this study, the immunogenicity of homemade human-bovine reassortant RV candidate vaccine was tested in an animal model. Rabbits were randomly distributed into eight experimental groups with three animals per group. Afterward, three rabbits in each test group designated as P1, P2, and P3 were experimentally inoculated with the 10^6, 10^7, and 10^8 tissue culture infectious dose 50 (TCID50) of the reassortant virus, respectively. The N1 group received the reassortant rotavirus vaccine containing 10^7 TCID50+zinc. The N2, N3, and N4 groups received rotavirus vaccine strain, RV4 human rotavirus, and bovine rotavirus strain, respectively, and the control group received phosphate-buffered saline. It is noteworthy that three rabbits have been included in each group. The IgA total antibody titer was measured and evaluated by non-parametric Mann-Whitney and Kruskal-Wallis tests. The antibody titer produced in the studied groups did not significantly differ. The candidate vaccine showed immunogenicity, protectivity, stability, and safety. The findings of this study indicated a critical role of IgA production, which can induce immunity against a gastroenteritis viral pathogen. Regardless of purification, candidate reassortant vaccine and cell adapted animal strains could be used as a vaccine candidate for production.

Keywords: IgA antibodies, Reassortment, Rotavirus, Vaccine, VP7 glycoprotein

1. Introduction

Despite the progress made in the production of effective vaccines, high mortality and increased susceptibility to various microbial infections occur during infancy due to the immature state of the immune system (1). Rotavirus (RV) is the most common cause of acute gastroenteritis in early childhood worldwide (2). Moreover, RV diarrhea contributes significantly to the infection and mortality of children under five years old (3). The virus genome contains 11 linear double-stranded RNA segments encoding viral structural proteins and non-structural proteins. The segmented genome of RV creates reassortant progeny viruses during co-infection (4). According to the antigenic differences of the inner capsid protein VP6, RV is classified into 10 different groups (A-J) (5). RVs that

cause infections in humans are predominant in group A (6). These viruses are serotyped based on the VP7 (glycoprotein, G-genotype) and the VP4 (protease-susceptible protein, P-genotype) proteins and form a binary classification system (7). RV serotypes and P-genotypes, which mainly cause gastroenteritis, include G1P [8], G2P [4], G3P [8], G4P [8], G9P [8], P [4], and P [8] (8, 9). It is noteworthy that the G1 serotype is one of the most commonly circulating serotypes in Iran (10). Gastroenteritis is a preventable disease by the vaccine, and vigorous efforts were made to produce attenuated oral RV vaccines. Currently, three live attenuated RV vaccines, namely Rotarix, monovalent human virus vaccine, and RotaTeq, which is a pentavalent human-bovine reassortant vaccine, have been approved. The pentavalent RotaTeq vaccine contains G1, G2, G3, G4, and P [8] RV serotypes. In addition, Rotasiil, which is a pentavalent bovine-human reassortant live attenuated vaccine, contains G1, G2, G3, G4, and G9 RV serotypes (11-14).

In recent years, despite the existence of these three types of vaccines, several countries, such as China and Vietnam, have intended to produce indigenous vaccines based on RV serotypes circulating among their populations (15), which have been licensed for consumption in their countries, including Rotavin-M1 in Vietnam (16) and Lanzhou Lamb Rotavirus (LLR-85) in China (17).

In this study, the human-bovine reassortant candidate vaccine from the authors' previous study (18) was tested for immunization in New Zealand white rabbits as a selected animal model. The RF bovine virus, which is of the G6 type, was used as a backbone virus due to relatively close genomic similarity to the WC3 bovine virus used in the commercial Rotateq vaccine. The virus replicates well in cell culture and generates a titer of 10^7 to 10^8 plaques forming units per milliliter. The human RV4 (a G1P [8] type), which is the most common genotype throughout the world (19) and Iran (10), was the other virus selected for the reassortant candidate RV vaccine. In the reassorted virus, the VP7 gene from the human RV4 strain was replaced in the backbone of the RF strain.

1.1. Objectives

In the previous study, the molecular characteristics of the reassortant strain were shown by genomic stability (18). The present investigation evaluated the immunogenicity, protectivity, and safety of the human-bovine RV candidate vaccine on the laboratory animal.

2. Materials and Methods

2.1. Study Design

In the previous study, the seeds of the reassortant virus have been produced, and the genetic stability of the VP7 segment of the human strain of the virus has been proved (18). In summary, characterization of the stability of reassortant RV strain was indicated by six times serial passage in cell culture, followed by RNA extraction, cDNA synthesis, and partial sequencing. Moreover, the sequencing was used to confirm the genetic stability of the reassortant virus after the first, third, and sixth passages. The rabbit was selected as the animal model, and the efficacy of IgA antibody production was evaluated due to its importance in clearance and protection from RV infection. In order to study the effectiveness of antibody production, the Rotarix vaccine as a standard and the existing vaccine was used along with the parental human (RV4) and the bovine strains (RF), as well as candidate reassorted RV vaccine in the rabbits.

2.2. Cell Culture

In the present study, the MA104 cells (epithelial monkey kidney cell line) were used for viral stock preparation and plaque assay for virus titration and purification. Confluent monolayers of MA104 cells were inoculated with human and bovine strains and RV candidate vaccine at a multiplicity of infection of 0.1 with a serum-free medium at 37°C and 5% CO_2. Trypsin (porcine pancreatic type IX: Sigma, 10 µg/mL) was added for virus activation and incubated for 1 h at 37°C. The infected cells were lysed by three freezing-thawing cycles to allow the release of cell-associated viral particles.

2.3. Seed and Reassortant Virus Propagation and Titration

The RV4 and RF as parental viruses and the human-bovine reassortant virus are propagated for *in vivo* assays. The second, fifth, and sixth passages were considered master seed, mother seed, and working seed, respectively. The titer of the sixth passage as a working seed was performed by the standard protocol of tissue culture infectious dose 50 (TCID50) endpoint assay and calculated by the Reed and Muench method, which was 10^8 TCID50. In order to investigate the possible effect of the virus titer on the stimulation of the immune system, a range of titers was prepared on the sixth passage 10^6 TCID50, 10^7 TCID50, and 10^8 TCID50 for consideration as a test group. As a standard control, the Rotarix vaccine was used to measure the response of the candidate reassortant vaccine antibody. In summary, the TCID50 was used for determining virus titers of RV4, bovine RF strain, and candidate RV vaccine using the Reed and Muench method (20). In addition, MA104 cells were cultivated in 96-well cell culture plates. After reaching the density of 90%, the plates were prepared to inoculate the virus stocks at dilutions from 10^{-1} to 10^{-10}. For each titer, five wells were considered for inoculation, and a row of five was also considered the control. An amount of 100 µl of the dilution of the trypsin activated virus was added to each well (in each titer, five wells were inoculated), and the plate was kept in an incubator at 37˚C. The cell's surface was cautiously washed with phosphate-buffered saline (PBS), and 200µl 1X serum-free Dulbecco's Modified Eagle's Medium (DMEM) was added to each well. The results of the cytopathic effect (CPE) were evaluated daily until the fourth day of post-inoculation.

2.4. Plaque Reduction Neutralization Test For Selection of Purified Non-Contaminated Reassortant Virus

This method was used to quantify and purify the reassortant RV candidate vaccine virus from its bovine parental virus. In this method, the guinea pig polyclonal anti-serum was used to neutralize the bovine virus for possible isolation of the pure and non-contaminated reassortant virus. The amount of 1 µl of the anti-serum was added to 4,999 µl of the 1X DMEM medium to obtain a dilution of 1/5000. Afterward, 100 µl of diluted anti-serum was added to the same volume of 100 TCID50 activated viruses and incubated for 1 h at 4˚C. The MA-104 cells were cultured in six-well culture plates overlaid with serial dilutions of the above-mentioned virus-antiserum mixture. After 1 h, unabsorbed viruses were removed by washing the cells twice with PBS. The cell monolayers were covered with a layer of 0.8% cell grade agar (Sigma, USA) in DMEM (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA) without serum. In addition, the plates were incubated at 37˚C for 48 h, and the second layer of agar (with the same content as the first layer) was added to the vital dye neutral red. Moreover, the inoculated plates were incubated at 37˚C for four days.

2.5. New Zealand White Rabbit Immunization with Candidate Vaccines

The rabbit immune system is relatively comparable to the human immune system, with slight differences in cellular and tissue organization (21). Furthermore, similarities between RV infection in rabbits and humans are apparent. For these reasons, four-week-old New Zealand white rabbits seronegative for RV were used in the present study. Rabbits were randomly disseminated into eight experimental groups containing three animals per group, and three rabbits were used in each experimental group. The rabbits were kept in the animal care department, with the approval of the animal ethics committee of Tarbiat Modares University, Tehran, Iran. All animals were maintained under a 12-hour dark and light cycle, with free access to food and water. A total of 24 rabbits were randomly divided into control and challenged groups (three rabbits per group): reassortant RV candidate vaccine (in three groups of P1, P2, and P3 received 10^6, 10^7, and 10^8 TCID50 of the reassortant virus, respectively), the N1 group inoculated with 10^7

TCID50 reassortant RV candidate vaccine+zinc, the N2 group receiving Rotarix monovalent vaccine strain (10^6 TCID50), the N3 group inoculated human rotavirus RV4 (10^4 TCID50 virus titer), the N4 group inoculated bovine RV strain RF (10^7 TCID50 virus titer), and the control group that received PBS.

At days 0, 30, and 70, an amount of 0.5 ml of the virus inoculum via gavage was inoculated using a sterile feeding tube. Since zinc has a booster effect on the mucosal immune response (22), it was administrated by reassortant RV candidate vaccine in their oral water. Animals were allowed to drink water supplemented with 50 mg zinc sulfate for one week after each administration in the N1 group. According to the published procedures, each rabbit was fed 0.5 ml of viral suspension with 0.5 ml 30% sorbitol for better swallowing.

2.6. Blood Sampling of Rabbits

During the study, animals were sedated with ketamine (10 mg/kg), and 2.5 ml of blood was taken from the rabbit's heart in four steps. The first step was performed before the oral inoculation of the virus, and the remaining three steps were 14 days after each oral inoculation of the virus at days 0, 30, and 70. In addition, serum was separated from the blood and placed in a -70°C freezer for long-term storage to perform IgA titration.

2.7. IgA Titration in Rabbit Serum

The Rabbit Immunoglobulin A ELISA kit (Cat. Num: E0249Rb) was used to measure IgA antibody titer in the rabbit sera, and the ELISA reaction was carried out according to the manufacturer's instructions.

2.8. Evaluation of Safety of Reassortant Rotavirus Candidate Vaccine

In order to assess the safety of the candidate vaccine, the rabbits were monitored daily in terms of the appearance of stool (color and texture of faces) and clinical signs, compared to the control group (receiving PBS).

2.9. Statistical Analysis

All statistical comparisons were performed using SPSS software (version 22), with one degree of freedom and an α level of 0.05. The serum IgA antibody titers among groups were analyzed using non-parametric Mann-Whitney and Kruskal-Wallis tests, and the values were expressed as the mean±SE. In addition, $P<0.05$ was considered statistically significant.

3. Results

3.1. Virus Seed Titer

A total of three 96-well cell culture plates inoculated with RV4, RF, and reassortant virus, were evaluated for CPE. The reassortant RV candidate vaccine, RF, and RV4 virus titer were calculated as 10^7, 10^8, and 10^4 TCID50, respectively.

3.2. Incapability of Purifying Non-Contaminated Reassortant Virus by Plaque Reduction Neutralization Test

The plaque reduction neutralization test (PRNT) is usually applied to quantify the titer of neutralizing antibodies for a virus. In this study, PRNT was used to select purified non-contaminated reassortant virus stock. Viral CPE was not observed on the microscopic and macroscopic scales in any of the inoculated virus dilutions, and the condition of the wells was similar to that of the control. Cell degradations were visible five days after RV inoculation in control infected cells. It was impossible to purify parental virus from the reassortant virus because the antibody produced in the Guinea pigs was a strong cross antibody to neutralize all infected cells.

3.3. Serum IgA Induction Indicated in Candidate Vaccine Similar to Approved Vaccine

After serum isolation, the antibody titer was investigated in animal groups by the ELISA method. The immune response results are presented in figure 1 and table 1. By comparing the results of the antibody titers, although all examined serum neutralizes RVs, there were no significant differences in different titers of reassortant RV candidate vaccine. Moreover, the results demonstrated that titer alteration is ineffective on

vaccine efficacy. Antibody titers in rabbits that received the Rotarix vaccine did not show any significant differences with the antibody titers in the rabbits that received candidate reassortant vaccine. It is noteworthy that the immunogenicity of the RV candidate vaccine was similar to the standard Rotarix vaccine. Moreover, the authors performed a comparison of IgA titers between N1 and each of the groups. According to the results of comparing IgA titer between N1 and each group, a statistically significant difference was found between the N1 and P2 groups. The only difference between N1 and P2 groups was the usage of zinc elements (Figure 2).

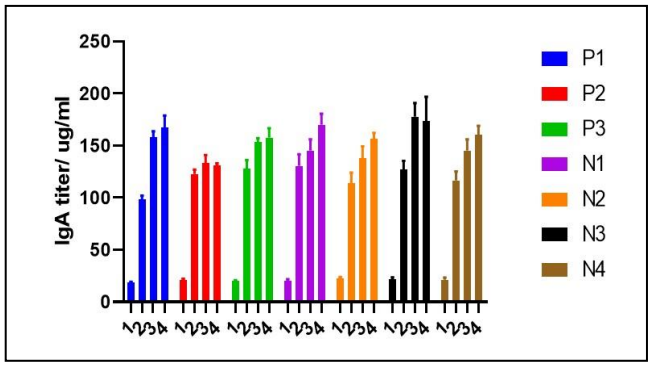

Figure 1. Antibody titer was shown in four stages of blood sampling in the studied groups. In all groups, there is an increasing trend in the antibody titer. There was a significant increase between the antibody titer in the first and the second specimens 14 days after the first dose of the virus administration. A similar pattern was observed in the change of the antibody among all groups. The statistical tests were performed to determine the differences among the groups using Kruskal-Wallis and Mann-Whitney statistical tests. 1: First blood collection, 2: Second blood collection, 3: Third blood collection, 4: Fourth blood collection.

Table 1. Results of the statistical analysis related to the animal groups receiving the studied viruses

Row	Group Comparison	*P*-Value	Statistical Test
1	P1, P2, P3	0.067	Kruskal-Walllis
2	(P1, P2, P3) with N2	0.084	Kruskal-Walllis
3	(P1, P2, P3) with N3	0.025	Kruskal-Walllis
4	(P1, P2, P3) with N4	0.144	Kruskal-Walllis
5	(P1, P2, P3) with N1	0.057	Kruskal-Walllis
6	P2, N1	0.100	Mann-Whitney
7	N3, N2	0.100	Mann-Whitney
8	N3, N4	1.000	Mann-Whitney

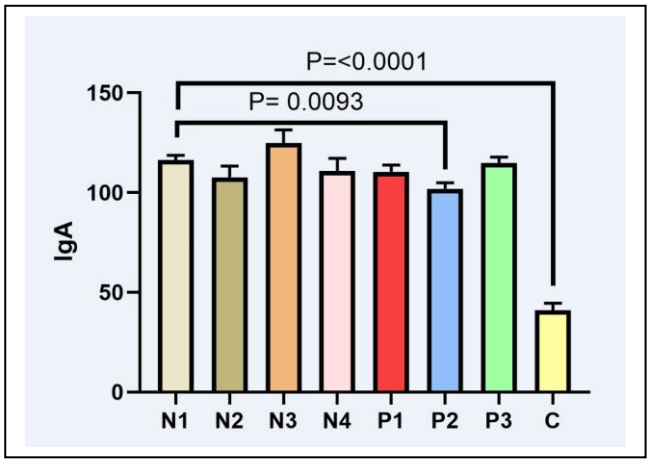

Figure 2. Comparison of IgA titers between N1 and each of the groups. A statistically significant difference was found between N1 and P2 groups. The N1 group received the reassortant RV candidate vaccine containing 10^7 TCID50 supplemented by zinc, the N2 group received rotavirus vaccine strain (10^6 TCID50), the N3 group received RV4 human rotavirus (10^4 TCID50 virus titer), the N4 group received bovine rotavirus strain (10^7 TCID50 virus titer), as well as P1, P2, and P3 groups were experimentally inoculated with the 10^6, 10^7 and 10^8 TCID50 of the reassortant virus, respectively, and finally, C group received PBS.

3.4. Results of the Safety of Reassortant Rotavirus Candidate Vaccine

The absence of clinical signs in vaccinated animals and no changes in the appearance of stool indicated the safety of the reassortant RV strain, although this should be further confirmed.

4. Discussion

Researchers and vaccine manufacturers are attempting to produce vaccines with appropriate criteria. These vaccines should meet both their regional and indigenous needs in protecting against RV strains that cause disease in their country and achieve global standards to introduce and obtain authorization for consumption in other countries (16).

The previous studies have examined the level of antibodies in the serum after immunization of various animal models against RV. Since the rabbit's immune system is relatively similar to humans and permits blood sampling readily, the rabbit was selected for the present study (23). The route and dose of immunization

have important roles in influencing immune responses at the initial site of pathogen entry. The oral route was used according to the World Health Organization (WHO) guidelines for RV vaccine administration. Since the administration of a 2-dose regimen (Rotarix) may not provide appropriate protection and antibody responses, three oral doses with an interval of at least four weeks between first and second doses and the two-month interval between second and third doses were considered for the development of efficient mucosal immune response. On the other hand, it was shown that zinc supplements could reduce the duration and severity of diarrhea management. The individuals who received the oral vaccine in combination with zinc had higher rates of seroconversion to RV. The WHO and the United Nations International Children's Emergency Fund propose that children with diarrhea receive 10-20 mg of zinc daily. Zinc affects the activity of more than 300 enzymes involved in DNA replication and RNA transcription. Furthermore, in the small intestine, it increases the integrity of the cells, as well as glycocalyx, and restores enzyme activity to the brush border enterocytes. On the other hand, it increases the production of mucosal antibodies and circulating immune cells against intestinal pathogens.

In this study, the RV vaccine titer is calculated at 10^6 TCID50, which is similar to the low titer of the reassortant virus and the titer of the bovine virus at 10^7 TCID50 used for the immunization of animals. In order to evaluate and compare the immunogenicity impacts of candidate vaccines between the reassortant RV and parental virus strain plus standard Rotarix vaccine, total IgA titration in the serum of rabbits were measured by ELISA assay.

The data of the present investigation provide experimental evidence that pathogen-specific IgA is essential for the development of protective intestinal immunity. The IgA is critical for the induction of immunity against pathogens, which invade and cause disease at any mucosal surface. Similarly, Ji-Tao Chang et al. constructed a bivalent vaccine candidate encompassing ovine RV strain LLR-85-based bovine RV reassortant and evaluated the immunogenicity of the candidate vaccine in the sera of inoculated calves. The results indicated that the candidate vaccine was well immunogenic to neonatal calves, and the highest titers of serum IgA were obtained at 21-day post-inoculation (24).

The results obtained in this study can support a better understanding of the antigenic effect of the reassortant RV vaccine and suggest that it can be selected as a candidate vaccine with the same effectiveness as a commercial vaccine for future use. Therefore, this vaccine candidate, along with zinc, can be used for passive immunization studies. In addition, it seems that this vaccine could cause protection against RV intestinal infection by IgA induction and pathogen clearance. Sarah E Blutt et al. developed mice lacking IgA ($IgA^{-/-}$) and exposed them to RV infection. Compared to wild-type, $IgA^{-/-}$ mice showed a significant delay in infection clearance and excreted RV in stool up to three weeks. It can be concluded from these results that IgA is a key component in the immune response to RV (25).

In conclusion, due to the immunogenicity of all strains, it seems that cell-adapted animal strains and reassortant vaccines, regardless of purification, could be used for vaccine production, and nations should invest in cost-effective manufacturing rather than large vaccine importation.

Authors' Contribution

Study concept and design: H. S.
Analysis and interpretation of data: A. T. and E. Z.
Drafting of the manuscript: E. Z.
Critical revision of the manuscript for important intellectual content: S. S. M. and H. S.
Performing the laboratory experiments: E. Z. and A. M.

Ethics

This study was conducted with the approval of the Animal Ethics Committee of Tarbiat Modares University, Tehran, Iran (IR.TMU.REC.1394.98).

Conflict of Interest

The authors declare that they have no conflict of interest.

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