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

Zafari, E1, Soleimanjahi, H1 *, Mohammadi, A2, Teimoori, A3, Shatizadeh Malekshahi, S1

1. Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Razi Vaccine and Serum Research Institute (RVSRI) Hessark Karadj, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran
3. Department of Virology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamedan, Iran

Corresponding author: soleim_h@modares.ac.ir

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, Vietnam and others have intended to produce indigenous vaccines on the basis of rotavirus serotypes circulating among their own 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 consisting of three animals per each group. Three rabbits in each test groups designated as P1, P2 and P3 were experimentally inoculated with the 10^6, 10^7 and 10^8 TCID50 of the reassortant virus, respectively. The N1 group received the reassortant rotavirus (RV) vaccine containing 10^7 TCID50+zinc. N2, N3 and N4 group received rotavirus vaccine strain, RV4 human rotavirus and bovine rotavirus strain, respectively. Control group received PBS. It should be
mentioned that in each group three rabbits has been included. 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 show any significant differences. The candidate vaccine showed immunogenicity, protectivity, stability and safety. Our findings indicate a critical role for IgA production which can induce immunity against a gastroenteritis viral pathogen. Regardless of purification, candidate reassortant vaccine, as well as cell adapted animal strains could be considered for usage as a vaccine candidate for production.

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

**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). Rotavirus diarrhea contributes significantly to the infection and mortality of children under the age of five years old (3). The virus genome contains 11 linear double-stranded RNA (dsRNA) segments encoding viral structural proteins (VPs) and non-structural proteins (NSPs). The segmented genome of RV creates reassortant progeny viruses during co-infection (4). Based on the antigenic differences of the inner capsid protein VP6, RV is classified into ten different groups (A-J) (5). Rotaviruses that cause infection in humans are predominantly in group A (6). Rotaviruses 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). Rotavirus serotypes and P genotypes that mainly cause gastroenteritis
include G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], P[4], and P[8] (8, 9). 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 rotavirus vaccines. Currently, three live attenuated RV vaccines have been approved: The Rotarix monovalent human virus vaccine and RotaTeq, a pentavalent human–bovine reassortant vaccine. The pentavalent RotaTeq vaccine contains RVs G1, G2, G3, G4, and P[8]. Rotasiil, a pentavalent bovine-human reassortant live attenuated vaccine, contains RV serotypes G1, G2, G3, G4, and G9 (11-14).

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

In this study, the human-bovine reassortant candidate vaccine from our previous study (18) was tested for immunization in New Zealand white rabbit as an selected animal model. In fact, RF bovine virus which is a G6 type was used as a backbone virus. It has been selected 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 other virus selected for the reassortant candidate RV vaccine was human RV4 (a G1P [8] type), the most common genotype throughout the world (19) and Iran (10). In the reassorted virus VP7 gene from human RV4 strain was replaced in the backbone of RF strain.

**Objective:**
In our previous study, the molecular characteristics of the reassortant strain were shown by genomic stability (18). Herein, the immunogenicity, protectivity, and safety of the human-bovine RV candidate vaccine were evaluated in the laboratory animal.

Materials and methods

Study design

In our 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 brief, characterization of the stability of reassortant rotavirus strain was shown by six times serial passage in cell culture, followed by RNA extraction, cDNA synthesis, and partial sequencing. The genetic stability of the reassortant virus after first, third and sixth passage was confirmed by sequencing. 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 rotavirus infection. To study the effectiveness of antibody production, 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.

Cell culture

The MA104 cells (epithelial monkey kidney cell line) were used in the present study 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 (MOI) of 0.1 with serum free medium at 37°C and 5% CO2. Trypsin (porcine pancreatic type IX: Sigma, 10 μg/mL) was added for virus activation and incubated for 1 hour at 37°C.
infected cells were lysed by three cycles of freezing-thawing to allow releasing of cell-associated viral particles.

**The seed and reassortant virus propagation and titration**

RV4 and RF viruses as parental viruses and human-bovine reassortant virus are propagated for *in vivo* assays. The second, fifth and sixth passage were considered as master seed, mother seed and working seed, respectively. The titer of the sixth passage as a working seed was done by the standard protocol of tissue culture infectious dose 50 (TCID50) endpoint assay and calculated by the Reed & Muench method which was $10^8$TCID50. To investigate the possible effect of the virus titer on 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 considering as a test groups. As a standard control, Rotarix vaccine was used to measure the candidate reassortant vaccine antibody’s response. Briefly, the TCID50 was used for determining virus titers of RV4, bovine RF strain and candidate RV vaccine using the Reed and Muench method (20). 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 of $10^{-1}$ to $10^{-10}$. For each titer, five wells were considered for inoculation, a row of five was also considered as control. To each well, 100 μl of the dilution of the trypsin activated virus was added (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 once with PBS, and 200μ1 1X serum-free DMEM was added to each well. The results of the cytopathic effect (CPE) were evaluated daily until the fourth day post-inoculation.

**Plaque reduction neutralization test (PRNT) for selection of purified non-contaminated reassortant virus**
This method was used to quantification and purification of 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. One μl of the anti-serum was added to 4999 μl of the 1X DMEM medium, to obtain a dilution of 1/5000. Then 100 μl of diluted anti-serum was added to the same volume of 100 TCID50 activated viruses and incubated for an hour 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), 1% penicillin/ streptomycin (Gibco, USA) without serum. Plates were incubated at 37 °C for 48 hours, and the second layer of agar (with the same content of the first layer) was added to the vital dye neutral red. Inoculated plates were incubated at 37 °C for four days.

**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 rotavirus infection in rabbit and human are apparent. For these reasons, four weeks old New Zealand white rabbits seronegative for RV were used in the current study. Rabbits were randomly disseminated into eight experimental groups containing three animals per each group. Three rabbits have been applied 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 are maintained under a 12-hour dark and light cycle, with free access to food and water. Twenty-four rabbits were randomly divided into control and challenged groups (3 rabbits/group): reassortant RV candidate vaccine (in 3 groups of P1, P2 and P3 receiving $10^6$, $10^7$, 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 rotavirus strain RF ($10^7$TCID50 virus titer) and finally, the control group received PBS.

At days 0, 30, and 70, 0.5 ml of the virus inoculum via gavage was inoculated using a sterile feeding tube. Because 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 50mg zinc sulfate up to one week after each administration in N1 group.

According to the published procedures, each rabbit was fed with 0.5ml of viral suspension with 0.5 ml 30% sorbitol for better swallowing.

**Blood Sampling of Rabbits**

During the study, animals were sedated with ketamine (10 mg/kg), and 2.5 ml 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. Serum was separated from the blood and placed in a -70°C freezer for long-term storage to do IgA titration.

**IgA titration in rabbit serum**

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

Statistical analysis

All statistical comparisons were performed using the statistical software programs SPSS v.22 software, with one degree of freedom and \( \alpha \) = level of 0.05. Analysis of serum IgA antibody titers between groups was performed by non-parametric Mann-Whitney and Kruskal-Wallis test. Values were expressed as the mean ± SE. \( p < 0.05 \) was considered statistically significant.

Results

Virus seed titer

Three 96-well cell culture plates, inoculated with RV4, RF, and Reassortant virus, were evaluated for CPE. Reassortant RV candidate vaccine, RF, and RV4 virus titer were calculated as \( 10^7 \), \( 10^8 \), and \( 10^4 \) TCID50, respectively.

The inability of purifying non-contaminated reassortant virus by PRNT

The PRNT is usually used to quantify the titer of neutralizing antibodies for a virus. In this study, we used PRNT for the selection of purified non-contaminated reassortant virus stock. In none of the inoculated virus dilutions, viral CPE was not observed on the microscopic and macroscopic scale, and the condition of the wells was similar to that of the control wells. In the control infected cells five days after RV inoculation, cell degradations were visible. We were not able to purify
parental virus from reassortant virus, because the antibody produced in the Guinea pigs were strong enough cross antibody to neutralize all infected cells.

The serum IgA induction shown 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 Table1. By comparing the results of the antibody titers, although all of examined serum neutralize Rota viruses but there were no significant differences in different titers of reassortant RV candidate vaccine and the results showed that titer alteration is ineffective on vaccine efficacy. Antibody titers in rabbits received Rotarix vaccine did not show any significant differences with the antibody titers in the rabbits received candidate reassortant vaccine. In fact, the immunogenicity of RV candidate vaccine was similar to the standard Rotarix vaccine. We performed a comparison of IgA titers between N1 and each of the groups, as well. According the results of comparing IgA titer between N1 and each groups statistical significant difference was found between N1 and P2 groups. The only difference between N1 and P2 groups was the usage of zinc element. (Figure 2).

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 rotavirus strain, although this should be further confirmed.

Discussion

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

Previous studies have examined the level of antibodies in the serum after immunization of various animal models against rotavirus. Because the rabbit’s immune system is relatively similar to the human immune system 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. According to the World Health Organization (WHO) guidelines for rotavirus vaccine administration, the oral route was applied. Since the administration of a 2-dose regimen (Rotarix) may not provide good protection and antibody responses, three oral doses with an interval of at least four weeks between first and second 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. Individuals who received the oral vaccine in combination with zinc had higher rates of seroconversion to rotavirus. The WHO and the United Nations International Children's Emergency Fund (UNICEF) propose that children with diarrhea should receive 10 to 20 mg of zinc daily. Zinc affects the activity of more than 300 enzymes involved in DNA replication and RNA transcription. In the small intestine, it increases the integrity of the cells as well as glycocalyx and restores enzyme activity to the brush border enterocytes. Besides, increases the production of mucosal antibodies and circulating immune cells against intestinal pathogens.

In this study, the rotavirus 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 was $10^7$ TCID50 which was used for 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.

Our data provide the experimental evidence that pathogen-specific IgA is essential for the development of protective intestinal immunity. IgA is critical to induce immunity against pathogens, which invade and cause disease at any mucosal surfaces. Likewise, 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 candidate vaccine in the sera of inoculated calves. The results showed that the candidate vaccine were well immunogenic to neonatal calves and the highest titers of serum IgA was 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. It seems that this vaccine could cause protection against Rotavirus 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 significant delay in infection clearance and excreted RV in stool up to three weeks. These results leading to the conclusion that IgA is a key component in the immune response to RV (25).

**Conclusion:**
In conclusion, due to the immunogenicity of the 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.

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**Competing interests**

None declared

**Ethical approval**

This study was conducted by an approval of the animal ethics committee of Tarbiat Modares University (Tehran, Iran)( IR.TMU.REC.1394.98).

**Author Contributions:**

Study concept and design: HS. Analysis and interpretation of data: AT, EZ. Drafting the manuscript: EZ. Critical revision of the manuscript for important intellectual content: SSM & HS. Performing the laboratory experiments: EZ & AM.

**Acknowledgment**

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References:


Figure 1: The 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 specimen and the second specimen, which is 14 days after the first dose of the virus administration. A similar pattern was observed in changing the antibody among all groups. Statistical tests were performed to determine the differences between the groups.

**Figure 2**: Comparison of IgA titers between N1 and each of the groups. Statistical 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. N2 group: received rotavirus vaccine strain ($10^6$TCID50). N3 group: received RV4 human rotavirus ($10^4$TCID50 virus titer). N4 group: received bovine rotavirus strain ($10^7$TCID50 virus titer). P1, P2 and P3 groups: were experimentally inoculated with the $10^6$, $10^7$ and $10^8$ TCID50 of the reassortant virus, respectively. C group: received PBS.
Table 1. Results of the statistical analysis related to the animal groups receiving the studied viruses

<table>
<thead>
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<th>Row</th>
<th>Group comparison</th>
<th>P-value</th>
<th>Statistical test</th>
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</thead>
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<td>1</td>
<td>P1, P2, P3</td>
<td>0.067</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
<td>2</td>
<td>(P1, P2, P3) with N2</td>
<td>0.084</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
<td>3</td>
<td>(P1, P2, P3) with N3</td>
<td>0.025</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
<td>4</td>
<td>(P1, P2, P3) with N4</td>
<td>0.144</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
<td>5</td>
<td>(P1, P2, P3) with N1</td>
<td>0.057</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
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<td>P2, N1</td>
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<td>Mann-Whitney</td>
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<tr>
<td>7</td>
<td>N3, N2</td>
<td>0.1</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>8</td>
<td>N3, N4</td>
<td>1</td>
<td>Mann-Whitney</td>
</tr>
</tbody>
</table>

-P1, P2 and P3 groups: were experimentally inoculated with the $10^6$, $10^7$ and $10^8$TCID50 of the reassortant virus, respectively. The N1 group: received the reassortant RV candidate vaccine containing $10^7$TCID50 supplemented by zinc. N2 group: received rotavirus vaccine strain ($10^6$TCID50). N3 group: received RV4 human rotavirus ($10^4$TCID50 virus titer). N4 group received bovine rotavirus strain ($10^7$TCID50 virus titer)