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Original Article

Preparation of an Inactivated Peste des Petits Ruminants Vaccine and Its Comparative Immunogenicity Evaluation in an Animal Model

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Abstract

Peste des petits ruminants (PPR) is a highly contagious disease that is considered a major threat to the small livestock industry. Although vaccination via live-attenuated PPR vaccine is a main controlling strategy in the endemic area, during PPR eradication process, the inactivated PPR vaccine (iPPRV) is recommended. This study aimed to compare the inactivation kinetics of the PPR virus via different inactivants and immunogenicity evaluations of the iPPRV formulated vaccine in mice. The vaccinal live PPR virus was inactivated by either H_2O_2 or binary ethylenimine (BEI) at two concentrations of 1 or 4 mM. Thereafter, the inactivated virus was formulated with different adjuvants, including aluminum hydroxide (AH), aluminum phosphate (AP), and a mixture of AH and AP that were intraperitoneally (IP) administrated (0.1 mL) to 90 BALB/c mice in a completely randomized design and 3×3 factorial arrangement (9 animals per group). The booster vaccination was carried out in all animals 21 days after the primary vaccination. Results showed that the PPR virus was successfully inactivated by all the inactivation agents; however, the time of complete virus inactivation was estimated to be 482, 295, and 495 min post-treatment initiation for 1 mM BEI, 4 mM BEI, and H_2O_2, respectively. The main effect of inactivant on antibody titers against PPR virus that was measured after 42days post-immunization in mice was significant ($P<0.05$); however, the adjuvant and interaction effect of inactivator×adjuvant were not effective($P>0.05$). Inactivation by 1 mM BEI was associated with a higher antibody titer against PPR virus ($P<0.05$) in comparison with both 4 mM BEI and H_2O_2 (2.51 vs. 2.25 and 2.22, respectively). Meanwhile, there were no significant differences among the used adjuvants in terms of eliciting antibody response against PPR virus. In conclusion, the use of 1 mM BEI in combination of AH, AP, or a mixture of AH and AP was associated with a higher immune response against PPR virus in mice. However, the appropriate inactivation kinetic of the virus and immunogenicity associated with the use of H_2O_2, as well as its biocompatibility property and better cost-benefit, nominated H_2O_2 to be used in iPPR preparation; however, more investigations are required in target animals.

Keywords: Immunogenicity; Inactivation; Peste des petits ruminants (PPR); Vaccination; Virus

1. Introduction

Peste des petits ruminants (PPR) is an acute or subacute and highly contagious viral disease of goats and sheep characterized by fever, stomatitis, gastroenteritis, pneumonia, and death. In 2011, the World Organization for Animal Health (OIE) and the United Nations Food and Agriculture Organization (FAO) started to evaluate the possibility of PPR progressive control leading to eradication. In March 2015, OIE and FAO officially launched a new program

to eradicate PPR by 2030. PPR is associated with high mortality and morbidity resulted in a considerable economic loss in the endemic area. This disease is caused by the PPR virus that belongs to the Morbilli virus genus of the Paramyxoviridae. Poor nutritional status and concurrent parasitic or bacterial infections enhance the severity of the PPR clinical signs (1).

As a global strategy, eradication of PPR up to 2030 is scheduled during four phases. Before and during phase1, the country does not have any structured response mechanism in place; however, the main controlling approach at phases 2 and 3 is programmed on the basis of vaccination. Immunization by a live-attenuated PPR vaccine, as a normal control component of stage 2, is targeted in a specific zone where PPR is endemic or at high risk, or a specific sub-population at higher risk or of higher commercial value. Meanwhile, inphase3, the vaccination strategy will depend on the outcomes of stage 2, and inactivated PPR vaccine is commonly applied. At the end of phase3, no clinical outbreaks should be detected in the whole territory and diagnostic tests, indicating that the virus is no longer circulating in the domestic and wild animals. In phase4, there is not any vaccination, and therefore, there is no more post-vaccination monitoring. All stocks of PPR vaccine (monovalent and polyvalent) should be safeguarded by the competent authorities or be removed from non-accredited sites (2).

The inactivated PPR vaccine contains viral antigen inactivated commonly by binary ethylenimine (BEI) (3). It has been shown that BEI reacts with viral nucleic acids while conformation and accessibility of epitopes are preserved (4). The BEI has been successfully inactivated various viruses for vaccine production (5). However, several limitations, such as being a carcinogen, having hazardous residual, as well as laboratory complicity associated with using BEI, increase the willingness to find an appropriate alternative (6), such as H_2O_2.

It has been shown that 3% of the aqueous solution of H_2O_2 inactivated both RNA and DNA viruses with up to a 6-log_{10}reduction in titer observed in less than 2 h (7). It was shown that the West Nile virus which causes mosquito-borne and epidemic encephalitis was efficiently inactivated by H_2O_2 (8). This vaccine could be used safely in at-risk populations and could induce effective neutralizing antibody responses in BALB/c mice (9). It has been postulated that hydroxyl radicals originated from H_2O_2 attack carbon double bonds in the nucleosides or obstruct the hydrogen atoms, thereby breaking single- or double strands in the genomic structure, leading to the inactivation of targeted organisms (10). During a reaction, H_2O_2 produces H_2O with no environmental contamination or human adverse effect. These properties nominated the H_2O_2 as a safer and more feasible inactivator component, compared to EBI. However, the knowledge about the effectiveness of the use of H_2O_2 during the inactivation of vaccine viruses is preliminary. Therefore, this study was conducted to investigate 1) the feasibility of inactivation PPR virus used in the vaccine production process; 2) compare the results of PPR virus inactivation by H_2O_2 and BEI, and 3) evaluate the immunogenicity of inactivated PPR virus in the animal model.

2. Material and Methods

2.1. Cell Culture

Lymphoid Cell line (F9) cells is a kind of suspension cell line, derived from cow (Bos tareus) lymph nodes established in Razi Vaccine and Serum Research Institute of Iran (11) were seeded in cell spin bottles at the density of 3-4×10^5 cell/ml. F9 was propagated in DMEM medium(Gibco, U.S.A.) and 10% fetal calf serum (Gibco, U.S.A.). Following that, it was supplemented by 100 IU/ml penicillin and 100 µg/ml Streptomycin (Sigma-Aldrich, U.S.A).

2.2. Preparation of Virus

PPR virus (Nigeria 75/1) with 0.03 MOI was co-infected to the F9 suspension cell line. The cell spin bottles were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell viability was evaluated daily by Trypan Blue (Sigma-Aldrich, U.S.A) staining on the Hemocytometer. When the

viability arrived at less than 15%, the suspension fluid was frozen at -20°C. Harvest was titrated (2) after freeze-thawing and clarification by centrifuge (10 min. at 1370g).Finally, harvest was aliquoted and frozen at-70°C.After each manipulation, PPR virus was tested for bacterial, fungi, and mycoplasma contamination (12).

2.3. Virus Inactivation

The PPR virus ($10^{6.3}$ TCID50/ml) was inactivated comparatively by BEI and H_2O_2. Briefly, the inactivation kinetics of the PPR virus was determined by the treatment of 1 and 4 mM BEI during different post-inactivation times at room temperature (3). Virus inactivation was evaluated by serial sampling and residual alive virus titration at 30-min intervals. Following the inactivation time, after each sampling and bulk, sodium thiosulfate (Sigma-Aldrich, U.S.A) at a final concentration of 2% (v/v) was used to neutralize the BEI. Therefore, for the H_2O_2 inactivator, a 30% stock solution of H_2O_2 (CarelRoth, Germany) was sterilized by membrane filtration and was kept in a dark sealed container. The virus suspension was treated with H_2O_2 stock solution to a final concentration of 3% H_2O_2 at room temperature (13). Finally, to remove residual H_2O_2, each aliquot was duplicity treated with 12.5 U/ml catalase (MP Biomedical. U.S.A) for10 min at room temperature (14). The titer of each aliquot was measured by microtitration method. For each inactivation agent, the process was repeated three times, and the average of the data was reported as the final outcome.

2.4. Safety Test

After the inactivation process, the inactivated virus suspension was assessed for any live virus by culture on the Vero cell.

2.5. Experimental Vaccine Formulation

The 300 ml of inactivated PPRV (iPPRV) product was divided into three aliquots. Each aliquot was used to prepare a different vaccine formulation with Aluminum hydroxide, Aluminum phosphate, or a mixture of Aluminum hydroxide and Aluminum phosphate (50/50 v/v). The formulated vaccines were stored at 5±3°C until future evaluation (2).

2.6. Quality Control Test of the Formulated Vaccines

2.6.1. Innocuity Test

An abnormal toxicity test was conducted in accordance with the European pharmacopeia monograph. Briefly, 20 BALB/c mice (17-22 g) were randomly divided into two groups (10 animals per group) that intraperitoneally (IP)received 0.1 ml of either BEI or H_2O_2 formulated vaccines. The animals were observed for any signs of ill-health during a 14-day period.

2.7. Vaccination

A total of 30 male BALB/c mice weighted 20-25g were randomly divided into three groups (10 animals per group) and vaccinated by IP administration of 0.1 mL (3) of each formulated vaccine with different inactivants (BEI and H_2O_2) and were boosted 21 days later. The control group received 0.1 mL of saline as a placebo. Blood samples of all animals were collected 42 days after the first immunization. No bodyweight loss or mortality was observed in the group of mice inoculated with either H_2O_2 or BEI inactivated virus suspensions.

2.8. Evaluation of Vaccine Immunogenicity in Mouse

After the second immunization, the serum antibody against the PPR virus was assessed by using a Serum Neutralization (SN) test, and the obtained culture was calculated by the Kerber method. Briefly, inactivated sera (56°C for one h) were twofold serially diluted and preincubated with 100 CCID50 PPR virus allowing one h for neutralization to occur prior to the inoculation of the mixture onto Vero cells. Detection of neutralizing antibodies is determined by the lack of cytopathic effect in Vero cells. The highest dilution of serum that prevents infectivity of the cell culture was considered the neutralizing antibody titer. The following SN assay provides a simple approach to assessing the presence

and quantity of anti-PPR neutralizing antibodies in serum.

2.9. Statistical Analysis

Data were analyzed in a completely random design with a 3×3 factorial arrangement by GLM procedure in SAS software (version 9.4). Before analysis, the normal distribution of the data was tested using the UNIVARIATE procedure, as well as the Shapiro-Wilk and Kolmogorov-Smirnov tests. Results were declared as mean±SEM, and a p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Virus Production and Inactivation Kinetics

At the beginning of the inactivation process, the PPR virus suspension contained $10^{6.4}$CCID50/ mL. An equation of Y= - 0.0121X+5.8540 with an R-square value (R^2) of0.9226 was fitted for the change in the PPR virus titer (Y) in response to inactivation with 1 Mm of BEI (Figure 1) during a different post-inactivation time (Y). Results showed that approximately 6h were required to reach the virus titer to detection threshold (LOD) of $10^{1.5}$CCID50/mL. However, complete virus inactivation was estimated 8 h after the initial inactivation process.

The equation of Y=-0.017X+5.0098 explained the PPRV titer response (Y) to inactivation with 4 mM BEI during different post-treatment times (X). Results showed an R^2value of 0.84 for the fitted equation (Figure 2). Following inactivation initiation, the PPRV titer reached LOD and complete inactivation point at 3.5 and 5 h, respectively.

The equation of Y=-0.0116X+5.7441 was obtained for the change of PPRV titer (Y) in response to H_2O_2 treatment during different times of the inactivation process. An R^2 value of 0.95 was obtained for the fitted equation. Although about 6 h was required to reach the PPRV titer to LOD level, the complete inactivation was estimated to be attained at 8 h post-initial treatment (Figure 3).

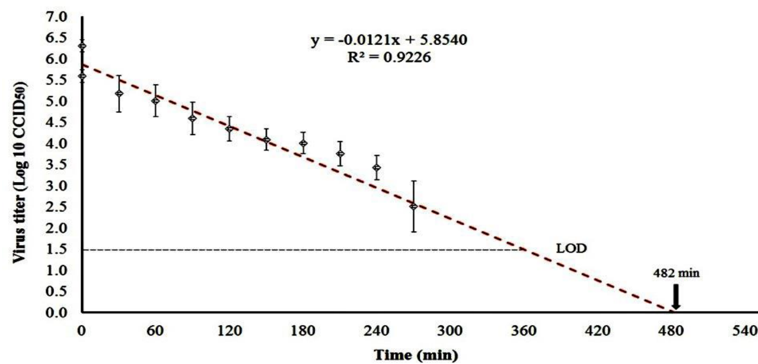

Figure 1. Inactivation kinetics of Peste des petits ruminants virus treated by 1 mM binary ethylenimine

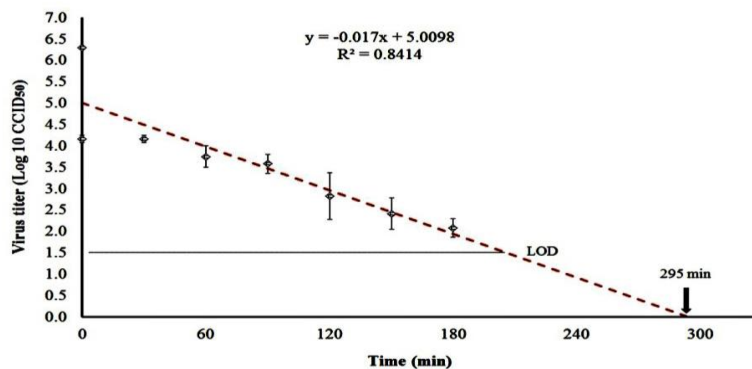

Figure 2. Inactivation kinetics of Peste des petits ruminants virus using4 Mmbinary ethylenimine

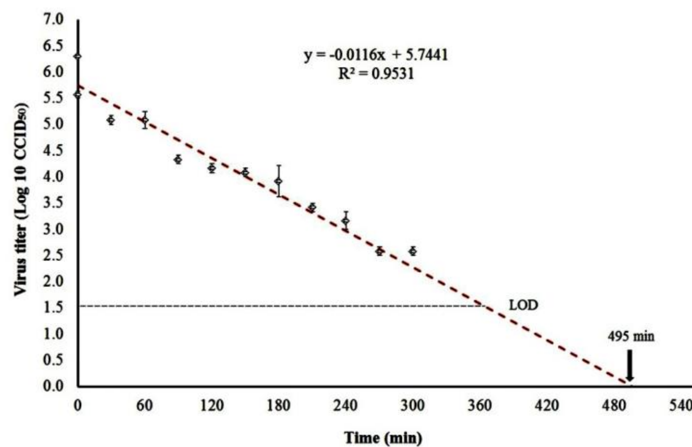

Figure 3. Inactivation kinetics of Peste des petits ruminants virus using H_2O_2

3.2. Innocuity and Abnormal Toxicity Test

The treated animals were alive with no signs of ill-health in abnormal toxicity tests throughout the test period. Moreover, no bodyweight loss or mortality was observed in the group of mice inoculated with either H_2O_2 or BEI inactivated virus suspensions, indicating the absence of the residual infectious virus.

3.3. Vaccine Immunogenicity in Mice

Results of sera antibody titer in response to the administration of various formulated iPPR vaccine with different inactivators and adjuvants were reported in figures 4-6. The results indicated a significant effect of inactivants (P<0.01) in sera antibody titer response (Figure 4), while the effect of adjuvant (Figure 5) and the interactive effect of inactivator×adjuvant (Figure 6) was not significant (P>0.05). Treatment of the PPRV with 1 mM BEI for the inactivation of the virus elicited a higher antibody response, compared to both 4 mM BEI and H_2O_2 (P<0.05). Meanwhile, no significant difference was noted between 4 mM BEI and H_2O_2 in terms of sera antibody titer against iPPR administration in mice (2.51, 2.25, and 2.22 for 1mM BEI, 4 mM BEI, and H_2O_2, respectively; P>0.05).

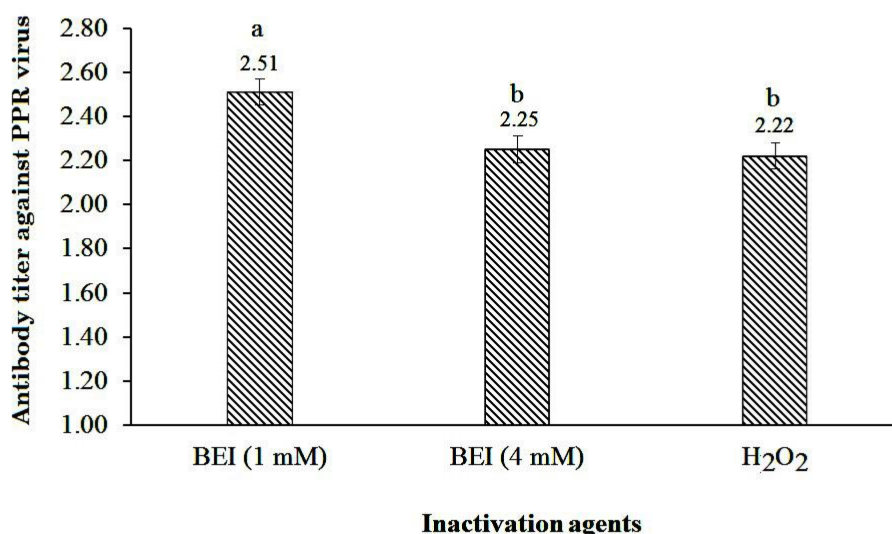

Figure 4. The main effect of different inactivant agents on antibody response against Peste des petits ruminants virus in mice
a,b: values with different superscripts are significantly different (P<0.05).

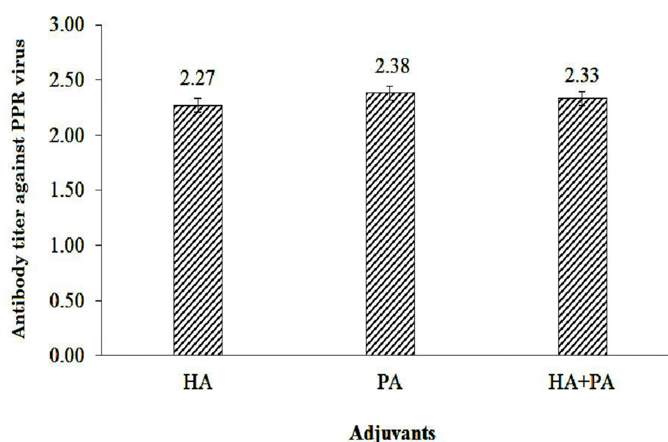

Figure 5. The main effect of different adjuvants on antibody response against Peste des petits ruminants virus in mice
Note: HA: Hydroxide aluminum; PA: phosphate aluminum.

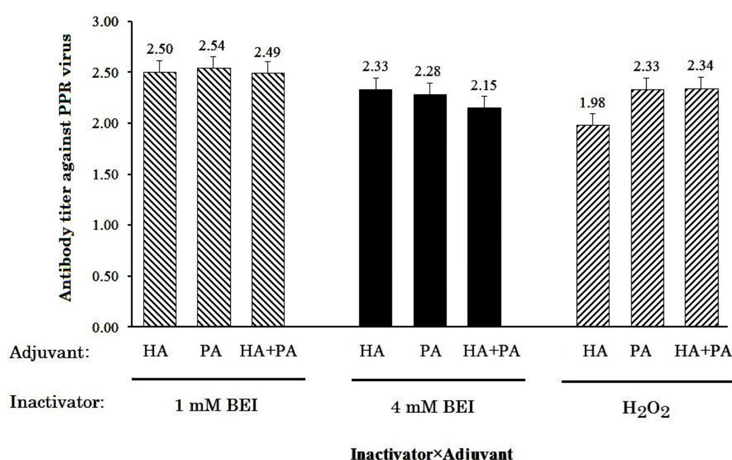

Figure 6. The interactive effect of inactivator×adjuvant on antibody response against Peste des petits ruminants virus in mice
Note: HA: Hydroxide aluminum; PA: phosphate aluminum.

4. Discussion

In this study, the PPR virus (Nigeria 75/1 strain) was used to prepare a new monovalent inactivated vaccine and its immunogenicity was assessed in mice. The iPPRV antigen was inactivated with 1 and 4 Mm BEI and 3% H_2O_2. The BEI acts only on the nucleic acids, not on the viral capsid proteins (4). BEI has been extensively used to inactivate other viruses, including foot-and-mouth disease (15), bluetongue (16), porcine parvovirus (17), and Newcastle disease (18), as well as PPR virus recently (3). In contrast, H_2O_2 has been successfully used to inactivate the rabies virus (13). For the first time, in this study, H_2O_2 was used for the inactivation of the PPR virus. Results of safety and abnormal toxicity test indicated that the formulated vaccines were safe with no abnormal signs of toxicity. The inactivated PPR vaccine with two inactivators BEI (1 and 4Mm) and H_2O_2 induced the high seroconversion in mice, achieving approximately 1/323 (2.51), 1/178 (2.25), and 1/166 (2.22) for BEI 1mM, 4mM, and H_2O_2, respectively. However, seroconversion cut-off for protection against PPR virus is at least 1/10 (2).

The results of comparing three adjuvants in each inactivator (BEI 1Mm, 4mM, and H_2O_2) showed that the average titers in different adjuvants had no statistically significant difference. However, a comparison between different inactivants revealed that the average antibody response was higher when 1 Mm of in BEI was used as the inactivator. The use of 4 Mm of BEIwith higher toxicity for PPR virus probably destroyed the viral structure and caused a lower antibody response than 1 Mm BEI. There are several studies indicating that the BEI is a good inactivating agent in the lower concentration (19). Despite the fact that BEI(1Mm) may be a better inactivator for eliciting antibody response in mice, the use of H_2O_2 is safer and cheaper than BEI. The BEI is a kind of aziridine that is formed by the cyclization of 2-chloroethylamine hydrochloride. BEI is known to alkylate nucleic acids; however, it does not damage the protein of inactivated virus in the concentration of 1 Mm. Some reports show the carcinogenic effects of BEI (6). Meanwhile, H_2O_2 as a famous oxidizing agent as considerable antimicrobial and antiseptic properties. Furthermore, it can be used for the sterilization of different surfaces and surgical tools due to its effectiveness against a wide range of viruses, bacteria, and bacterial spores (20). H_2O_2 inactivation of range of DNA and RNA viruses showed minimal damage to epitopes, compared to BPL and formalin; additionally, the superiority of H_2O_2 inactivated vaccines was demonstrated by the elicitation of a strong neutralizing antibody response, effective T cell responses, and protection in mice (9).

In addition, it is worth noting that according to US FDA, even 30% H_2O_2 solution is considered an environmentally safe chemical as it is decomposed to oxygen and water. Therefore, the use ofH_2O_2in the inactivation process may not require complicated purification processes to be removed from the prepared biological process. Moreover, seroconversion following BEI inactivated PPR vaccine is more than H_2O_2; however, the use of H_2O_2 is recommended because of its safety, price, and no adventitious residue in the vaccine. This may nominate H_2O_2 for the inactivation of iPPRV during the vaccine manufacturing process; however, the immunogenicity response from the provided iPPRV vaccine should be evaluated in target animals (sheep and goat).

5. Conclusion

In this study, the immunogenicity of formulated inactivated PPR vaccine that was prepared by different inactivant agents and adjuvants was investigated in mice. The present findings indicated that the inactivation of the PPR virus by 1 mM BEI was associated with higher antibody response against PPRV than 4 mM BEI and H_2O_2. However, the appropriate inactivation kinetic of the virus and immunogenicity associated with the use of H_2O_2, as well as its biocompatibility property and better cost-benefit, nominated H_2O_2 to be used in iPPR preparation; however, more investigation in target animals are required.

Authors' Contribution

Study concept and design: M. L., H. R. V. and S. M. A. D.

Acquisition of data: M. A.

Analysis and interpretation of data: H. K., M. L. and M. A.

Drafting of the manuscript: M. A.

Critical revision of the manuscript for important intellectual content: M. A., H. K., M. L. S. M. A. D. and H. R. V.

Statistical analysis: M. A.

Administrative, technical, and material support: M. A., H. K., M. L. S. M. A. D. and H. R. V.

Ethics

All the procedures were approved by the Ethics Committee at the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization in Karaj, Iran (No. 2-18-18-061-971034).

Conflict of Interest

The authors declare that they have no conflict of interest.

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