Preparation of an inactivated Peste des Petits Ruminants (PPR) vaccine and its comparative immunogenicity evaluation in animal model

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Abstract

Peste des petits ruminants (PPR) is a highly contagious disease that is considered as a major threat to 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 was aimed to compare the inactivation kinetics of the PPR virus via different inactivant and immunogenicity evaluation of the iPPRV formulated vaccine in mouse. The vaccinal live PPR virus was inactivated by either H₂O₂ 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 mixed of AH and AP, and were intraperitoneally (IP) administrated (0.1 mL) to 90 BALB/c mice in a completely randomized design and 3×3 factorial arrangement (n= 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 minutes 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, measured 42d post-immunization in mouse, was significant (P<0.05), but the effect of adjuvant and interaction effect of inactivator×adjuvante were not affected (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 the 4 mM BEI and H_2O_2 (2.51 vs. 2.25 and 2.22, respectively). Meanwhile, there were no significant different between 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 mix of AH and AP was associated with a higher immune response against PPR virus in mouse. 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 preparation iPPR; however, more investigation in target animals are required.

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

**Introduction**

Peste des petits ruminants (PPR) is an acute or sub acute 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 a high mortality and morbidity resulted in a considerable economic loss in the endemic area. This disease caused by PPR virus that belong to the Morbillivirus genus of the Paramyxoviridae. Poor nutritional status and concurrent parasitic or bacterial infections enhances the severity of the PPR clinical signs (Kitching, 1988).

As a global strategy, eradication of PPR up to 2030 is scheduled during four phases. Before and during phase 1, the country does not have any structured response mechanism in place; however, the main controlling approach at the phases 2 and 3 is programmed on the base of vaccination. Immunization by a live-attenuated PPR vaccine, as a normal control component of the stage 2, is targeted in specific zone where PPR is endemic or at high risk, or a specific sub-population at higher risk or of higher commercial value. Meanwhile, in the phase 3, the vaccination strategy will depend on the outcomes of the stage 2 and inactivated PPR vaccine is commonly applied. At the end of the phase 3, 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 the phase 4, there is not any vaccination and therefore no any post vaccination monitoring. All stocks of PPR vaccine (monovalent and polyvalent) should be safeguarded by the competent authorities or remove from non-accredited sites (OIE, 2017).

The inactivated PPR vaccine contain viral antigen inactivated commonly by binary ethylenimine (BEI) (Ronchi et al., 2016). It has been shown that BEI reacts with viral nucleic acids while conformation and accessibility of epitopes is preserved (Bahnemann, 1990). The BEI has been successfully inactivated various viruses for vaccine production (Razmaraii et al., 2012, Dilovski and Tekerlekov, 1983). However, several limitations such as being
carcinogen, having hazardous residual as well as laboratory complicity associated with using BEI, increases the willing to find an appropriate alternative (Delrue et al., 2012) such as H$_2$O$_2$.

It has been shown that 3% aqueous solution of H$_2$O$_2$ inactivated both RNA and DNA viruses with up to a 6-log$_{10}$ reduction in titer observed in less than 2 h (Abd-Elghaffar et al., 2016). showed that West Nile virus which cause mosquito-borne and epidemic encephalitis was efficiently inactivated by H$_2$O$_2$ (Pinto et al., 2018). This vaccine could be used safely in at-risk population and could induce effective neutralizing antibody responses in BALB/c mice (Amanna et al., 2012). It has been postulated that hydroxyl radicals originated from H$_2$O$_2$ attack carbon double bonds in the nucleosides or obstruct the hydrogen atoms and thereby break single- or double strands in the genomic structure, leading to inactivate targeted organisms (Paik et al., 2000). During reaction, H$_2$O$_2$ produces H$_2$O with no environmental contamination or human adverse effect. These properties nominated the H$_2$O$_2$ as a safer and more feasible inactivator component as compared to EBI. However, the knowledge about the effectiveness of the use H$_2$O$_2$ during inactivation of vaccinal viruses is preliminary. Therefore, this study was conducted to investigated 1) the feasibility of inactivation PPR virus used in vaccine production process; 2) comparison the results of PPR virus inactivation by H$_2$O$_2$ and BEI and 3) evaluation of immunogenicity of inactivated PPR virus in the animal model.

Material and method

Cell culture
Lymphoid Cell line (F9) cells is kind of suspension cell line, derived from cow (Bos tareus) lymph nodes established in Razi Vaccine and Serum Research Institute of Iran (Mofrad et al., 2016) were seeded in cellspin 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.) and supplemented by 100 IU/ml penicillin and 100 µg/ml Streptomycin (Sigma-Aldrich, U.S.A.).

**Preparation of virus**

PPR virus (Nigeria 75/1) with 0.03 MOI was co-infected to F9 suspension cell line. The cellspin bottles were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cell viability was evaluated daily by Trypan Blue (Sigma-Aldrich, U.S.A.) staining on the Hemocytometer. When the viability arrived to less than 15 %, suspension fluid was freeze in – 20 °C. Harvest was titrated (OIE, 2017) after freeze-thawing and clarification by centrifuge (10 min. at 1370g). Finally, harvest was aliquoted and freeze in -70°C. So, after each manipulation, PPR virus were tested for bacterial, fungi and mycoplasma contamination (Commission, 2019).

**Virus inactivation**

The PPR virus (10^{6.3} TCID50/ml) was inactivated comparative by binary ethylenimine (BEI) and H_2O_2. Briefly, the inactivation kinetics of the PPR virus was determined by treatment of 1 and 4 mM BEI during different post-inactivation times, at room temperature (Ronchi et al., 2016). 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. So, for H$_2$O$_2$ inactivator, a 30% stock solution of H$_2$O$_2$ (Carel Roth, Germany) was sterilized by membrane filtration and was kept in a dark sealed container. The virus suspension was treated with H$_2$O$_2$ stock solution to a final concentration of 3% H$_2$O$_2$ at room temperature (Abd-Elghaffar, 2015). Finally, to remove residual H$_2$O$_2$, each aliquot was duplicity treated with 12.5 U/ml catalase (MP Biomedical. U.S.A.) for 10 min at room temperature (Martins et al., 2013). The titer of each aliquot was measured by micro titration method. For each inactivation agents, the process was repeated three times and average of the data was reported as the final outcome.

Safety test

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

Experimental vaccine formulation

The 300 ml of inactivated PPRV (iPPRV) product was divided into 3 aliquots. Each aliquot used to prepare different vaccine formulation with Aluminum hydroxide, Aluminum phosphate or mixture of Aluminum hydroxide and Aluminum phosphate (50/50 v/v). The formulated vaccines were stored at 5 ±3°C until future evaluation (OIE, 2017).

Quality control test of the formulated vaccines

Innocuity test
Abnormal toxicity test was conducted in accordance with European pharmacopoeia monograph. Briefly, twenty 17–22 g BALB/c mice were randomly divided into two groups (n=10/group) and intra-peritoneal (i.p.) received 0.1 ml of either BEI or H\textsubscript{2}O\textsubscript{2} formulated vaccines. The animals were observed for any signs of ill-health during a 14 days period.

**Vaccination**

Thirty male BALB/c mouse weighted 20-25 g were randomly divided into three groups (n=10 per group) and vaccinated by i.p. administration of 0.1 mL (Ronchi et al., 2016) of each formulated vaccine with different inactivant (BEI and H\textsubscript{2}O\textsubscript{2}), and were boosted 21 days later. The control group received 0.1 mL of saline as placebo. Blood samples of all animals were collected 42 days after first immunization. No body weight loss or mortality was observed in group of mice inoculated with either H\textsubscript{2}O\textsubscript{2} or BEI inactivated virus suspensions.

**Evaluation of vaccine immunogenicity in mouse**

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

Data were analyzed in a completely random design with a 3×3 factorial arrangement by GLM procedure of SAS 9.4 software. Before analyze, normal distribution of the data were tested using UNIVARIATE procedure and Shapiro-Wilk and Kolmogorov–Smirnov testes. Results were declared as mean ± SEM and P<0.05 was considered as the level of statistically significant difference between the means.

Results

Virus production and inactivation kinetics

At the beginning inactivation process the PPR virus suspension contained $10^{6.4}$ CCID50/ml. An equation of $Y = -0.0121X + 5.8540$ with R-squer value (R²) of $= 0.9226$ was fitted for the change in the PPR virus titer (Y) in response to inactivation with 1 Mm of BEI (Figure 1) during different post-inactivation time (Y). Results showed that about 6 hrs were required to reach the virus titer to detection threshold (LOD) of $10^{1.5}$ CCID50/ml. However, complete virus inactivation was estimated 8 hrs after initial inactivation process.
Figure 1. Inactivation kinetic of Peste des petits ruminants virus treated by 1 mM binary ethylenimine (BEI).

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

Figure 2. Inactivation kinetic of Peste des petits ruminants virus using 4 Mm binary ethylenimine (BEI).
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 inactivation process. An $R^2$ value of 0.95 was obtained for the fitted equation. Although about 6 hrs was required to reach the PPRV titer to LOD level, the complete inactivation was estimated to attain at 8 hrs post initial treatment (Figure 3).

![Figure 3. Inactivation kinetic of Peste des petits ruminants virus using $H_2O_2$.](image)

**Innocuity and abnormal toxicity test**

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

**Vaccine immunogenicity in mouse**
Results of sera antibody titer in response to administration of various formulated iPPR vaccine with different inactivators and adjuvants was reported in Figure 4-6. Results indicated a significant effect of inactivant (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 inactivation of the virus elicited a higher antibody response compared with both 4 mM BEI and H$_2$O$_2$ (P<0.05). Meanwhile, no significant difference was noted between the 4 mM BEI and H$_2$O$_2$ 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$_2$O$_2$, respectively; P>0.05).

![Bar chart showing antibody titer against PPRV with different inactivants](image_url)

**Figure 4.** The main effect of different inactivant agents on antibody response against Peste des petits ruminants virus in mouse.

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 mouse.

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 mouse.

Note: HA: Hydroxide aluminum; PA: phosphate aluminum.
Discussion

In this study, PPR virus (Nigeria 75/1 strain) was used to prepare a new monovalent inactivated vaccine and its immunogenicity was assessed in mouse. The iPPRV antigen was inactivated with 1 and 4 Mm BEI and 3% H$_2$O$_2$. The BEI acts only on the nucleic acids and not on the viral capsid proteins (Bahnemann, 1990). BEI has been extensively used to inactivate other viruses including foot-and-mouth disease (Dilovski and Tekerlekov, 1983), bluetongue (Stott et al., 1979), porcine parvovirus (Buonavoglia et al., 1988) and Newcastle disease (King, 1991) and recently, PPR virus (Ronchi et al., 2016). In contrast to, H$_2$O$_2$ has been successfully used to inactivate rabies virus (Abd-Elghaffar, 2015). For the first time, in this study, H$_2$O$_2$ was used for inactivation of PPR virus. Results of safety and abnormal toxicity test indicating that the formulated vaccines were safe with no abnormal signs of toxicity. The inactivated PPR vaccine with two inactivators BEI (1 and 4 Mm) and H$_2$O$_2$ induced the high seroconversion in mice, achieving approximately 1/323 (2.51), 1/178 (2.25), 1/166 (2.22) for BEI 1mM, 4mM and H$_2$O$_2$ respectively. While, seroconversion cut-off for protection against PPR virus is at least 1/10 (OIE, 2017).

However, comparison between three adjuvants in each inactivators (BEI 1Mm, 4mM and H$_2$O$_2$) showed that the average titers in different adjuvants does not have a statistically significant difference. However, comparison between different inactivant revealed that the average antibody response was higher when 1 Mm of in BEI was used as the inactivator. Probably the use of 4 Mm of BEI with higher toxicity for PPR virus and probably destroy the viral structure and caused lower antibody response than the 1 Mm BEI. There are several studies indicating that the BEI is a good inactivating agent in the lower concentration (Mondal et al., 2005). Despite the fact that BEI (1Mm) may be a better inactivator for eliciting antibody response in mice, but the using of H$_2$O$_2$ is safer and cheaper than BEI. The
BEI is a kind of aziridines and formed by the cyclization of 2-chloroethylamine hydrochloride (BEA). BEI is known to alkylate nucleic acids, but do not damage the protein of inactivated virus in the concentration of 1 Mm. However, there is some reports that show carcinogenic effects of BEI (Delrue et al., 2012). Meanwhile, H$_2$O$_2$ as a famous oxidizing agent, have a considerable antimicrobial and antiseptic properties. It can be used for the sterilization of different surfaces and surgical tools due to its effectiveness against wide range of viruses, bacteria and bacterial spores (Ogden et al., 2008). H$_2$O$_2$ inactivation of range of DNA and RNA viruses showed minimal damage to epitopes compared to BPL and formalin and superiority of H$_2$O$_2$ inactivated vaccines was demonstrated by the elicitation of a strong neutralizing antibody response, effective T cell responses, and protection in mouse (Amanna et al., 2012). In addition, it is worth noting that according to US FDA even 30% H$_2$O$_2$ solution is considered as an environmentally safe chemical as it is decomposed to oxygen and water. Therefore, the use of H$_2$O$_2$ in the inactivation process may not require complicated purification processes to be removed from the prepared biological. Also, seroconversion following BEI inactivated PPR vaccine is more than H$_2$O$_2$, but use of H2O2 is recommended because of its safety, price and no adventitious residue in vaccine. This may nominate H$_2$O$_2$ for 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).

Conclusion

In this study, immunogenicity of formulated inactivated PPR vaccine that was prepared by different inactivant agents and adjuvants was investigated in mouse. The present findings indicated that inactivation of PPR virus by 1 mM BEI was associated with a higher antibody
response against PPRV than 4 mM BEI and H$_2$O$_2$. However, the appropriate inactivation kinetic of the virus and immunogenicity associated with the use of H$_2$O$_2$ as well as its biocompatibility property and better cost-benefit, nominated H$_2$O$_2$ to be used in preparation iPPR; however, more investigation in target animals are required.

**Ethics**

We hereby declare all ethical standards have been respected in preparation of the submitted article.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

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**Authors’ Contribution**

Study concept and design: Dr. M. Lotfi; Dr. H.R. Varshovi and Dr. S.M. Azimi; Experiment was conducted by Dr. M.Akbarian; Data was analyzed and interpreted by Dr. H. Keyvanfar, M. Lotfi and Dr. M.Akbarian. All the authors was contributed in manuscript writing and reviewed written.
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References


