



Immunogenicity and Efficacy of Formaldehyde-Inactivated and γ -Irradiated Vaccines against Hemorrhagic Septicemia in Mice

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Abstract

Background: Hemorrhagic septicemia (HS) is an acute and fatal disease of livestock caused by the bacterium *Pasteurella multocida*. Prophylactic vaccination is the most effective method for controlling this disease. Formaldehyde-inactivated HS vaccines are commonly used; γ -irradiated vaccines show potential. **Objectives:** This study aimed to evaluate the immune efficiency and protective immunity of formaldehyde-inactivated and γ -irradiated vaccines for HS in mice. **Methods:** Vaccines were compared through mouse bioassays. Two oil-based experimental vaccines, each 6.4×10^{18} CFU/ml, to potentiate the immune response. Physical (gamma radiation) and chemical (formaldehyde) methods inactivate bacterial cultures. Twenty mice were divided into four groups; Groups 1 and 2 were vaccinated with a formaldehyde-inactivated vaccine and γ -irradiated, while Group 3 was inoculated with the reference HS vaccine. Group 4 served as the negative control. Swelling, mortality rate, and body temperatures of all mice were recorded from days 1 to 14. The antibody titers of both vaccines were assessed using an indirect hemagglutination assay (IHA). All vaccinated animals were challenged with virulent *P. multocida*. **Results:** Experimental vaccines had no adverse effects on the mice, except for slight swelling at the injection site, which faded within 3-4 days. The IHA showed the irradiated vaccine produced early antibody, more pronounced compared to the formaldehyde-treated vaccines. The oil-based vaccine formulations showed a slower, steady immune response that continued throughout the experiment. The observed challenge protection rates were 95% for Group 1 (19/20 mice), 80% for Group 2 (16/20 mice), 85% for Group 3 (17/20 mice), and 0% for the control group (0/20 mice). An ANOVA *p*-value of 0.001 indicated a statistically significant difference ($p < 0.05$). **Conclusion:** Efficacy and safety of the irradiated vaccine were found to excel; a suitable candidate compared to formaldehyde-inactivated vaccines. However, vaccine trials in cattle or buffaloes must be set up so that the safety and protective properties seen in mice are also applicable to the target species.

Introduction

Hemorrhagic septicemia (HS) is an acute and lethal disease of bovine caused by the bacterium *Pasteurella multocida* serotype B:2 (Arif et al., 2013; OIE et al., 2013b) which is usually found in the upper respiratory tract of animals (De Alwis et al., 1992). The infection occurs via direct animal-to

-animal contact (transmission via infected animals) or by ingesting/inhaling it (contact through important disease with periodic outbreaks taking place in Southern Europe (Henning et al., 1949). The confirmed outbreaks of HS in America occurred in bison (Yellowstone National Park), most recently from 1965 to 1967. HS caused significant economic loss in

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Pakistan to the livestock sector, which is estimated at Rs. 1.887 billion per annum, and case fatality is 70% and is responsible for an annual loss of several hundred million dollars for animal production (May et al., 2001). HS is thought to exist in urbanized nations with an abundant population of water buffalo, where the climatic conditions resemble those of tropical Africa and Asia (Benkirane & De Alwis, 2002). In Asian countries, HS outbreaks are more common during the monsoon season, which has high levels of humidity, and the temperature is also high (D. Alwis, 1999a). Usually, this pathogenic infection of bacteria can be cured with the help of a range of antibiotics, but the probability of saving the animal rises if it is cured during the initial phases of the disease (Benkirane & De Alwis, 2002). As an indication that antibiotics are ineffective, vaccination is required for control when illness returns after therapy (Ali et al., 2014). Active immunity against *P. multocida* infection is primarily a humoral immune response. HS bacterin has been utilized in the past for the immunization of animals (Wasti et al., 2015). However, the time of immunity conferred by bacterin was short. Thus, bacterin was converted to adjuvant vaccines. Routinely, alum-precipitated HS vaccine is used at a mass scale in Pakistan; it provides immunity only for three to four months and is used as a primary vaccine against HS (IQBAL & Habib, 2014; Israil & Quader, 1960; Iyer et al., 1955). The protective efficiency of alum-precipitated vaccines is just 60% (FAO et al., 1991). For these reasons, oil-based vaccines are used in many regions as they provide long-term immunity for one year (Patten et al., 1993; Verma et al., 1998). However, oil-based vaccines are difficult to administer because of high viscosity and inflammation at the injection site, as well as reactions after vaccination, which have frequently been documented (Ali et al., 2000). The basic approach in the development of hemorrhagic septicemia vaccine is to inactivate *P. multocida* with physical (gamma radiation) or chemical (formaldehyde) techniques, which results in the retention of antigens that are responsible for the production of the host's defensive immune response (Verma et al., 1998).

A live HS vaccine, which is derived from an avirulent *P. multocida* strain B:3,4 (a fallow deer strain), was introduced to the bovines of more than six months of age via intranasal aerosol. There are safety and efficacy concerns relating to the use of live vaccines that have led to investigations into the use of inactivated vaccines instead. According to the Food and Agriculture Organization (OIE et al., 2012) Radiation-based pathogen inactivation is a potentially useful technique in the development of safe vaccines and the sterilization of biological materials. Gamma radiation has been of particular interest since the 1940s in inactivating pathogens and retaining antigenic protein structures. In contrast to chemical treatment procedures, including formaldehyde fixations and exposure to ultraviolet light, gamma radiation penetrates the biological substance, thus targeting DNA and RNA without

affecting the surface antigen significantly, and there is no need to clear chemical residues. With this advancement in technology, researchers have come up with inactivated vaccines that tend to stimulate a unique and possibly better immune response than that induced by heat or chemically inactivated vaccines (Radiation et al., 1982b).

Gamma-irradiated *Streptococcus agalactiae* vaccines were also found to induce significantly higher CD4⁺ T-cell responses and opsonophagocytic antibody titers ($P < 0.01$) than formalin-inactivated vaccines, and thus, this indicated that immunogenic structures would be better preserved by using irradiation. In contrast to formalin, gamma irradiation, with its mechanism of action of affecting nucleic acids, does not lead to cross-linking of surface proteins or to diminishing the availability of epitopes. The challenged gamma-irradiated *S. agalactiae*-vaccinated mice had an 80 percent survival rate, contrasted with 50 percent in formalin-inactivated vaccines, indicating the most significant protective efficacy (Zhi et al., 2023).

The rationale behind this study is that the existing vaccines against HS are problematic mainly because live vaccines have significant safety issues, and some inactivated vaccines, i.e., those killed using formaldehyde, have shown reduced immunogenicity. This would mean that developing a gamma-irradiated HS vaccine would be a cost-effective option, which is safer and more effective than continuing to use the existing HS vaccine, thereby saving the financial impact of incurring losses and improving the health of the livestock on a global scale. In the current study, we managed to develop gamma-irradiated and formaldehyde-inactivated HS vaccines, keeping the same concentration of the antigen, and performed an experimental study in the mouse to compare their humoral immune responses. The refined vaccine could help in controlling HS and will help both veterinary practitioners and farmers. This study hypothesizes that a gamma-irradiated oil-based vaccine, compared to a formalin-treated vaccine and reference NIAB® HS vaccine, provides better protection against HS.

Materials and Methods

Sample collection

For the isolation of *P. multocida*, the infected heart of a buffalo was collected from Marzi pura, Faisalabad, Pakistan, which showed characteristic signs of HS. The sample was transported (on ice packs) to the Vaccine Development laboratory of the Animal Science Division, Nuclear Institute of Agriculture and Biology, Faisalabad. Blood was collected aseptically from the heart. A direct blood smear was prepared and stained with Giemsa stain. *P. multocida* is not present in blood before the last stage of infection and is not constantly present in nasal discharge and body fluid of diseased animals. All animal experiments were performed according to the ethical aspects approved by the institution's animal ethics committee located at NIAB, Faisalabad.

Isolation and Identification of *P. multocida*

BHI media was made ready, and blood was inoculated on it. Streaks were made on blood agar, Casein/Sucrose/Yeast (CSY) agar, and nutrient agar plates to allow isolation and purification of *P. multocida*. The identification of colonies isolated on blood agar and nutrient agar plates was done based on morphological features after incubation of the plates at 37 °C for 24 hours. There is a pleomorphism in the length of the bacillus in older culture. They vary in terms of size (i.e., larger grayish colonies on blood agar and bluish transparent colonies on nutrient agar plates). Old cultures had produced rough colonies. Non-hemolytic colonies of Gram-negative bacteria were identified by use of biochemical tests (IMViC and catalase test) and PCR specific to HS-causing type B.

Hemorrhagic septicemia type-B specific PCR Technique

Preliminary identification of *P. multocida* type B:2 was done by PCR amplification assay using type B-specific primers KTT72 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3' and KTSP61 5'-ATC-CGC-TAA-CAC-ACT-CTC-3' (Townsend et al., 1998). 25µl PCR mixture was prepared; 2.5µl of 10X Taq polymerase Buffer, 2.5µl of 25mM MgCl₂, 0.5µl dNTPs of 10mM, 0.1µl Taq Polymerase and 17µl ddH₂O to adjust the volume. The PCR amplification was carried out with an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 9 min. A 1.5 % agarose gel was used to run the PCR product along with a 100bp ladder.

Seed Culture; Antigen Preparation

P. multocida cultured in BHI broth incubated at 37°C for 8 hours. 0.1 ml broth was inoculated into mice intraperitoneally to confirm pathogenicity. Seed culture was prepared for antigen preparation used in the vaccine. The purified colony of B:2, confirmed by the above-mentioned procedure, was inoculated into the CSY broth and incubated at 37°C for 24h. 1 mL from the seed culture was then inoculated into 1000 mL of CSY broth and incubated at 37°C for 24h. After incubation, the broth was centrifuged at 4000 rpm for 20 min, and the supernatant was discarded.

The pellet was then mixed with normal saline and divided into two parts. The 1st pellet was inactivated by 0.3% of 37% formaldehyde. The second pellet was inactivated by gamma radiation for 1 hour (660 Gy/hour). According to literature studies on inactivation thresholds of infections by *P. multocida*, using gamma irradiation, values have been reported to vary between strains, media, and conditions, and the dose at which there is complete inactivation of replication

but the organism still retains immunogenicity are estimated in the range of 1 and 10 kGy (1000 and 10,000 Gy) (Ahmed et al., 2024).

At about 45-50 minutes, the bacteria were inactivated. Formalinized and irradiated bacterial suspensions were taken for sterility tests: these vaccines were cultured on Blood agar, Nutrient agar, and MacConkey agar. Optimization of CFU/ml was perfected at 640 nm using a spectrophotometer and adjusted in normal saline to 20% transmittance. It is equivalent to 2×10^9 CFU/ml (Mohiuddin et al., 2014). The vaccines were prepared as the no. of inactivated bacteria /unit volume of vaccine remained equal, such as 6.4×10^8 CFU /ml (Muneer et al., 2005).

Vaccine preparation

For the preparation of both formalin-killed and γ -Irradiated oil-based HS vaccine, in solution I, 31.5 mL of antigen (formalin-killed) was mixed with 3 mL of Tween-80 (3%). Solution I mixed for 30 minutes at 1200 rpm. In solution II, 7.5 mL Span-80 (7.5%) was mixed with 58 mL paraffin oil (58%). After mixing for 30 minutes at 1200 rpm, Solution I was poured into Solution II and homogenized for 3 hours at the same speed. The sterility test of the vaccine was also performed. To confirm the stability of the vaccine, emulsions were kept at 40°C, 37°C, and 4°C for half a month.

Immunization and efficacy of the HS vaccine

For the evaluation of the efficacy of the vaccines, a total of 20 mice were divided into four groups, with 5 mice in each group. Group 1-2 were given experimental vaccines, and Group 3 was inoculated with the NIAB® HS vaccine. Group 4 was kept as a negative control. The booster dose is given on the 15th day after primary vaccination. The body temperature, inflammation at the site of inoculation, and the mortality rate were recorded in the vaccination trial. IHA was performed to assess the antibody production against *P. multocida* (Ashfaq & Hur, 1994).

Statistical Analysis

All the data were analyzed statistically by using Statistix version 8.1. The mean antibody titer among the different vaccinated groups was analyzed by one-way analysis of variance (ANOVA). A value of $p \leq 0.05$ was considered significant (Ruzauskas et al., 2005). The power calculation utilized a difference in survival rates of 20%, a power of 80%, and a significance level of 0.05 to subsequently determine the sample sizes of 20 mice per group.

Results

Bacterial Identification

P. multocida showed smooth, grayish in color, glistening, nonhemolytic, and translucent colonies on blood agar, transparent bluish colonies on CSY media, and no growth on MacConkey agar (Hasan, Ali, Siddique, Rahman, & Islam, 2010). The organism tested positive for the Indole and catalase tests, and negative for the Methyl-red, Voges-Praskauer, and citrate tests (Buxton & Fraser, 1977). PCR results, Figure 1 showed a size of ~618- 620 bp of primer product using F-KTT-72 and R-KTSP-61 primers.

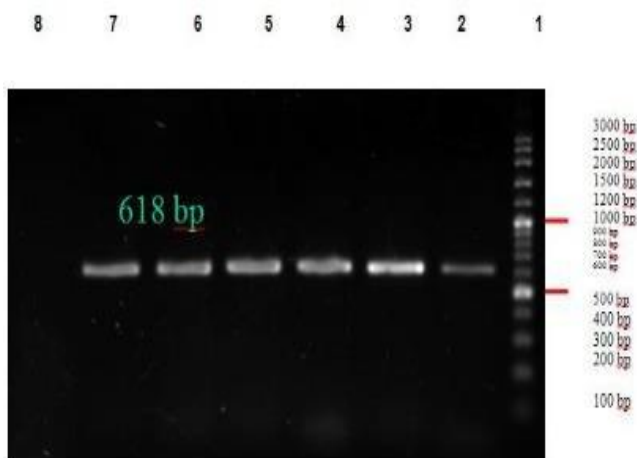


Figure 1. Agarose gel electrophoresis of *P. multocida* serotype B:2 (Lane-1: 1000 bp DNA ladder; Lanes 2-7 Agarose Gel Electrophoresis (1.5%) of PCR product 618 bp obtained with KTSP61, KTT72; Lane-8: Negative sample)

Sterility and stability of the Vaccine

The vaccine was cultured on nutrient, CSY, blood, and MacConkey media, showing no growth. The Cannon viscometer measured the white color and viscosity. The emulsion traveled 110-130 mm/sec at 25 °C.

Post-vaccination Observation and Safety Assessment

After the vaccination, the mortality rate was recorded in groups 1 to 4 from day 1-14. There was no mortality rate recorded in this duration (0.00%). All mice stay healthy. Vaccines are secured and have no adverse effect except for slight inflammation at the injection site (Ruzauskas 2005). Post-vaccination means body temperature of Groups 1-4 from day 1-14 was 36.81, 36.80, 36.80, 36.79, respectively. vaccination mean body temperature of Groups 1-4 was non-significant. Whereas Group 4 was kept as a control.

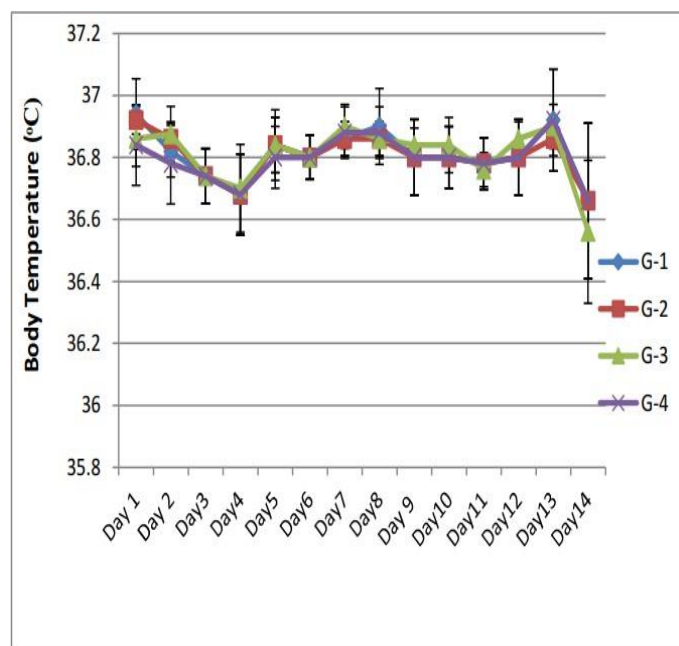


Figure 2. Graphical representation of post-vaccination body temperature (°C).

Vaccine Efficacy and Immune Response Evaluation

Survival rates in all the G-1, G-2, G-3, and G-4 groups were 60% (3/5 mice), 100% (5/5 mice), 80% (1/5 mice), and 100% (5/5 mice), respectively, as shown in Table 1. On the 27th day, the IHA titer shown in Figure 3, and its respective geometric mean titer (GMT) were calculated. The results are shown in Table 2, which shows G-1 has GMT 4 display lower tiers, G-2 has the highest titers with GMT 32, followed by G-3 with GMT 8.

At 27 days post-vaccination, using one-way analysis of variance (ANOVA) and Tukey HSD post-hoc multiple comparisons, groups 1-4 were compared. The ANOVA result shows a highly significant difference among the groups ($F = 25.9^{**}$, $p < 0.01$). The 95% CI for mean antibody titer was group 1 (3.92–5.68), group 2 (31.94–44.86), group 3 (9.24–13.16), and group 4 (0.00–0.00). These results suggest that G-2 has a higher antibody titer level as compared to others and has a statistically stronger immune response, as shown in Table 2, graphically represented in Figure 4.

Table 1: Percentage survival rate of mice after experimental infection with pathogenic *P. multocida*

Group (n=20)	Type of HS Vaccine	Infection Result	
		%Survived	%Died
G-1	Vac-1 (Formalin treated)	60%	40%
G-2	Vac-2 (Irradiated HS Vaccine)	100%	0%
G-3	Oil-based NIAB HS Vaccine (Reference Vaccine)	80%	20%
G-4	Negative Control	100%	0%

Table 2: IHA Antibody Titres of Vaccinated Mice along with GMT and Means Comparison (Tukey HSD All-Pairwise Comparison test)

Group (n=5)	Titres expressed as Log ₂ X	GMT	Mean ± SD
G-1	2,2,3,2,2	4	4.8000 ± 0.80
G-2	5,5,5,6,5	32	38.400 ± 6.40
G-3	3,4,3,3,4	8	11.200 ± 1.96
G-4	-	-	0.0000 ± 0.00

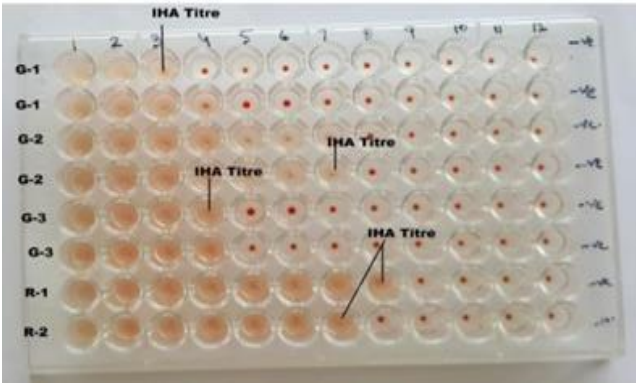


Figure 3. IHA Antibody titres

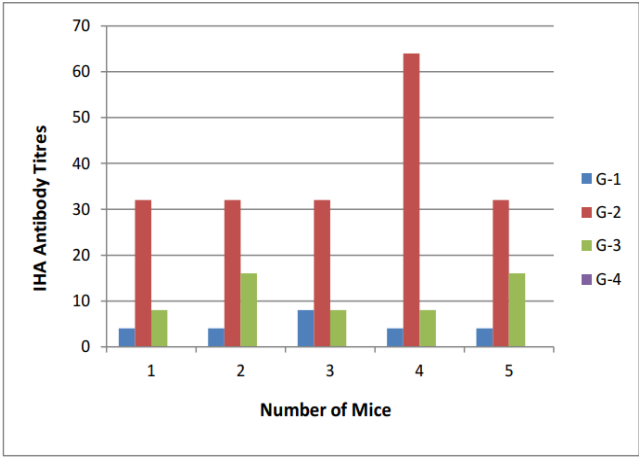


Figure 4. Graphical representation of IHA antibody titres of vaccinated mice

Discussion

In the present study, attempts were made to successfully isolate and characterize *P. multocida*. The organism was isolated from naturally affected buffalo in the Faisalabad district of Punjab, Pakistan. The isolation was confirmed as serotype B:2 through PCR and biochemical tests, aligning with the earlier report. The isolate showed no growth on MacConkey agar and formed non-hemolytic colonies on blood agar. During the present study, the pathogenicity of *P. multocida* was confirmed by inoculation of *P. multocida* into mice that died 24-36 hours post-inoculation. The bipolar organism was seen in blood smears. Pure cultures were grown from heart blood cultures of the mice (De Alwis et al., 1999a). It is reported that the type B strain of *P. multocida* is highly virulent for mice and rabbits, whereas the susceptibility of the Guinea pig is considerably lower (Biswas et al., 2004).

In this study, to potentiate the immune response, physical and chemical methods were used to inactivate bacterial cultures. The physical source of inactivation in this experimental trial was gamma radiation, and the chemical source was 0.3% of 37% formaldehyde. Paraffin oil was used as an adjuvant, and it produces a W/O emulsion vaccine that is stable, well tolerated, and induces a long-term immune response. These promising vaccines were compared with the reference NIAB HS[®] vaccine that was already used for HS in bovines. The 20% transmittance of inactivated cultures was adjusted (Mohiuddin et al., 2014).

The gamma-irradiated vaccine may be performing better as it preserves antigenic structures, as well as its ability to perform better as compared to the formaldehyde-inactivated one. DNA and RNA are prime targets of ionizing radiation, but surface proteins, essential in the development of a powerful

humoral immune response, are spared. Such preservation increases the vaccine capacity to trigger an antibody response twofold, as GMT (32) in Group 2 is more than that of Group 1 (GMT 4) and Group 3 (GMT 8). Also, the lack of chemical products in the irradiated vaccine removes the possibility of interfering with the immune recognition that is present in formaldehyde-inactivated vaccines (Radiation et al., 1982).

Gamma irradiated and formalin-inactivated HS vaccines were known to be sterile and safe due to strict testing in compliance with previous literature. The potency was determined by vaccination of rabbits, which were then directly challenged with *P. multocida*, showing excellent protective ability. Each vaccine was found stable during 12 months at 4°C, which made applicability to field conditions practicable (Anonymous et al., 2008b). In this work, we chose subcutaneous vaccination in mice, which was safe and did not provoke adverse effects in the local area around the injection site, as occurs with intramuscular and intraperitoneal administration (Mohi-ud-din et al., 2014). This selection is in line with the fact that a convenient and safe method of administration is needed due to the possible veterinary application, which promotes the translational potential of the vaccine.

The immune response was assessed through post-vaccination checking of antibody titer and body temperature. Except for a transient increase in rectal or mouth temperature six hours after vaccination, no further systemic or local reactions were observed (Shah et al., 2001). The early rise in antibody response in the case of the irradiated vaccine was more pronounced than that of the formaldehyde-treated vaccine. This can be attributed to the ability of ionizing radiation to prevent bacterial multiplication but retain the antigenic structures that generate a higher humoral immune response and protection against both extracellular and intracellular pathogens (Sheikh et al., 1996). Oil-based vaccines were mostly used to induce a sustained immune response in animals, which increased till the end of the experiment (Mohi-ud-din et al., 2014).

A survival rate of 60%, 100%, 100%, and 80% was observed in groups 1, 2, 3, and 4, respectively; both groups 2 and 3 recorded survival rates of 100% within 26 and 30 days after the challenge. The GMTs of groups 1 through 4 were calculated on day twenty-seven. Statistical analysis placed the groups in the sequence of significant GMT difference: group 2, group 3, group 1, and group 4. According to these findings, the gamma-irradiated vaccination performs exceptionally well, and it can exhibit promising candidacy, subject to additional validation on target species, which could result in a safer and more potent HS vaccine for cattle.

Conclusion

These observations provide evidence that the γ -irradiated HS vaccine is more immunologically effective (conferred 100% protection in the mouse model) than the formaldehyde-inactivated (less than 100% protection) and reference NIAB HS vaccine. Another positive feature of the γ -irradiated vaccine was a good safety profile since no adverse effects except for a transient inflammation at the point of injection were encountered. These findings indicate that the γ -irradiated HS vaccine, prepared with *P. multocida* type B:2 strains, can be a promising candidate subjected to further development. Although the current study may provide valuable understanding, the protective efficacy and safety observed herein should be confirmed in vaccine tests in target livestock species, i.e., cattle or buffaloes, to elucidate whether this will be applicable in field situations.

Author Contributions

Conceptualization, Muhammad Salahuddin Shah; Writing—original draft preparation, Sana Riaz, Aroobah Ashraf, Iqra Shafiq; Writing—review and editing, Muhammad Salahuddin Shah. The authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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