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DEVELOPMENT OF COST EFFECTIVE OIL-BASED HEMORRHAGIC SEPTICEMIA VACCINE USING LIQUID PARAFFIN ADJUVANT

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Abstract

Hemorrhagic septicemia (HS) is a contagious bacterial disease that primarily affects livestock, especially buffaloes and cattle, leading to substantial economic losses in the livestock industry. This research explores the development of an oil-based vaccine for Hemorrhagic Septicemia using liquid paraffin as an adjuvant. The vaccine was prepared using the Robert strain B2 as the antigen. After harvesting the biomass, it was inactivated by adding 0.5% formaldehyde. Tween 20 and Span 80 were emulsified in liquid paraffin as adjuvants, and 0.03% thimerosal sodium was added to the final formulation. The homogenized mixture then passed all quality-control checks before proceeding to in vitro clinical trials in rabbits. Following a primary dose and a booster at day 14, blood samples were collected on day 30, and sera were tested for antibodies by IHA. The results showed that Montanide induced the highest antibody titer (GMT = 256), followed by liquid paraffin (GMT = 64) and Eolane 150 (GMT = 16). The oil-adjuvanted vaccine demonstrated satisfactory immunogenicity and could be deployed in endemic areas.

Keywords: *Brain Heart Infusion (BHI), Casine sucrose yeast (CSY), Eolane, Hemorrhagic septicemia, Indirect hemagglutination assay, Liquid paraffin , Montanide, Pasturella multocida, Vaccine*

INTRODUCTION

The places where HS is presently most prevalent and economically significant disease are in Asia and Africa (1). *Pasteurella multocida* (*P. multocida*) is a Gram-negative bacteria within the Pasteurellaceae family, identified as facultatively anaerobic, fermentative, and chemo-organotrophic (2). Members of this family have been linked to many diseases in both domestic and agricultural species (3). Bovine respiratory disease, transit fever, and shipping fever all fall under bovine pasteurellosis. Previous HS outbreaks were mistakenly identified as "shipping fever." In this context, *P. multocida* serves as an opportunistic secondary infection following a respiratory viral infection comprising infectious bovine rhinotracheitis (IBR), parainfluenza-3 virus (PI3V), bovine viral diarrhea virus, and related stress factors (4).

Oil-based vaccines, also known as emulsion vaccines, are comprised of an antigen, in this case, *P. multocida* —suspended in an oil phase mixed with an aqueous phase. The main advantage of these vaccines is their ability to provide the antigen in a slow, regulated release, so enhancing and extending the animal's immunological response. The oil in the vaccination formulation stabilizes the component elements of the vaccination and enhances the body's immune response to the infection acting as an adjuvant. Adding oil to vaccines as an adjuvant has been shown to boost their efficacy, so enabling the induction of immunity with less dosage required (5).

Liquid paraffin, a type of mineral oil, is a popular choice in vaccine formulations because it's not only affordable but also safe and reliable. Its inert nature and ability to create stable emulsions make it perfect for slowly releasing the vaccine's active ingredients into the body. This gradual release keeps the

immune system stimulated for a longer period, boosting the chances of a lasting immune response. What's more, liquid paraffin is non-toxic and doesn't react with other components, making it a great fit for veterinary vaccines where safety and effectiveness are key. The oil phase in the vaccine acts as a protective barrier for the antigen, allowing it to stay at the injection site longer. This extended presence gives the immune system more time to recognize and interact with the antigen, which leads to a more robust immune memory. By continuously exposing immune cells to the antigen, both antibody production (humoral response) and the body's cell-based defense mechanisms (cell-mediated response) are activated, ensuring stronger and longer-lasting protection (6). This study was aimed to develop cost effective oil based vaccine using liquid paraffin as adjuvant and evaluate the humoral immunity induced.

MATERIALS AND METHODS

SEED COLLECTION

The research lab facility of Center For Advance Studies in Vaccinology & Biotechnology provided the seed for hemorrhagic septicemia, serotype B:2. The pre characterized seed (biochemically and PCR) confirmed was collected from the seed bank of CASVAB.

SEED ACTIVATION, BIOMASS PRODUCTION AND BIOMASS WASHING

The bacterial seed was revived in brain–heart infusion (BHI) broth and incubated at 37 °C for 8 hours. Then, 0.2 mL of this culture was injected intraperitonially into mice. After the mice death, their heart blood was collected and streaked onto fresh BHI and casein–sucrose–yeast (CSY) agar for further culturing. The biomass grown in CSY was harvested after 24 hours of incubation and inactivated with 0.5 % formaldehyde.

The biomass and culture media were centrifuged in large tubes at 6,000 rpm for 10 minutes. The resulting pellet was collected, washed three times with PBS, and re-pelleted by centrifugation. Finally, the pellet was resuspended in 100 mL of normal saline. The next day, the stock suspension was streaked onto various media to confirm sterility.

VACCINE FORMULATION

The vaccine was formulated using liquid paraffin as the adjuvant. Two mixtures were prepared: Mixture A (antigen and Tween 20) and Mixture B (liquid paraffin and Span 80). Both mixtures were combined in a homogenizer following the established protocol (7). Finally, 0.5% formaldehyde and 0.03% thiomersal sodium were added to the emulsion.

STERILITY AND SAFETY TESTING

The vaccine underwent sterility and stability testing, and a safety test was conducted by injecting 0.2 cc of the vaccine into mice. The mice were observed for five days to monitor for any untoward effects (8).

ORGANOLEPTIC CHARACTERISTIC AND DROP TEST

The developed vaccine underwent an organoleptic evaluation, displaying a uniform milky appearance with no phase separation and remaining stable at both room temperature and under refrigeration for several weeks. Next, a drop test was performed following the protocol of (9) a single drop of vaccine was introduced into cold water, and its dispersal and ability to remain afloat without alteration were observed.

STERILITY AND SAFETY TESTS

The final product of vaccine prepared was inoculated with different culture media, Sabourauds's agar, Macconkey agar and Nutrient agar in order to check its sterility during in process control as per requirement of Quality control parameters (10).

The prepared vaccine underwent safety testing: 0.2 mL was administered intramuscularly to two albino mice, and 4 mL to two calves. All animals were then observed closely for 14 days for any adverse effects (10).

COMPARATIVE ADJUVANT STUDY IN RABBITS

Afterward, other commercially available adjuvants, such as Montanide and Eolane 150, were also used in the desired amounts for use in experimental vaccines to evaluate their efficacy in rabbits. A total of 8 rabbits were used as experimental animals. Each pair of rabbits was assigned to different adjuvants: Group A was injected with liquid paraffin, Group B with Montanide, and Group C with Eolane 150. A control group was also kept. All three groups received 0.5 cc of the vaccine on day 0, followed by a booster dose on day 14. Blood was collected on day 30 (11).

INDIRECT HEMAGGLUTINATION ASSAY (IHA) FOR ANTIBODY TITER

On 30th day, blood was collected and serum was separated to conduct the Indirect Hemagglutination Assay (IHA) to determine and compare the antibody titers of the Vaccine prepared and its comparison with other vaccines as described by (12).

RESULTS

Results revealed comparable efficacy of liquid paraffin with that of montanide, it is recommended to use the liquid paraffin adjuvant vaccine with promising results to curtail disease. The IHA test was used to evaluate the antibody titers in the serum of rabbits. A U-shaped 96-well plate was used to determine the GMT values for these adjuvants. The antigen from VRI Lahore was used to carry out the test. 5 mL of sheep red blood cells (RBCs) were sensitized using the antigen. Then, 50 µL of saline was added from the 1st to the 12th well, and 50 µL of each rabbit serum sample with different adjuvants, including liquid paraffin, Montanide, and Eolane 150, were loaded into the first row and mixed using a micropipette. Two-fold serial dilutions were performed using a multichannel micropipette until the 10th well. Well 11 was kept as a positive control with serum + normal saline, and the 12th well was kept as a negative control with RBCs + normal saline. Sensitized RBCs were then added to each well up to the 10th well, and the plate was incubated for half an hour at 37. Results were recorded, and the plate was then transferred to the refrigerator at 4°C overnight to take the readings again and confirm the results (13).

Table I. Titer values of different adjuvants, liquid paraffin, montanide and eolane$_{150}$ for HS vaccine in rabbits

	1:1	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**	**1:1024**	
Wells	1	2	3	4	5	6	7	8	9	10	11	12
L1							•	•	•	•		•
L2.							•	•	•	•		•
M1									•	•		•
M2									•	•		•
E1					•	•	•	•	•	•		•
E2					•	•	•	•	•	•		•

The GMT value indicates the average titer of a group of serum samples. To calculate the GMT values, the sample titers are first transformed into logarithmic values and then summed. The result is calculated by taking the anti-logarithmic values. The GMT values help minimize the effect of very high and very low titers, providing a more accurate representation of the titer in a given population (14).

To find the GMT take the anti-logarithm (or exponential) of the average. Each titer was converted to its logarithmic value. The average of each logarithmic value was determine by adding the number of different logarithmic values of the titer results, and then divided by the number of samples which is

basically taking an arithmetic means of those logs. Then the antilogarithmic value was taken to get the final GMT value.

Positive results were indicated by the agglutination of red blood cells (RBCs), while negative results were characterized by the formation of a button at the bottom of the well with sharp margins. The GMT value for the Eolane150 titer was 16 which were the lowest among the tested adjuvants, yet it still produced a significant titer. Montanide yielded the highest response, with a GMT value of 256, followed by Liquid Paraffin with a GMT value of 64. Based on the antibody titers, these results suggest that Montanide is the most effective adjuvant of the three evaluated during the trials (Fig. 1)

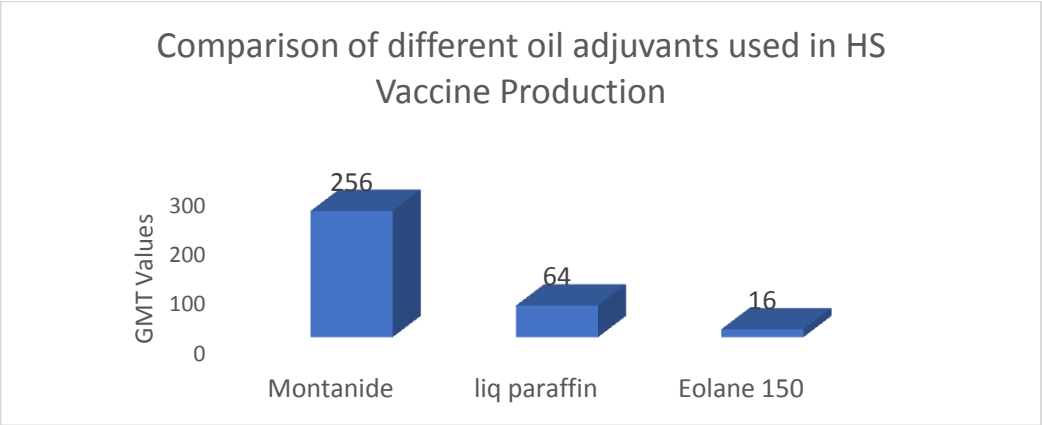

Fig. 1. Comparison of different oil adjuvants used in HS vaccine production

DISCUSSION

This study aimed to develop an affordable formulation for the hemorrhagic septicemia (HS) vaccine, using liquid paraffin as an adjuvant. It also evaluated the vaccine's ability to stimulate an immune response compared to traditional adjuvants, such as Montanide and Eolane 150. Hemorrhagic septicemia, caused by *Pasteurella multocida*, is a serious health threat to cattle, particularly in regions where the disease is endemic. This highlights the importance of finding practical and cost-effective vaccination solutions to protect livestock populations (15).

The cost analysis revealed a significant reduction in production costs when liquid paraffin was used as the adjuvant, in contrast to Montanide, which is commercially produced and imported in different regions. Our findings suggest that liquid paraffin, a mineral oil-derived adjuvant, offers an affordable alternative to expensive commercial adjuvants without sacrificing immune efficacy. When compared to Montanide, a well-established oil-based adjuvant known for its strong performance and sustained antigen release, the liquid paraffin-based vaccine triggered a strong and long-lasting antibody response. In contrast, Eolane 150 showed a less consistent immune response than both liquid paraffin and Montanide, despite still eliciting some degree of immunity (9).

CONCLUSION

In conclusion, this study justifies the use of liquid paraffin, a common adjuvant in the preparation of veterinary vaccines and verified that it could be better cost effective alternative for the development of the hemorrhagic septicemia (HS) vaccine. In comparison to other expensive adjuvants like Montanide and Eolane150, the liquid paraffin-based formulation showed an equally robust and long-lasting immune response. This makes the liquid paraffin an economically viable option, particularly in developing countries such as Pakistan, where the high cost of veterinary vaccines is a significant barrier to widespread use and control of disease. By utilizing locally available and affordable ingredients, this approach helps maintain high-quality standards.

RECOMMENDATION

Extensive field studies are recommended to assess the long-term protective efficacy of the liquid paraffin-based HS vaccine in different environmental and management conditions. A thorough cost-benefit

analysis at the field level would help to evaluate the economic advantages of using this vaccine over conventional formulations that rely on more expensive adjuvants. While liquid paraffin adjuvants offer a cost-effective option for routine vaccination. However, Montanide a comparatively, costly ingredient based vaccine may be considered in case of emergency or outbreaks due to its super robust action to generate high antibody titer more quickly.

Authors' contribution:

RM Carried out the research work, manuscript writing and data analysis; MS & AS supervision and conceptualize the research; IF, SAK, MN, FA & RN critical analysis; AR & MSK experimentation.

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Conflict of interest:

All authors declare no conflict of interest.

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