Ann. For. Res. 65(1): 7889-7897, 2022
ISSN: 18448135, 20652445

ANNALS OF FOREST RESEARCH
https://www.e-afr.org/

THE ADJUVANT POTENTIAL OF DEUTERIUM IN SUBUNIT VACCINE PREPARED FROM OUTER MEMBRANE PROTEIN OF *PASTEURELLA MULTOCIDA*

J. Jogi[1]*, A. Nayak[1], A. Rai[1], P. Shakya[1], R. Gangil[2], R. Sikrodia[2], D. Chhabra[2], R. Sharda[2], K. Rawat[2], and S. Bordoloi[3]

Corresponding author:
***Dr. Joycee Jogi**
Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, India
Email: drjoycee@gmail.com
[1] Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, India
[2] Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Mhow NDVSU, Jabalpur, India
[3] Dr. Smita Bordoloi, Research Scientist I, ICMR-VRDL, Mahatma Gandhi Medical College, Indore, India

Abstract:
The present research work aimed to determine the adjuvant potential of deuterium in a subunit vaccine prepared from the outer membrane protein of *Pasteurella multocida*. *P. multocida* type B:2 causes Haemorrhagic Septicaemia (HS) in cattle and buffaloes. Humoral immunity gained through vaccination may play an important role in protection against HS. The Porin H (OmpH) is a major immunodominant outer membrane protein in the envelope of *P. multocida.* Deuterium / heavy water (D2O) might be utilised to increase the thermostability and the shelf life of the vaccines. Prepared subunit vaccine (recombinant OmpH) was tested for its immunogenicity by using deuterium as an adjuvant in adult albino mice. The animals were divided into five groups were immunized with recombinant OmpH with different concentrations of deuterium as 25, 50, 75 and 100%. The antibody titre and humoral immunity were assessed at a predetermined time interval starting from day 0 to 28 days. Antibody titres across groups showed a very significant difference ($p \leq 0.01$) in statistical analysis. Compared to the other four groups, the antibody titre in Group II (recombinant OmpH with 50% deuterium) was much higher. Similarly, Group II (recombinant OMPH with 50% deuterium) showed the maximum humoral immune response and was considered for the immunization trial of the thermostable property of deuterium. The overall study concluded deuterium is an excellent candidate for the enhancement of antibody titre and humoral response and it may play a pivotal role in the development of vaccines against the treatment of HS.

Keywords: *Pasteurella multocida,* Deuterium, Haemorrhagic Septicaemia, Porin H, Outer membrane protein

Introduction:

Pasteurella multocida (*P. multocida*) is a gram-negative coccobacillus found in the respiratory tracts of warm-blooded mammals as a commensal. It infects a variety of species, including cattle, pigs, rabbits, and poultry. *P. multocida* type B:2 causes Haemorrhagic Septicaemia (HS), an acute, deadly septicemic disease in cattle and buffaloes **[1]**. Humoral immunity plays an important role in protection against HS. Vaccination has a larger impact on reducing HS mortality than any other intervention**.** Broth bacterins, alum precipitated, aluminium hydroxide gel, and oil adjuvant vaccines are among the vaccinations used to prevent HS. The oil adjuvant vaccine has not been popular because of the difficulty in syringing and occasional adverse local tissue reactions **[2]**.

P. multocida outer membrane proteins (OMP) have been discovered as powerful immunogens and recognized as immunodominant antigens, and are assumed to be responsible for cross-protective immunity in the pathogenesis of pasteurellosis **[3]**. Apart from that, capsules of P. multocida have been tested for their immunogenic capabilities to create a high-quality vaccine with antigenic components, such as lipopolysaccharides (LPS). *P. multocida* outer membrane proteins are immunogenic in rabbits, calves, and chickens **[4].** The proteins of the outer membrane also defend against HS. On the cell surface, *P. multocida* expresses heat-modifiable (OmpA) and porin (OmpH) proteins. Heat-modifiable and porin proteins are two types of OMPs that are surface-exposed and have a wide range of molecular mass and antigenic diversity **[5]**.

Although immunisation with these vaccines has resulted in a significant reduction in disease-related fatalities, there are still issues with giving total protection. Quality of vaccine, immunity breakdown, immunity length, post-vaccination shock, temperature sensitivity, and maintaining a strict cold chain throughout production, storage, and shipping are some of these issues. Many authors have observed H.S. vaccine instability, which might be one reason for vaccination failure owing to a lack of cold chain management. According to the existing literature, deuterium / heavy water (D2O) might be utilised to increase the thermostability of vaccinations, extending their shelf life **[6, 7]**. The maximal concentration of D2O should not exceed 70%, and they hypothesized that the inhibitory action of D2O is related to delayed cell metabolism and the structural stability of molecules **[8]**. As a result, it may use the characteristic of D2O for thermostabilized vaccinations. Heavy water has been recommended as a thermostabilized for polio vaccination. D2O improves thermal/microbiological stability and prevents specific compounds from disaggregating **[9]**.

Taking these factors into account, the current work was designed to look into the thermostabilizing properties of heavy water in H.S. vaccination to make a D2O-based thermostable subunit vaccine.

Materials and methods:

Materials:

Deuterium was purchased from Merck India, Mumbai. The recombinant OmpH protein was expressed as a protein with *E. coli* BL21 (DE3) cells transformed with recombinant plasmid pQE30-Xa-*ompH*.

Animals:

Adult albino mice (Mus musculus, 25-30 g, n=30) of either sex were procured from a laboratory animal house facility and used as the experimental host and housed in a lab animal house. The animal handling, management and care of the mice were done as per the standard guidelines for lab animals and they were given feed and water ad libitum. All animal experiments were performed with prior approval of the institutional animal ethical committee of the Institutional Animal Ethical Committee, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh.

Methods:

Deuterium as an adjuvant in subunit vaccine:

The prepared subunit vaccine (recombinant OmpH) was tested for its immunogenicity by using deuterium as an adjuvant. Five groups of mice consisting of six in each group were taken for immunization trial with OmpH and deuterium as an adjuvanted vaccine. The animals of groups II to V (recombinant OmpH) were immunized subcutaneously (S/C) with different concentrations of deuterium as 25, 50, 75 and 100%, respectively. Group I was considered the control group without deuterium. The details of the animal groups and their respective treatment of vaccines with varying concentrations are presented in **Table 1.**

Table 1: Evaluation of adjuvant potential of deuterium with recombinant OmpH at different concentrations

Groups	Conc. of deuterium + OmpH	No. of lab animals	Route	Dose (0.2ml*)
Group I	25%+ OmpH	06	S/C	100µg +0.05ml D
Group II	50% + OmpH	06	S/C	100µg +0.1ml D
Group III	75% + OmpH	06	S/C	100µg +0.15ml D
Group IV	100% + OmpH	06	S/C	100µg +0.2ml D
Group V	0% + OmpH	06	S/C	100 µg of OMP

** Volume/ dose 0.2 ml was adjusted accordingly.*

Mice were inoculated with 0.2 ml of subunit vaccine on days 0 and 14 by the S/C route. Serum samples were withdrawn at a predetermined time interval of day 7, 14, 21 and day 28 post-inoculation for the evaluation of antibody response. Immunized mice of all the groups were observed for any untoward signs and symptoms during the entire immunization trial. Blood samples were collected aseptically by an intraorbital route at 10% body weight of lab animals at weekly intervals starting from day 0 to day 28. Serum samples were separated and pooled for evaluation of antibody response. The antibody titers were assessed with the help of I-ELISA. The group showing the highest antibody response was considered for further evaluation of the thermostable property of deuterium.

Results and Discussion:

In this current research work, the adjuvant potential of deuterium was evaluated, and we found it an excellent candidate for enhancing the humoral response and mean antibody titre and may play an important role in the development of vaccines against the treatment of HS.

Adjuvant potential of deuterium with recombinant OmpH:

Table 2 and **Figure 1** show the antibody titres assessed by I-ELISA in serum from mice inoculated with recombinant OmpH and various deuterium concentrations.

Table 2: Adjuvant potential of deuterium with recombinant OmpH at different concentrations

Days post-vaccination	Mean antibody titres				
	Group-I (25% deuterium)	Group-II (50% deuterium)	Group-III (75% deuterium)	Group-IV (100% deuterium)	Group-V (Control)
07	$1.940^{d} \pm 0.021$	$1.983^{d} \pm 0.003$	$1.543^{c} \pm 0.009$	$1.233^{d} \pm 0.012$	$1.507^{c} \pm 0.009$
14	$2.397^{b} \pm 0.033$	$2.427^{c} \pm 0.012$	$1.827^{b} \pm 0.007$	$1.537^{c} \pm 0.009$	$1.950^{b} \pm 0.017$
21	$2.547^{a} \pm 0.020$	$3.170^{a} \pm 0.014$	$1.930^{a} \pm 0.005$	$1.860^{a} \pm 0.011$	$2.187^{a} \pm 0.023$
28	$2.267^{c} \pm 0.022$	$3.027^{b} \pm 0.012$	$1.837^{b} \pm 0.012$	$1.640^{b} \pm 0.007$	$1.947^{b} \pm 0.043$

Means with different superscripts differed significantly ($p \leq 0.01$) at different time intervals

It has been observed that In Group I (recombinant OmpH with 25% deuterium), the mean antibody titres showed a significant ($p \leq 0.01$) increase in titres from day 07(1.940 ± 0.021), day 14 (2.397 ± 0.033), day 21 (2.547 ± 0.020) and a non-significant increase at day 28 (2.267 ± 0.022) post-immunization while in Group II (recombinant OmpH with 50% deuterium), the mean antibody titres showed a significant increase in titres from day 07(1.983 ± 0.003), day 14 (2.427 ± 0.012) and day 21 (3.170 ± 0.014), and there was a non-significant decrease in mean antibody response at day 28 (3.027 ± 0.012) post-immunization. A similar trend was observed in Group III (recombinant OmpH with 75% deuterium) in which there was a significant ($p \leq 0.01$) increase in titres from day 07(1.543 ± 0.009) and day 14 (1.827 ± 0.007) whereas there was a non-significant increase at day 21 (1.930 ± 0.005) followed by a non-significant decrease at day 28 (1.837 ± 0.012) with mean values post-immunization while in Group IV (recombinant OmpH with 100% deuterium), the mean antibody titres showed a significant ($p \leq 0.01$) increase from day 07(1.233 ± 0.012), day 14 (1.537 ± 0.009), day 21 (1.860 ± 0.011) and non-significant decrease at day 28 (1.640 ± 0.007) post-immunization. In Group V (control, recombinant OmpH without deuterium), the values of the mean titre of antibodies from day 07 to day 28 showed a significant ($p \leq 0.01$)

increase. The antibody titres were from day 07(1.507 ± 0.009), day 14 (1.950 ± 0.017), day 21 (2.187 ± 0.023) and nonsignificant decrease on day 28 (1.947 ± 0.043), respectively.

Antibody titres across groups showed a very significant difference ($p \leq 0.01$) in statistical analysis. Compared to the other four groups, the antibody titre in Group II (recombinant OmpH with 50% deuterium) was much higher. The antibody titres in groups III (recombinant OmpH with 75% deuterium) and V were not significantly different (Control, recombinant OmpH without deuterium). Group-IV (recombinant OmpH with 100% deuterium) had the lowest antibody titre, which was substantially different from the other groups, as stated in Figure 02. These observations showed the excellent adjuvant potential of deuterium with recombinant OmpH.

Humoral immune response in deuterium adjuvanted groups:

The overall humoral response measured by I-ELISA is presented in Table 3 and Figure 2.

Table 3: Overall humoral immune response in deuterium adjuvanted groups measured by I-ELISA

Sr. No.	Groups	Mean antibody titre
1	Group-I (recombinant OmpH with 25% deuterium)	$2.287^{B} \pm 0.067$
2	Group-II (recombinant OmpH with 50% deuterium)	$2.652^{A} \pm 0.144$
3	Group-III (recombinant OmpH with 75% deuterium)	$1.784^{CD} \pm 0.044$
4	Group-IV (recombinant OmpH with 100% deuterium)	$1.567^{D} \pm 0.068$
5	Group-V (Control, recombinant OmpH without deuterium)	$1.897^{C} \pm 0.075$

Means with different superscripts differed significantly ($p \leq 0.01$) at different time intervals

Group II (recombinant OMPH with 50% deuterium) showed the maximum humoral immune response and was considered for the immunization trial of the thermostable property of deuterium at 4ºC and 37ºC for 180 days and it was further compared with HS alum precipitated vaccine at 4ºC and 37ºC for 180 days.

Recombinant OmpH is a decent option for immunisation in-field vaccine investigations. In a mouse protection investigation, recombinant OmpH was employed. Mice vaccinated subcutaneously and immunised intraperitoneally had 100% protection against *P. multocida*, but mice immunised subcutaneously and challenged intraperitoneally only had 80% protection **[9, 10]**. Heavy water, also known as deuterium oxide or D2O, is a kind of water that includes the hydrogen isotope deuterium (D) rather than the usual hydrogen-1 isotope (H), which accounts for most of the hydrogen in ordinary water (H2O). D2O has two different impacts on living systems or biological macromolecules. The "solvent isotope effect," which affects the structure of water and biological macromolecules, is the first while "deuterium isotope effect," in which D2O replaces H with D in biological molecules, is the other. The C–D bond is many times stronger than the C–H bond, making it more resistant to cleavage by enzymes and even chemicals. This characteristic has

been used to improve the thermostability of oral polio vaccination, showing that the D2O reconstituted vaccine stays biologically active even when the cold chain is disrupted for a short period **[11]**. Later, a similar method was used to stabilise the influenza vaccine and HS vaccine **[7, 12].**

A lot of effort has been done throughout the world to produce new-generation vaccinations as diagnostics using recombinant DNA technology. A huge number of plasmids are used in research for cloning foreign genes, as well as sequencing and recombinant protein production. Plasmid DNA vaccines for infections have been produced, and they may be stored for short or extended periods **[13]**. Many experiments have been carried out since the discovery of deuterium (D) to determine its effect on living cells/organisms, with several investigators reporting inhibitory effects of the isotopes on a variety of biological systems and reporting that D has the thermostabilizing property. Deuterium promotes thermal, and microbiological stability and slows the disaggregation of specific macromolecules. For macromolecular medications such as vaccines, the presence of 95% D was shown to be equal to a 4-5ºC drop in storage temperature when compared to H2O; as little as 7-25% D2O helps to prevent protein denaturation. They proposed D2O may increase the thermostability of vaccinations, extending their shelf life. Contrary to the claim, their research showed that incorporating D2O in 50%, 70%, and 90% concentrations had no significant influence on *Salmonella Gallinarum* development **[9]**.

The cold chain is critical in protecting the integrity of any vaccination. The development and maintenance of the cold chain is a considerable cost to vaccination programs, and maintaining the cold chain in underdeveloped nations is regarded to be a 'Herculean task' owing to irregular power sources and issues with equipment repair and maintenance **[14]**. This emphasises the critical necessity to produce vaccination formulations that are stable both at ambient temperature and at high temperatures. Different ways of improving the thermal stability of vaccines have been developed to address the issues connected with the cold chain. Adebayo et al. (1998) investigated several stabilisers for the reconstitution of the freeze-dried vaccine. 0.9 % NaCl, double distilled water (ddH2O), and different amounts (10–90 %) of D2O were used as stabilising agents **[15]**. With 0.9 % NaCl and 10% D2O, the infectivity titer was dramatically reduced. When ddH2O was utilised instead of 0.9% NaCl solution for reconstitution, the reconstituted vaccine was shown to be more stable. Under heat treatment at 37°C for up to 24 hours, 90 % D2O provided the best stability of the reconstituted vaccine among the three stabilising agents utilised for reconstitution **[15]**.

Conclusion:

The antibody titre in Group II (recombinant OmpH with 50% deuterium) was much higher compared to the other four groups. Group II showed the maximum humoral immune response and can be considered for the immunization trial of the thermostable property of deuterium. We found deuterium to be an excellent candidate for enhancing the humoral response and mean antibody titre and may play an important role in the development of vaccines against the treatment of HS.

Acknowledgements
The authors would like to thank the Dean, College of Veterinary Sci. & A.H., Jabalpur for providing the necessary facilities to carry out the research work.

Authors' Contribution:
JJ carried out sample collection, lab experiments, and writing of the manuscript. AN gave design, supervision, and revision of the manuscript. AR and PS supervised the research work and revised the article. SB contributed in lab experiments and RG, RS and DC contributed in manuscript writing. All authors have read and approved the final manuscript.

Funding: The present research work was not financially supported by any funding agency
Data Availability: All datasets are presented in the main manuscript.
Declarations
Conflict of interest: Authors do not have any competing interests.

References:

1) Jogi J, Nayak A, Shukla PC, Dubey A, Singh R, Rai A, Shakya P, Bordoloi S (2020) Molecular characterization of Pasteurella multocida serotype B: 2 strain. J Entomol Zool Stud 8(4): 1307-1312.
2) Plotkin SA (2009) Vaccines: the fourth century. Clin Vaccine Immunol 16:1709-1719. https://doi.org/10.1128/CVI.00290-09
3) Shivachandra SB, Viswas KN, Kumar AA (2011) A review of hemorrhagic septicemia in cattle and buffalo. Anim Health Res Rev 2:67-82. https://doi.org/10.1017/S146625231100003X
4) Zhang H, Ainsworth AJ, Montgomery RD (1994) Use of a 35.5 kDa cell membrane composition of Pasteurella multocida and an anti-idiotype antibody to induce protective immunity in leghorn chickens. Vet Immunol Immunop 41:89-100. https://doi.org/10.1016/0165-2427(94)90059-0
5) Lee JM, Kim YB, Kwon MS (2007) Outer membrane protein H for protective immunity against Pasteurella multocida. J Microbiol 45:179-84.
6) Sheikh MA, Tasneem K, Zafar MS, Butt IA, Shakoori AR (1995) Effect of Storage on the Prevalent Alum-precipitated Haemorrhagic Septicaemia Vaccine in Pakistan and Preparation of a More Efficient Oil Adjuvant Vaccine using Dense Culture of Pasteurella multocida Roberts Type 1 on an Improved Culture Medium. J Vet Med B 42:28-34. https://doi.org/10.1111/j.1439-0450.1995.tb00678.x
7) Gandge RS, Sherikar AA (2010) Thermostabilizing property of deuterium oxide in haemorrhagic septicaemia vaccine. Indian J Comp Microbiol Immunol Infect Dis 31:59-61.
8) Gandge RS, Sherikar AA (2016) Effect of Deuterium Oxide on Growth of Clostridium chauvoei. J Bom Vete Colle 23: 4-7.

9) Gandge RS, Sherikar AA (2008) Effect of deuterium oxide on growth of Salmonella Gallinarum. Indian J Comp Microbiol Immunol Infect Dis 29: 63-64.
10) Tan HY, Nagoor NH, Sekaran SD (2010) Cloning, expression and protective capacity of 37 kDa outer membrane protein gene (ompH) of Pasteurella multocida serotype B: 2. Trop. Biomed. 27:430-41.
11) Milstien JB, Lemon SM, Wright PF (1997) Development of a more thermostable poliovirus vaccine. J Infect Dis 175:S247-S253. https://doi.org/10.1093/infdis/175.supplement_1.s247
12) Ikizler MR, Wright PF (2002) Thermostabilization of egg grown influenza viruses. Vaccine. 20:1393-1399. https://doi.org/10.1016/s0264-410x(01)00466-2.
13) Krishnan BR (2000) Current status of DNA vaccines in veterinary medicine. Adv Drug Deliv Rev 43:3-11. https://doi.org/10.1016/s0169-409x(00)00073-9.
14) Clements CJ, Aguado MT, Jódar L (2001) Technologies to improve immunisation safety. Drug Saf 14:1019-1026. https://doi.org/10.2165/00002018-200124140-00001.
15) Adebayo AA, Sim-Brandenburg JW, Emmel H, Olaleye DO, Niedrig M (1998) Stability of 17D yellow fever virus vaccine using different stabilizers. Biologicals 26:309-316. https://doi.org/10.1006/biol.1998.0157

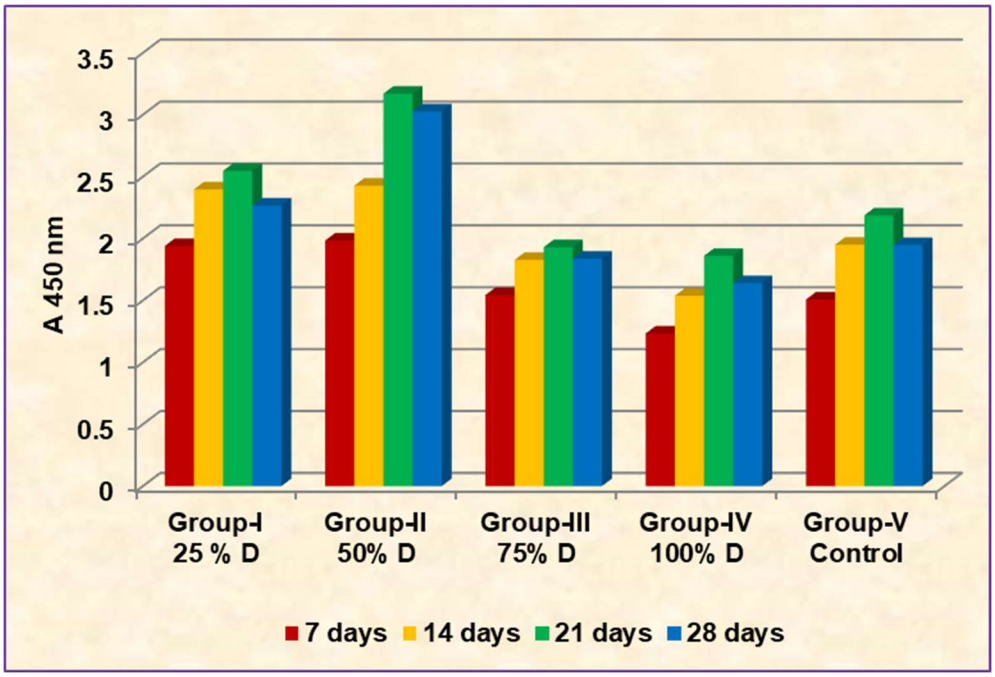

Figure 1: Adjuvant potential of deuterium with recombinant OmpH at different concentrations

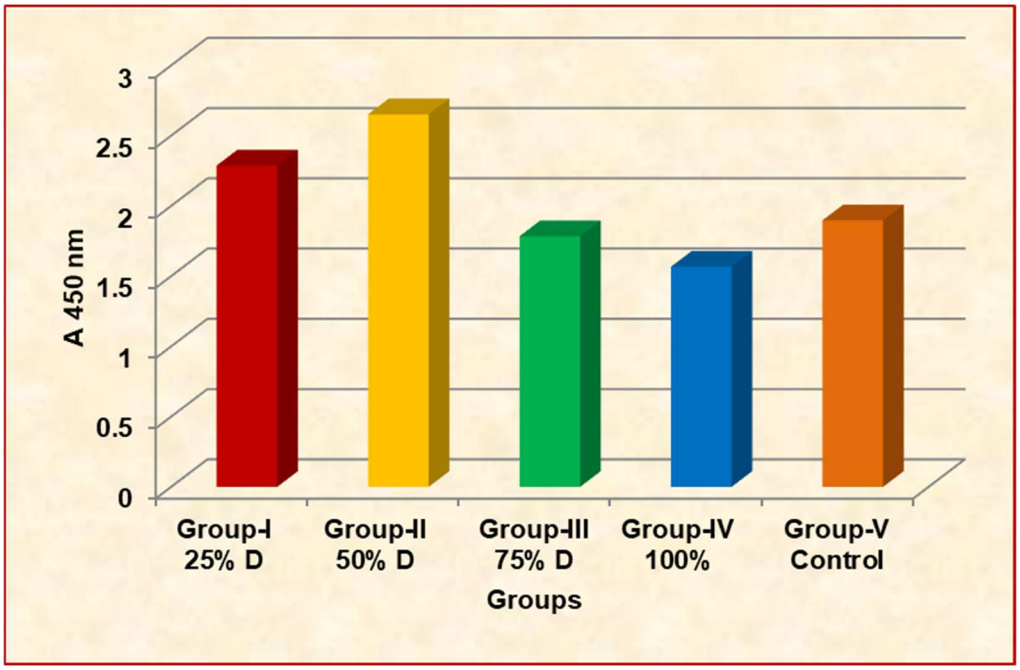

Figure 2: Overall humoral immune response in deuterium adjuvanted groups measured by I-ELISA