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RESEARCH ARTICLE

Immunomodulatory Potential of Montanide-Adjuvanted Anti-Idiotype Antibodies Against *Babesia bigemina* and *Babesia bovis* in Bovine Babesiosis

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ABSTRACT

Bovine babesiosis is a vector-borne parasitic disease of cattle that causes significant economic losses worldwide. Disease control is largely hampered by factors such as tick resistance and the lack of an effective vaccine. Other preventive methods, such as chemoprophylaxis, have high costs and resistance issues. Thus, to address this research gap, anti-idiotype antibodies against *Babesia* were prepared, and their immunomodulatory potential was compared with chemoprophylaxis. The current study was designed to analyze the immunomodulatory potential of Montanide-adjuvanted anti-idiotype antibodies against experimental bovine babesiosis infection. Blood samples from PCR-confirmed *Babesia (B.) bigemina* and *B. bovis*-infected cattle were taken, and those parasitized erythrocytes were subjected to gamma-ray (γ-rays) irradiation. The irradiated, parasitized erythrocytes were inoculated into experimental goats to induce idiotype antibodies (IgG) against *B. bigemina* and *B. bovis*. The $F(ab')_2$ fragments of these antibodies were injected into experimental layer hens to produce anti-idiotype antibodies (IgY). The anti-idiotype antibodies were subsequently evaluated for immunogenicity in experimental mice and immunomodulatory potential in calves. In experimental mice, group M Big showed mean antibody titers ranging from 76% to 83%, while M Bov showed titers of 74% to 82%. In experimental calves, the ELISA results showed that group C calves elicited a greater immune response at 21 days post-infection (DPI) for *B. bigemina* (optical density (OD) value=1.709) and *B. bovis* (OD value=1.698) as compared to calves of group D, which showed mean OD values for *B. bigemina* and *B. bovis* as 1.344 and 1.264, respectively. The results confirmed that anti-idiotype antibodies are an effective candidate for immunomodulation against bovine babesiosis.

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INTRODUCTION

Bovine babesiosis is an economically important and highly fatal parasitic disease of bovines. It poses significant risks to the livestock industry worldwide (Asrar *et al.,* 2022). Apicomplexan protozoans, particularly *Babesia (B.) bigemina* and *B. bovis*, cause disease in tropical regions, whereas *B. divergens* is more prevalent in temperate regions (Springer *et al*., 2020). The disease is transmitted by tick species such as *Rhipicephalus (R.) microplus* and *R. annulatus,* which act as biological vectors (Yousef *et al*., 2024). The tick prevalence across all animals at different farms in Punjab, Pakistan, was reported as 36%, whereas the prevalence of just *R. microplus* was 14% (Batool *et al.,* 2019).

A wide range of *Babesia* species have been reported with different host specificities (Ulucesme *et al*., 2025). They can infect a wide range of vertebrates, including humans, wildlife, and domestic animals (He *et al*., 2021; Baker *et al*., 2024). The most important piroplasmosis infections in camels include Babesiosis and Theileriosis, which can be fatal and cause significant losses (Aslam *et al.,* 2023). The disease is present in canines too, with different species such as *B. canis*, which is larger, and *B. gibsoni*, which is smaller (Bashir *et al.,* 2009; Abdoli *et al*., 2024). The disease is considered of public health significance and has attracted considerable attention in human medicine due to its zoonotic potential (Farooqi *et al.,* 2017). The parasites complete their life cycle in two stages, i.e., sexual reproduction in ticks as definitive hosts

and asexual reproduction in mammals as intermediate hosts (Jerzak *et al*., 2023). The transmission of *Babesia* via tick vectors usually occurs through the transovarial route and is a significant factor in disease transmission between vector and host (Ozubek *et al*., 2020; Ahmad *et al*., 2025).

Bovine babesiosis is characterized by parasitemia, intravascular hemolysis, hemoglobinuria, anemia, and high mortality (Milošević *et al*., 2024). *B. bigemina* is more prevalent than other species but is generally associated with less severe disease (Rimal *et al*., 2025). In untreated animals, the mortality rate in *B. bigemina* infections may reach up to 30%, whereas in *B. bovis* infections it can be as high as 70-80% due to sequestration of erythrocytes in cerebral capillaries (Ahmed *et al.,* 2024). Furthermore, *B. divergens* has been reported to be of zoonotic significance in endemic regions (Suarez *et al*., 2019). *B. bigemina* and *B. bovis* possess self-survival strategies to evade the immune responses of the host. Antigenic variation has been widely reported as the most efficient mechanism of parasite survival (Bastos *et al*., 2022; Santos *et al*., 2023). Moreover, environmental factors play a pivotal role in disease incidence in tick-endemic regions. *B. bigemina* and *B. bovis* harboring ticks find favorable conditions during summer, when high temperatures and high humidity support enormous tick multiplication (Mir and Qureshi, 2022; Selim *et al*., 2026).

The control of babesiosis depends heavily on appropriate diagnostic facilities and effective intervention strategies (Atif *et al*., 2025). Serological and molecular techniques provide accurate findings and are hence instrumental in designing effective control strategies in a region. In addition, molecular biology is highly significant in determining antigenic variation (Alvarez *et al*., 2019; Cossu *et al*., 2025). Effective disease control usually requires prompt vector control and herd-level immunization of healthy cattle. Vaccine development against bovine babesiosis is difficult because of complex interactions among vector, parasite, and host, which are further exacerbated by uncontrolled climate change (Silva *et al*., 2023). In vitro culture-based live attenuated vaccines against bovine babesiosis have been developed in many countries. Still, their success is subject to factors such as very short shelf life, storage limitations, and immune-protective capacity. Calf-derived live vaccines pose risks of reversion to acute infection, contamination with other pathogens, and the risk of hemolytic anemia (Alvarez *et al*., 2020). There are no effective control measures for bovine babesiosis, a major research gap that must be addressed to prevent disease outbreaks and associated economic losses. Therefore, this study was designed to develop anti-idiotype antibodies and determine their immunomodulatory potential in experimentally infected babesiosis calves through antibody titers and hematological investigations. The concept was derived from their experimental evaluation against pathogens such as *B. gibsoni*, *Theileria sergenti*, *Plasmodium falciparum,* and *Aphthovirus* in previous literature.

MATERIALS AND METHODS

Development and purification of idiotype antibodies (IgG): Erythrocytes from PCR-confirmed cattle having natural infection of *B. bigemina* and *B. bovis* were taken and irradiated with γ-rays (60krad) to attenuate the parasites (Bishop and Adams, 1974). The irradiated parasitized erythrocytes were injected into experimental female Beetal goats of 10 months of age to develop idiotype antibodies. A total of 12 experimental goats were divided into 3 groups (GI, GII, and GIII), with 4 goats randomly assigned to each group via simple randomization. The goats were kept under standard farming conditions at the livestock experiment station, UAF, and the study was conducted in a single-blind manner. The goats were tested by physical and clinical examination to confirm their disease-free status. The GI control group was inoculated with 2×10^8 normal bovine erythrocytes/mL (3mL) through the intramuscular (IM) route. The groups GII and GIII were injected IM with 3mL of irradiated parasitized erythrocytes (IPR) containing 2×10^8 infected erythrocytes/mL of *B. bigemina* and *B. bovis*, respectively, during the 1st week. The same concentration (2×10^8 infected erythrocytes/mL) in a 4.5mL inoculum in the 2nd week and 6mL in the 3rd week, inoculated into GII and GIII goats, respectively. Serum samples were collected from the experimental goats at weekly intervals, on days 14, 21, 28, and 35 post-inoculation, and IgG antibody titers were determined through ELISA (Fig. 1). The samples with the highest antibody titers were further processed as pooled samples of each group for antibody purification using 2M ammonium sulfate (0.25mg/mL), and the $F(ab')_2$ fragments were subsequently separated by pepsin digestion using a pepsin-to-protein ratio of 100:1 (Rea and Ultee, 1993). SDS-PAGE was used to detect the fragmented IgG $F(ab')_2$. The concentrations of idiotype antibodies (IgG) were adjusted to 5mg/mL and 10mg/mL using a NanoDrop, and they were mixed with Montanide adjuvant at a 1:1 ratio.

Development and purification of anti-idiotype antibodies (IgY): For IgY production, specific pathogen-free (SPF), White Leghorn layer hens (n=75) of 20 weeks of age were kept at the Poultry Experiment Station, UAF, under standard laying conditions in a single-blind manner. The hens in the control group LC (n=15) were inoculated with 0.25mL of Montanide adjuvant through the IM route. The Montanide-adjuvanted idiotype antibodies were injected at concentrations of 5mg/mL in group LA hens and 10mg/mL in group LB hens through the IM route at a dose rate of 0.25mL per hen, biweekly on days 3rd and 6th of every week for a total of 4 weeks. The purpose of using both concentrations was to investigate which group would produce a higher concentration of IgY. Each group (n=30) was further subdivided into 2 groups, i.e., LA Big and LA Bov, LB Big and LB Bov, with respective antibody inoculations on days 3 and 6 of each week for a total of 4 weeks (Fig. 1). The egg yolks from the experimental hens were taken and treated with ammonium sulfate (0.25mg/mL) for purification of anti-idiotype antibodies (Deignan *et al*. 2000). The $F(ab')_2$ components from pooled samples with the highest antibody titer were separated by pepsin digestion (100:1) from all experimental groups (LA Big, LA Bov, LB Big, LB Bov), and the specificity of the $F(ab')_2$-fragmented IgY was determined by SDS-PAGE.

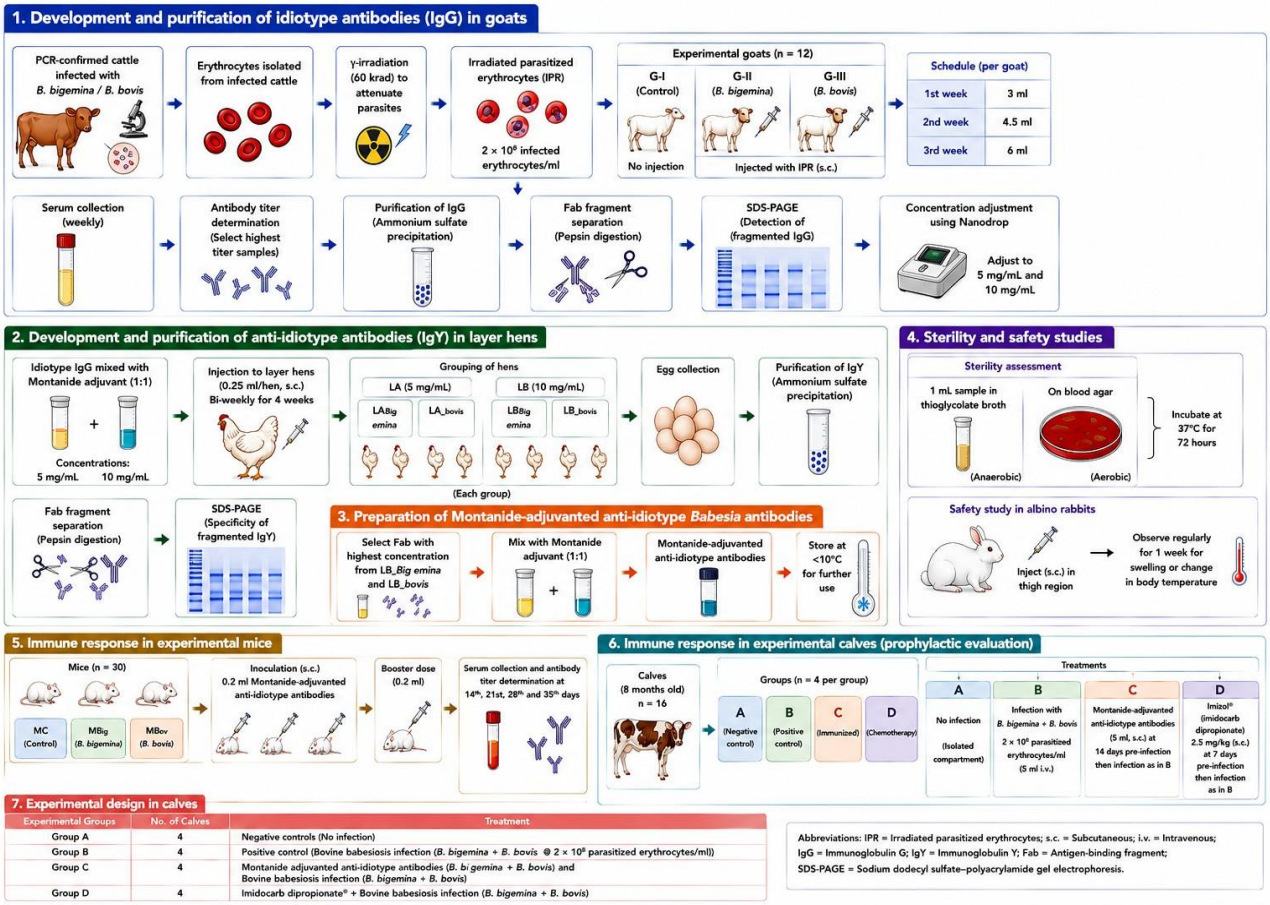

Fig. 1: Schematic illustrations of the materials and methods of anti-idiotype antibody production and experimental design for prophylactic evaluation.

Preparation of Montanide-adjuvanted anti-idiotype *Babesia* antibodies: The F(ab')$_2$ components having the highest antibody concentration from LB Big and LB Bov were added with Montanide adjuvant in a ratio of 1:1 to develop Montanide-adjuvanted anti-idiotype antibodies against *B. bigemina* and *B. bovis,* respectively.

Sterility and safety studies of Montanide-adjuvanted anti-idiotype *Babesia* antibodies: For sterility assessment of the Montanide-adjuvanted anti-idiotype antibodies against *B. bigemina and B. bovis*, a 1mL sample was incubated in thioglycolate broth and on blood agar at 37°C for 72hrs. Thioglycolate broth was used for anaerobic growth detection, and blood agar for aerobic growth. For safety studies, 40 experimental male albino rabbits of 4 months of age were kept and randomized into 2 groups using simple randomization in a single blind experiment. Montanide-adjuvanted anti-idiotype antibodies at a dose of 2mL were injected subcutaneously (SC) into the thigh region of experimental rabbits in group RS2 (n=20), and only 2mL of Montanide adjuvant was SC injected in group RS1, control rabbits (n=20). The rabbits were observed regularly for 4 weeks to monitor for swelling or changes in body temperature.

Immunogenicity of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in experimental mice: For immunogenicity, 30 male albino mice of 4 months of age were randomly divided into 3 groups: MC, M Big, and M Bov using simple randomization (Microsoft Excel). The mice were kept in individual standard cages, and the study was done in a single-blind manner. The MC group served as a control, with only 0.25mL SC Montanide adjuvant inoculation, and 0.25mL of Montanide-adjuvanted anti-idiotype antibodies against *B. bigemina* and *B. bovis* were inoculated SC in groups M Big and M Bov, respectively. Following a booster dose of 0.25mL, serum samples were collected on the 14th, 21st, 28th, and 35th days post-immunization (PI) and processed by IgG Sandwich ELISA (AFG Bioscience® EK240904) to determine antibody titer.

Immune response of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in experimental calves: The anti-idiotype antibodies were evaluated for immune response in experimental calves by using Sandwich ELISA (AFG Bioscience® EK760717, EK760604) to determine the antibody titers (Alvarez *et al.,* 2019). The ELISA plates were read on an ELISA reader at 450nm wavelength. For this purpose, a total of 16 Holstein-Friesian cross-breed bovine male calves of 8 months of age were kept at the livestock experimental station, UAF, under standard rearing conditions, and a 3-week acclimatization period was given. A simple block randomization in Microsoft Excel was used to divide the calves into 4 equal groups, i.e., A, B, C, and D (Table 1). The experiment was carried out in a single blind manner, and the animals were monitored twice on a daily basis. The negative control (Group A) calves were inoculated

with 5mL of Montanide adjuvant through the SC route and kept in a completely isolated compartment to avoid disease transmission. The calves in the positive control (Group B) were experimentally infected with bovine babesiosis using an infectious dose of 2×10^8 parasitized erythrocytes/mL (5mL) of *B. bigemina* and *B. bovis* by simultaneous inoculation. The infection was given by intravenous (IV) inoculation to each calf. Furthermore, the calves in group C were immunized with Montanide-adjuvanted anti-idiotype *Babesia* antibodies at a concentration of 10mg/mL (5mL per calf) via the SC route at 14 days pre-infection. Group D received chemoprophylactic therapy with Imizol® (Imidocarb dipropionate) at a dose of 5mL/calf having 2.5mg/kg concentration through the SC route at 7 days pre-infection. Both groups C and D were infected with bovine babesiosis using an infectious dose of 2×10^8 parasitized erythrocytes/mL of *B. bigemina* and *B. bovis* by simultaneous inoculation of 5mL/calf through the IV route.

Clinicopathological and Hematological studies of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in experimental calves: The calves were observed twice daily for behavioral changes, i.e., anorexia, and clinical parameters such as fever, hemoglobinuria, and anemia. The findings were recorded in terms of severity scoring (Absent (-), Mild (+), Moderate (++), Severe (+++), and very severe (++++)), and weekly means were calculated at 7th, 14th, 21st, and 28th DPI. Parasitemia percentage (%) was calculated for all groups of calves using thin blood smear microscopy (100X). The morbidity and mortality (%) were also recorded for all groups based on the gross clinical findings. The morbid calves with severe anemia were given blood transfusions to support the time period and comparative evaluation of the study. The experimental infection in the positive control group was also validated through blood smear microscopy and conventional PCR. The primers and conditions used in the PCR are described in Tables 2 and 3, respectively. Blood samples with a volume of 5mL each were collected from the calves of all the groups for hematological studies. In hematology, total erythrocytic count (TEC), packed cell volume (PCV), and hemoglobin concentration (Hb) were determined (Aziz *et al.,* 2020).

Statistical analysis: The obtained data were analyzed through the mixed repeated-measures ANOVA (analysis of variance) using Greenhouse-Geisser correction, and group means were compared for significant differences by Tukey-adjusted pairwise comparisons among the groups within each time point via R Statistics version 4.5.3 ($P<0.05$).

RESULTS

Purification of idiotype antibodies (IgG): The SDS-PAGE results revealed a 110kDa band for the fragmented IgG samples on the acrylamide gel. The samples from both groups, i.e., G-II and G-III, showed bands of equal color density, indicating equal concentrations of the detected $F(ab')_2$ fragments, as shown in Fig. 2. The lanes GI-A to GI-D were from the control group, GII-A to GII-D were from the *B. bigemina*-associated group, and GIII-A to GIII-D were from the *B. bovis*-associated group.

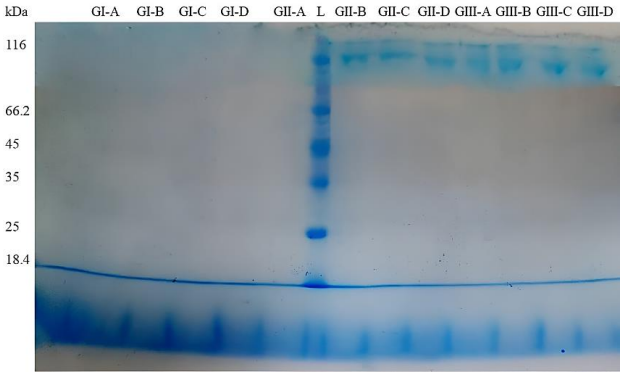

Fig. 2: SDS-PAGE image showing fragment antibody $F(ab')_2$ components of idiotype antibodies (IgG). L is the protein ladder, whereas lanes GII-B to GII-D indicate $F(ab')_2$ fragments of goat IgG at 110 kDa obtained post-immunization with *B. bigemina,* and lanes GIII-A to GIII-D indicate those of *B. bovis*.

Purification of anti-idiotype antibodies (IgY): The ammonium sulfate precipitation of the egg yolks provided yellow precipitates of IgY, and a subsequent treatment with ammonium sulfate yielded clear white precipitates of IgY. Most of the samples from the LB Big and LB Bov groups yielded IgY concentrations >10mg/mL on NanoDrop analysis, and those samples with maximum IgY concentrations from each group were further utilized for development of the final product. Following pepsin digestion, the band size obtained in SDS-PAGE analysis on an acrylamide gel is depicted in Fig. 3. The band density indicates the concentration of fragmented immunoglobulins (IgY), and all samples showed a narrow range of 55-65kDa for fragmented anti-idiotype antibodies $F(ab')_2$ of IgY. The $F(ab')_2$ anti-idiotype antibodies (IgY) from the LB Big group showed a slightly higher concentration, with sample A having the highest within the group. While the LB Bov group showed a slightly lower $F(ab')_2$ anti-idiotype antibody (IgY) concentration than the LB Big group, sample H had the highest concentration within the group.

Table 1: Experimental design to evaluate the efficacy of Montanide-adjuvanted anti-idiotype antibodies against bovine babesiosis in calves

Groups	No. of Calves	Treatment and Dosage	Protocol Administration	Evaluation Parameters
Group A	4	Negative control (Montanide adjuvant (5mL))	Treatment 5mL (SC)	Clinical, Hematological & Immunological
Group B	4	Positive control (Bovine babesiosis infection (*B. bigemina* + *B. bovis* @ 2×10^8 parasitized erythrocytes/mL (5mL))	Challenge 5mL (IV)	Clinical, Hematological & Immunological
Group C	4	Montanide-adjuvanted anti-idiotype antibodies (*B. bigemina* + *B. bovis*) and Bovine babesiosis infection (*B. bigemina* + *B. bovis* @ 2×10^8 parasitized erythrocytes/mL (5mL))	Treatment 5mL (SC) + Challenge 5mL (IV)	Clinical, Hematological & Immunological
Group D	4	Imidocarb dipropionate @ 2.5mg/kg (5mL) + Bovine babesiosis infection (*B. bigemina* + *B. bovis* @ 2×10^8 parasitized erythrocytes/mL (5mL))	Treatment 5mL (SC) + Challenge 5mL (IV)	Clinical, Hematological & Immunological

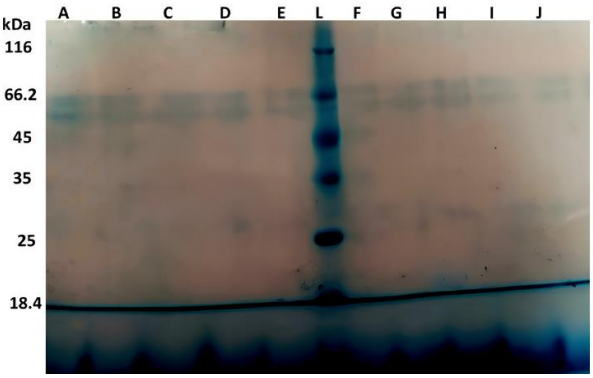

Fig. 3: SDS-PAGE image showing fragment antibody $F(ab')_2$ components of anti-idiotype antibodies (IgY). L is the protein ladder, whereas lanes A-E indicate group LB Big and lanes F-J indicate group LB Bov $F(ab')_2$ fragments of egg yolk IgY at 55-65kDa.

Sterility and Safety studies of Montanide-adjuvanted anti-idiotype *Babesia* antibodies: The Montanide-adjuvanted anti-idiotype antibodies against *B. bigemina and B. bovis* inoculated into culture media showed no bacterial or microbial growth for 1 week; hence, the preparation was considered sterile. Moreover, the blood agar medium inoculated with the same preparation also revealed no bacterial growth. The experimental rabbits inoculated with Montanide-adjuvanted anti-idiotype antibodies against *B. bigemina* and *B. bovis* showed a slight decrease in feed intake (60±5g/kg) and water intake (55±5mL/kg), while a slightly elevated body temperature (102.6°F±0.7 to 103.4°F±0.6) and local inflammation at the injection site persisted for up to 48hrs (Table 6). All these parameters were recorded as normal after 48hrs.

Immunogenicity of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in mice: The experimental mouse groups, i.e., M Big and M Bov, showed a similar pattern of titer development and decline for *B. bigemina* and *B. bovis,* respectively. However, the mean antibody titers in M Big were comparatively higher than those in the M Bov group. The M Big group showed a range of antibody titers between 76% and 83% (Fig. 5A). The antibody titer was 76% at 14 days (PI), followed by an increase to 83% at the 21st day PI. However, there was a gradual decrease in the antibody titer afterwards, i.e., 78% on the 35th day PI. Similarly, group M Bov achieved a mean antibody titer of 74%, with a maximum of 82%, over 35 days. Initially, at the 14th day PI, the mean antibody titer was 74%, then increased to 82% at the 21st day PI, followed by a decrease to 79% at the 28th day PI. The mean antibody titer declined gradually thereafter, reaching 77% at the 35th day PI.

Clinicopathological studies of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in experimental calves: The experimental infection and parasitemia in group B were confirmed through the presence of parasites in blood smear microscopy for *B. bigemina* (Fig. 4A) and *B. bovis* (Fig. 4B). Furthermore, PCR products of the "SS rRNA gene A" also confirmed the presence of *B. bovis* at 541bp, (Fig. 4C) and *B. bigemina* at 1124bp (Fig. 4D). In the positive control group, the parasitemia (%) of *B. bigemina* was highest at 14 DPI, whereas that of *B. bovis* was at 7 DPI. In groups A, C, and D, no parasitemia (0%) was present from 7 DPI to 28 DPI, as shown in Table 4. Anorexia and fever were severe (+++) at 7 DPI in group B, moderate (++) in group D, and very mild (+) in group C. Hemoglobinuria and anemia of moderate intensity were consistently found in group B from 7 DPI to 28 DPI, whereas group D showed mild intensity till 14 DPI, and group C showed neither hemoglobinuria nor anemia. The morbidity was recorded highest in group B (100%), followed by group D (75%) and group C (50%), whereas no morbidity was observed in group A. Group B showed the highest mortality (50%), where two of the calves died at 28 DPI, and groups A, C, and D showed no calf mortality, as shown in Table 5. The results of the study included all calves for sampling and parameters, as the mortality of the two calves occurred on the last day of the study at 28 DPI, and all the samples were taken before the mortality.

Table 2: Oligonucleotide sequences to detect and target SS rRNA genes of *B. bigemina* and *B. bovis* in positive control calves

Target Gene	Oligonucleotides sequences	Product size	Reference
SS rRNA gene A (*B. bigemina*)	GAU5(F): 5'- TGGCGGCGTTTATTAGTTCG- 3'	1124bp	Chaudhry *et al.*, 2010
	GAU6(R): 5'- CCACGCTTGAAGCACAGGA- 3'		
SS rRNA gene A (*B. bovis*)	GAU9(F): 5'- CTGTCGTACCGTTGGTTGAC- 3'	541bp	
	GAU10(R): 5'- CGCACGGACGGAGACCGA- 3'		

Table 3: Thermocycler conditions for the detection of bovine babesiosis in positive control calves by conventional PCR

Process	Temperature	Time	Reference
Initial denaturation	94°C	5min	Chaudhry *et al.*, 2010
Denaturation	94°C	30sec	
Annealing	50°C	30sec 35 Cycles	
Extension	72°C	45sec	
Final extension	72°C	7min	

Table 4: Clinical signs and symptoms in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies

Duration	Clinical Signs	Group A	Group B	Group C	Group D
7 DPI	Parasitemia%	0%	12% *B. bigemina* 3% *B. bovis*	0%	0%
	Anorexia	-	++++	+	++
	Fever	-	+++	+	++
	Hemoglobinuria	-	++	-	+
	Anemia	-	++	-	+
14 DPI	Parasitemia%	0%	19% *B. bigemina* 2% *B. bovis*	0%	0%
	Anorexia	-	+++	+	+
	Fever	-	++	-	+
	Hemoglobinuria	-	++	-	+
	Anemia	-	++	-	+
21 DPI	Parasitemia%	0%	16% *B. bigemina* 2% *B. bovis*	0%	0%
	Anorexia	-	++	-	-
	Fever	-	+	-	-
	Hemoglobinuria	-	++	-	-
	Anemia	-	++	-	-
28 DPI	Parasitemia%	0%	9% *B. bigemina* 1% *B. bovis*	0%	0%
	Anorexia	-	++	-	-
	Fever	-	+	-	-
	Hemoglobinuria	-	++	-	-
	Anemia	-	++	-	-

Severity Scoring: Absent (-); Mild (+); Moderate (++); Severe (+++); Very severe (++++). DPI: days post-infection; Group A: Negative control; Group B: Positive control; Group C: Challenge+Montanide-adjuvanted anti-idiotype *Babesia* antibodies; Group D: Challenge+Imidocarb dipropionate

Table 5: Morbidity and mortality in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies

	Group A	Group B	Group C	Group D
Morbidity	0	4	2	3
Morbidity%	0%	100%	50%	75%
Mortality	0	2*	0	0
Mortality%	0%	50%	0%	0%

*Mortality: 2 calves in group B at 28 DPI.

Immune response of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in experimental calves: The optical density (OD) values obtained from ELISA indicate the antibody titer developed in the experimental calves. The positive control group showed significantly higher mean OD values than the negative control group for both *B. bigemina* and *B. bovis*. Group B showed the highest mean OD value among all the groups. For *B. bigemina*, the mean OD value of group C was highest, i.e., 1.709±0.01 at 21 DPI, followed by a gradual decline to 1.623±0.03 at 28 DPI. Similarly, the mean OD value for *B. bigemina* in group D was highest, i.e., 1.344±0.02 at 21 DPI, followed by a gradual decline to 1.274±0.02 at 28 DPI as shown in Fig. 5B. For *B. bovis*, the mean OD value of group C was highest, i.e., 1.698±0.02 at 21 DPI, followed by a gradual decline to 1.615±0.04 at 28 DPI. Similarly, the mean OD value for *B. bovis* in group D was highest, i.e., 1.264±0.03 at 21 DPI, followed by a gradual decline to 1.184±0.03 at 28 DPI (Fig. 5C). So, the anti-idiotype-immunized group (Group C) showed a higher antibody titer against *B. bigemina* and *B. bovis* as compared to the chemoprophylaxis-treated group (Group D).

Hematological studies of Montanide-adjuvanted anti-idiotype *Babesia* antibodies in experimental calves: The positive control group showed a significantly lower TEC than the anti-idiotype-administered group at 7th, 14th, 21^{st,} and 28th DPI (Fig. 6A). Moreover, no significant difference in TEC was observed between groups C and D. Similarly, the group B showed significantly lower PCV% than the group C and D at 7th, 14th, 21^{st,} and 28th DPI (Fig. 6B). There was no significant difference in the PCV of group C and D. The Hb concentration was significantly higher in group C and D than that of positive control group as shown in the Fig. 6C.

Table 6: Safety analysis of Montanide-adjuvanted anti-idiotype antibodies in experimental rabbits

Day PI	Safety analysis in control (RS1) and immunized (RS2) rabbit groups									
	Feed intake (g/kg)		Water intake (mL/kg)		Body Weight (g)		Body temperature (°F)		Local Inflammation	
	RS1	RS2	RS1	RS2	RS1	RS2	RS1	RS2	RS1	RS2
01	90±5	45±5	80±5	50±5	1650±10	1645±10	101.6±0.7	102.6±0.7	-	Present
02	85±5	60±5	80±5	55±5	1655±10	1650±10	101.4±0.6	103.4±0.6	-	Resolved
03	90±5	70±5	85±5	60±5	1660±10	1655±10	101.3±0.9	102.3±0.9	-	-
04	95±5	75±5	85±5	65±5	1665±10	1660±10	101.3±0.8	101.8±0.8	-	-
05	95±5	90±5	90±5	70±5	1675±10	1665±10	101.5±0.5	101.7±0.5	-	-
06	100±5	100±5	90±5	90±5	1680±10	1673±10	101.2±0.3	101.4±0.3	-	-
07	110±5	110±5	90±5	90±5	1685±10	1680±10	101.2±0.2	101.2±0.2	-	-
14	140±5	140±5	95±5	95±5	1740±10	1725±10	101.2±0.2	101.3±0.2	-	-
21	150±5	150±5	95±5	95±5	1780±10	1760±10	101.4±0.2	101.5±0.2	-	-
28	160±5	160±5	95±5	95±5	1805±10	1790±10	101.3±0.2	101.4±0.2	-	-

PI: Post-immunization; Absent: (-); Group RS1: Montanide inoculation; Group RS2: Montanide-adjuvanted anti-idiotype antibodies inoculation.

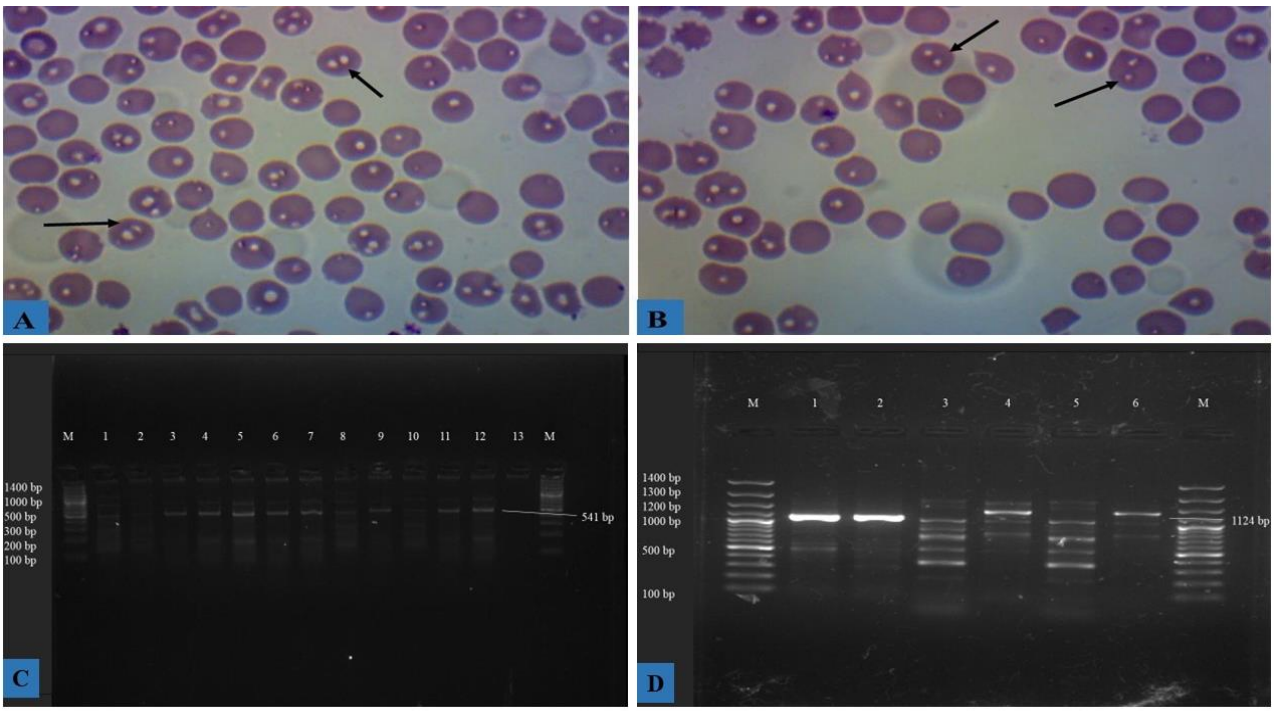

Fig. 4: Parasitemia in positive control group; (A) Calves erythrocytes showing piriform bodies of *B. bigemina* in the cytoplasm (100X), (B) Calves erythrocytes showing piriform bodies of *B. bovis* in the cytoplasm (100X), (C) Amplified PCR products of *B. bovis* "SS rRNA gene A" at 541bp; lane M indicates a molecular weight marker of 1000bp, and lanes 3, 4, 5, 6, 7, 9, 11, and 12 show positive PCR products of *B. bovis* in positive control group, and (D) Amplified PCR products of *B. bigemina* "SS rRNA gene A" at 1124bp; lane M indicates a molecular weight marker of 1000bp, and lanes 1, 2, 4, and 6 show positive PCR products of *B. bigemina* in positive control group.

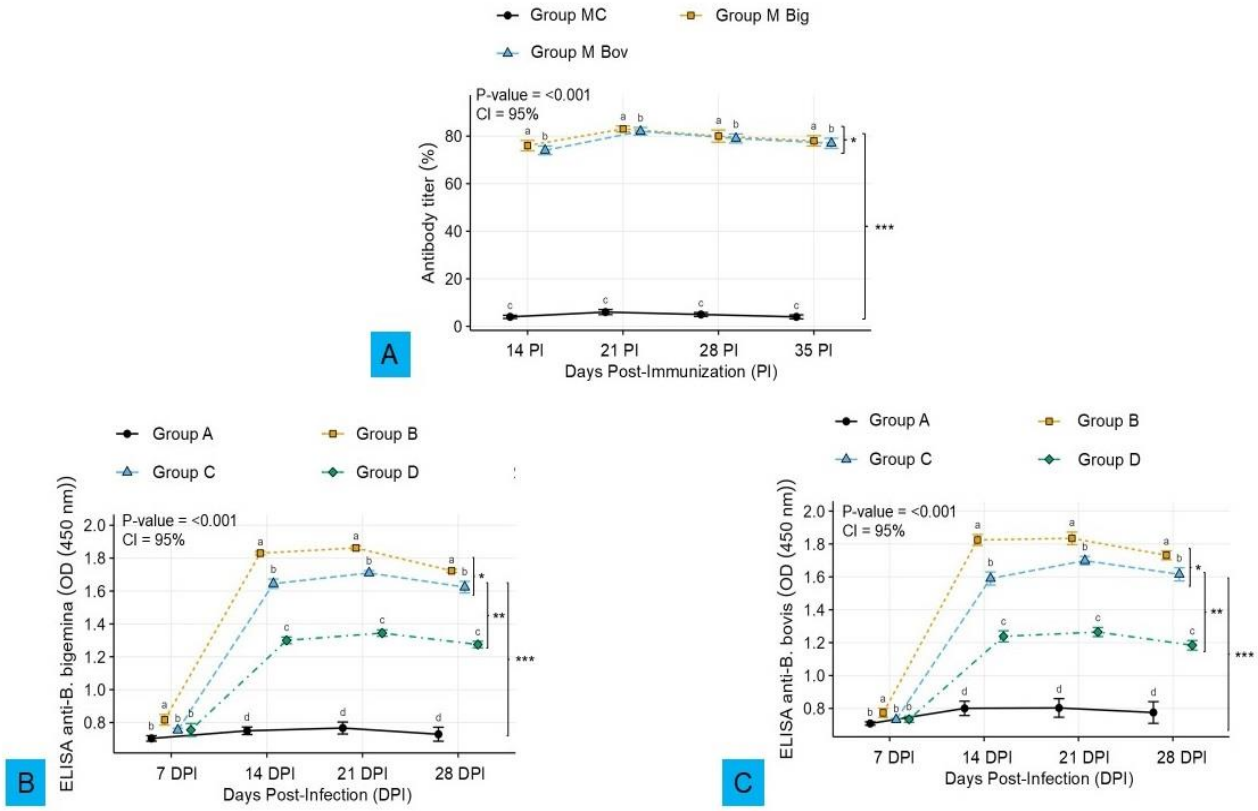

Fig. 5: Antibody titers in the immunized mice and calves. (A) Level of antibody titer (%) in the serum of immunized mice against *B. bigemina* and *B. bovis* through ELISA, (B) Optical density (OD) values of ELISA at 450nm for *B. bigemina* in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies, and (C) Optical density (OD) values of ELISA at 450nm for *B. bovis* in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies. Bars indicate Mean±SD, and letters indicate significant differences between groups (*P<0.001, **P<0.01, ***P<0.05).

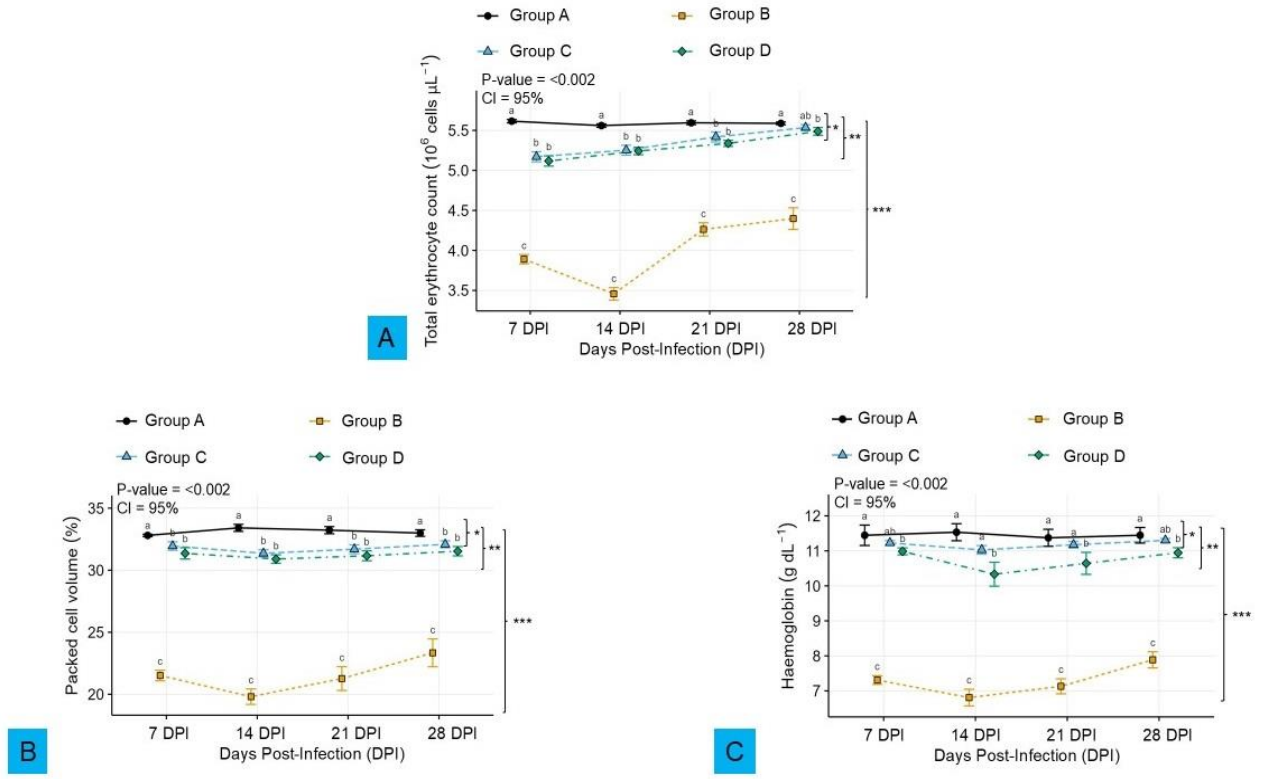

Fig. 6: Hematological parameters in experimental calves received Montanide-adjuvanted anti-idiotype *Babesia* antibodies. (A) Total erythrocyte count (10^6/µL) in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies, (B) Packed cell volume (%) in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies, and (C) Hemoglobin concentration (g/dL) in calves experimentally infected with bovine babesiosis and immunized with anti-idiotype antibodies. Bars indicate Mean±SD, and letters indicate significant differences between groups (*P<0.002, **P<0.02, ***P<0.05).

DISCUSSION

The study was performed to analyze the immunomodulatory potential of anti-idiotype antibodies developed for *B. bigemina* and *B. bovis.* The anti-idiotype antibodies were developed in the egg yolks of experimental layer hens after retrieving idiotype antibodies from caprine sera. The protective potential was assessed in experimental laboratory animals, and the calves were used for a comparative immunomodulatory study with a chemoprophylactic agent. Immunological parameters were compared across groups to evaluate anti-idiotype antibodies.

The findings of anti-idiotype purification were in accordance with those of Deignan *et al*. (2000). They also found that the purification and yield of IgY via ammonium sulfate precipitation are better than those obtained by chromatography. Furthermore, the extraction of IgY from egg yolk using ammonium sulfate treatment is more efficient in terms of yield and purity than octanoic acid or other methods. The results of the study were also consistent with those of Hodek *et al*. (2013), where sodium chloride precipitation was employed for the separation of IgY from the egg yolk. In our study, the purified anti-idiotype antibodies (IgY) were subjected to pepsin digestion, which retrieved $F(ab')_2$ fragments of 55-65kDa. Similarly, Akita and Nakai (1993) reported $F(ab')_2$ fragments of 44kDa obtained through pepsin digestion of IgG. Moreover, in a similar study, Naveed *et al.* (2018) reported $F(ab')_2$ fragments of 55-70kDa for anti-idiotype antibodies of Foot and Mouth Disease (FMD).

The development of anti-idiotype antibodies as vaccine agents against various parasites is a significant breakthrough, as no vaccines are currently available against many of the parasites. The anti-idiotype antibodies thus serve as prophylactic agents against diseases that are otherwise uncontrollable, such as those caused by the dengue virus and *Plasmodium* (Moore *et al.*, 1991; Wang *et al*., 2017). Moreover, anti-idiotype antibodies can generate protective immunity against self-antigens in the body in various cancerous conditions, where the host cannot mount a defense (Pan *et al*., 2020). Anti-idiotype antibodies developed against the hepatitis B virus surface antigen are immunogenic and elicit a protective immune response against viral infection. Gazzinelli *et al.* (1988) reported stimulation of the cellular immune response and the release of T-lymphocytes following the inoculation of anti-idiotype antibodies in patients with Chagas disease.

In an idiotype-anti-idiotype interaction, the antigen submerged in the idiotype only supports attachment of β anti-idiotype antibodies. Hence, a robust immune response always requires an abundance of β anti-idiotype antibodies (Pan *et al*., 1995). The idiotype antibodies (IgG) were employed to develop anti-idiotype antibodies (IgY) in the current study, which comprises β anti-idiotype and other antibody types. In viral infections, Chiang and Nicholson (1996) reported disruption of viral adsorption to host cells, which they attributed to high levels of β anti-idiotype antibodies. In the current study, the mean antibody titer observed in experimental calves is consistent with Arif *et al*. (2013). It not only demonstrates the efficacy of anti-idiotype antibodies in achieving high antibody titers but also confirms the bioavailability of β-anti-idiotypes in the product, as this isotype is primarily involved in the development of protective immunity. Thus, the anti-idiotypes developed in the study were specifically raised against $F(ab')_2$ fragments of idiotype antibodies.

Monoclonal anti-idiotype antibodies targeted the cellular receptors of Reoviruses as described by Gaulton *et al*. (1985), where a strong neutralizing effect was observed. The immune response induced by anti-idiotype antibodies was also assessed without adjuvant, and it was observed that anti-idiotype antibodies inhibit antigen binding to host cells and elicit a robust immune response, depending on dosage. In the current study, the immunomodulatory potential of Montanide-adjuvanted anti-idiotype *Babesia* antibodies was analyzed, and higher antibody titers were recorded.

The production and development of monoclonal anti-idiotype antibodies were carried out in vitro in experimental mice by Garmendia *et al*. (1989). In the experiment, the mice were immunized with an anti-idiotype (Ab2) and an anti-anti-idiotype antibody (Ab3) to compare immune responses, with Ab2 showing a higher response than Ab3. The capability of an anti-idiotype antibody to behave as a surrogate antigen (Ag) was confirmed in our experiment by the antibody (Ab) titration results obtained after immunization with the anti-idiotype antibodies. In our study, the dynamics of the mean antibody titer were recorded in the mice injected with anti-idiotype antibodies prepared from IgY. The initial response to Montanide-adjuvanted anti-idiotype antibodies was delayed, then increased over time, followed by a gradual decline. The findings of the study are in accordance with the results of Baxt *et al.* (1989). Here, first, monoclonal idiotypes (Ab1) were generated in the experimental rabbits following antigen exposure, and the immune response against anti-idiotypes (Ab2) was analyzed in mice. The results showed a stronger immune response by Ab2 than by Ab1 or Ab3. The distinct behaviors of these antibodies can be correlated with the magnitude and diversity of Ab1, Ab2, and Ab3. The Ab3 antibodies are clinically less significant because their production levels are approximately half those of Ab2.

In the current study, the attenuated *Babesia* antigens injected into goats produced the highest idiotype antibody (Ab1) titer. The mean antibody titer recorded during this study in the experimental calves can be correlated with a similar report by Rehan *et al.* (2020), who compared immune responses elicited by IgY from serum and probiotics. The results of our study are also supported by the study of Arif *et al*. (2013), where *P. multocida*-specific anti-idiotype antibodies were compared with bacitracin for immune behavior in experimental rabbits, and a stronger immune response was observed with anti-idiotype antibodies than with bacitracin. Presently, the mean antibody titer of Montanide-adjuvanted anti-idiotypes was compared with that of a babesicide in the experimental animals. The mean antibody titer of the Montanide-adjuvanted anti-idiotype antibodies ranged between 73.33% and 78.66%. The maximum mean antibody titer was observed at day 21 PI, followed by a gradual decrease at day 28 PI. The

research results are consistent with previous studies. Hence, anti-idiotype antibodies can serve as potential vaccine antigens for developing vaccines against *B. bigemina* and *B. bovis*.

Conclusions: Anti-idiotype antibodies have shown promising immunomodulatory potential against bovine babesiosis in experimental calves. The anti-idiotype-immunized calves showed higher antibody titers as compared to chemoprophylaxis-treated calves, and improved hematological parameters were recorded against both *B. bigemina* and *B.bovis*. Therefore, the anti-idiotype antibodies produced a higher immunomodulatory response as compared to chemoprophylaxis. Hence, they can be used for further research to determine their immunoprophylactic potential against bovine babesiosis.

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Conflict of Interest: The authors declare that they have no competing interests.

Data Availability: The data of the study will be provided on demand.

Ethics statement: The research study was conducted in accordance with the ethical recommendations of the Institutional Biosafety Committee (IBC), University of Agriculture, Faisalabad (UAF), vide letter No. 3137/ORIC, dated 30-05-2023.

Authors contributions: FR and STG supervised and designed the experiment. MSS conducted the experiment and wrote the manuscript. MIA assisted in the experiment and reviewed the manuscript.

Generative AI Statement: Artificial intelligence (AI) tools were used during manuscript preparation for language editing, grammatical correction, and improvement of text clarity. Google Gemini® was also used to develop Fig. 1, which provides a graphical representation of the Materials and Methods. AI tools were not used for data generation, data analysis, interpretation of results, or formulation of scientific conclusions. The authors reviewed and verified the final content and take full responsibility for the manuscript.

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