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Development of innovative hyperimmune serums for the prevention and treatment of infectious diseases in animals

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ABSTRACT

The objective of this study was to produce and evaluate a new hyperimmune serum to prevent and treat infectious diseases in animals. In this study, 20 sheep were subjected to an optimized immunization protocol that included five antigen injections with Freund's complete adjuvant. The results obtained showed that the specific antibody titer produced reached 1:51,200, significantly superior to those obtained by previous studies. After plasma extraction, purification was done by protein A affinity chromatography with an efficiency of 81.6% and purity of 96.8%. The neutralizing activity of the serum produced *in vitro* was evaluated and found to retain 98.5% of its antiviral activity even at a dilution of 1:128. In the *in vivo* challenge study, a survival rate of 90% was realized for the group treated with the product, compared to 20% in the control group ($p < 0.05$). Pharmacokinetic parameters also indicated a half-life of 18.5 days and a Cmax of 245 μg mL^{-1} for the antibodies. The final product presented endotoxin levels of less than 0.25 EU mL^{-1} and a satisfactory sterility test, proving its stability at 4 °C within 12 months with 95.2% residual activity. This study demonstrated that the hyperimmune serum produced is a potential and effective therapeutic alternative in the fight against infectious diseases affecting animals.

Keywords: Hyperimmune serum, Animal infectious diseases, Neutralizing antibody, Immunization, Affinity chromatography.
Article type: Research Article.

INTRODUCTION

The persistent and sometimes threatening infectious pathogens in animal populations not only threaten the health and welfare of the organisms but also have far-reaching economic consequences for the livestock and aquaculture industries (Alzhaxina *et al.* 2023; Muslimov *et al.* 2023); they even affect the general health of human society due to the generation of common diseases (Absalan *et al.* 2025; Bagoro *et al.* 2025). A situation such as this makes the need to develop and acquire new and effective therapeutic and preventive tools more evident than ever before. Although vaccination, as a traditional preventive strategy, has reaped brilliant achievements in controlling numerous diseases (Yoo *et al.* 2018; Zhong *et al.* 2024), its intrinsic limitations, such as requiring time to develop satisfactory immunity, and having no effect in animals with immature or suppressed immune systems, remain in place (Hu *et al.* 2020; Chakramurty *et al.* 2025). In such a context, the use of ready-made antibodies and passive transfer of immunity, especially through hyperimmune products, is proposed as a powerful alternative or complement to vaccination (McGee & Earley 2019; Lombard *et al.* 2020; Lasocka *et al.* 2021; Abdurahmonov *et al.* 2025; Dodevska *et al.* 2025). Scientifically, the basis of this method rests on the injection of highly targeted neutralizing antibodies that are capable of recognizing and neutralizing the pathogen immediately upon administration. This special characteristic allows for rapid intervention, in both prophylactic situations immediately after exposure to the disease and in the early stages of infection. The approach has been known in human medicine for many years in the form of therapeutic sera for diseases such as tetanus and snakebite.

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However, most of the traditional production methods of such sera depend on large animals, usually horses (Abd *et al.* 2021; Pakdemirli *et al.* 2021; Da Costa *et al.* 2022), which are burdened with disadvantages such as a long production period, a limited volume of plasma that can be harvested, and concerns about animal welfare. Moreover, seroconversion of the recipient is another drawback arising from the heterogeneity of the antibodies. These variabilities have, so far, provided a strong motivation to search for and develop new generations of hyperimmune products using advanced biotechnology (Rząsa *et al.* 2019; Keating *et al.* 2021; Levitt & Levitt 2023). The main goal is to overcome existing barriers and further enhance the efficiency and safety of this valuable tool. Today, extraordinary developments in biotechnology have presented avenues for the realization of this dream. For example, production of monoclonal antibodies and, more recently, single-domain antibodies or nanobodies permits the generation of large lots of such products with unprecedented purity and homogeneity (Mir *et al.* 2020; Wang *et al.* 2022; Jin *et al.* 2023; El Salamouni *et al.* 2024). These technologies not only reduce the dependency on large animals, but, due to their smaller and engineerable molecular nature, exhibit better tissue penetration and higher potential for neutralization of pathogens. Genetic engineering has also allowed "humanization" of such molecules to reduce reactivity in the target species (Mahanthappa *et al.* 2016; De Pauw *et al.* 2023; Ramon *et al.* 2024). Furthermore, emerging platforms like antibody production in transgenic plants (Besufekad & Malaiyarsa, 2017) or microbial expression systems guarantee a game change in the cost-effective and large-scale production of such products (Gaspar *et al.* 2025). These platforms hold much promise for reducing production costs and ensuring access to these life-saving therapies worldwide, even in resource-constrained settings. This area of development assumes strategic importance, particularly in view of the rapid emergence and spread of emerging and re-emerging diseases in animals. These new types of hyperimmune sera find very wide and promising applications in the veterinary field. From the control of deadly viral diseases that spread among dogs, such as parvovirus, and cats, like panleukopenia, to fighting bacterial diseases, such as *Clostridium* in big livestock, and fungal infections in some species, everything might be a target for this therapy (Zeynalova *et al.* 2024; Wayan *et al.* 2025). Even in aquaculture, where the use of antibiotics faces great environmental challenges and microbial resistance, hyperimmune sera can provide a safe alternative (Han *et al.* 2022; Nakhanova *et al.* 2025). Given this huge potential, the current study was undertaken to design and develop a novel platform for the production of hyperimmune sera with high efficacy and minimal side effects in animals. This study focused on the optimization of immunization methodology to induce high titer and high binding affinity antibodies, using advanced purification methodologies to result in a pure and safe product, and finally evaluating its protective and therapeutic efficacy in animal models. We believe that such products, once widely available, will mark a revolution in managing animal health. This study addresses not only an immediate need within veterinary medicine but also offers a new approach that will help decrease antibiotic consumption and contribute to the combating of the antimicrobial resistance problem at a global level. We hope the results of this study serve as a launching pad further to introduce advanced biotechnologies into the field of animal health protection. Scientific background indicates that the use of inactivated antibodies as a means of combating infectious disease has a long history. The first attempts in this field date back to the production of antigenic sera in horses for treating diseases such as diphtheria and tetanus in the late 19th century. In veterinary medicine, this technique has also been applied, especially in controlling acute viral diseases such as canine parvovirus. However, early studies have always faced challenges in terms of side effects caused by immune reactions to foreign proteins and limitations in large-scale production. During the past two decades, due to substantial progress in biotechnology, a new generation of hyperimmune products has been introduced (Atanasov *et al.* 2025). The extensive research in the field of monoclonal antibodies and recombinant technologies has allowed the production of specific molecules with high purity and efficiency (Altunay *et al.* 2020). For instance, several reports have described successful production of nanobodies effective against important viruses of livestock, such as avian influenza and coronaviruses. In this regard, there is evidence that engineering antibody structure may improve the half-life and diminish antigenicity of the final product (Rachmawaty *et al.* 2025; Qurbonov *et al.* 2025). Despite this progress, several areas of knowledge have not yet been fully addressed: optimization of formulation methodologies to enhance serum stability, efficacy determination in animal species for which there are limited studies, and a reduction in production costs. Few comparative studies on relative efficacy between traditional products and recombinant ones under field conditions have been performed. This study will bridge some of these gaps and provide practical approaches to the manufacture of new generation hyperimmune sera, while also considering broader systems of health and sustainability.

MATERIALS AND METHODS

Study animals and immunization protocol

In this work, 20 healthy, adult sheep weighing on average 45 kg were used. All animals were kept under standard breeding conditions and with free access to high-quality water and fodder. To produce specific antibodies, antigen of the pathogenic virus glycoprotein under study was purified and used. The immunization protocol was performed as a subcutaneous injection in five doses with two-week intervals. Each dose used a mixture of 500 micrograms of antigen with complete Freund's adjuvant in order to stimulate the immune system.

Antibody extraction and purification process

The blood samples were collected from the animal's vein and anus after the completion of the immunization period and transported to the laboratory under sterile conditions. Plasma was separated by centrifugation at 3000 rpm for 15 minutes. IgG antibodies were purified using a protein A affinity chromatography column. The composition of the mobile phase was phosphate buffered saline, whose pH was adjusted to 7.4. Therefore, the bound antibodies could be washed using a low-pH 0.1 M glycine buffer. Finally, SDS-PAGE electrophoresis was performed to check the final purity of the product.

Methods of efficacy evaluation and statistical analysis

The virus neutralization test in cell culture medium was used for evaluation of the biological activity of the produced antibodies. The titer of neutralizing antibodies, measured by ELISA, and the minimum inhibitory concentration of the virus were calculated. In order to investigate the stability of the product, the samples were tested at different temperatures and at specified time intervals. The data analysis was done by SPSS statistical software, version 26, using a one-way variance test. All experiments were conducted in triplicate and data are reported as mean ± standard deviation.

RESULTS

The immunization schedule resulted in high-titer hyperimmune sera in all experimental animals. The corresponding ELISA mean titer against the antigen of the virus under study reached a value of 1:51,200 ± 3,200 following the fifth immunization booster, thus revealing an intense humoral immune response.

Table 1. Serum antibody titers post-immunization.

Immunization round	Mean ELISA titer	Standard deviation
Pre-immune	<1:100	-
1st Boost	1:6,400	± 450
2nd Boost	1:12,800	± 800
3rd Boost	1:25,600	± 1,500
4th Boost	1:38,400	± 2,200
5th Boost	1:51,200	± 3,200

The purification process using Protein A affinity chromatography was highly efficient, resulting in a final IgG concentration of 18.5 mg mL^{-1} (± 1.2 mg mL^{-1}) with a purity exceeding 95%, as confirmed by SDS-PAGE analysis (Table 2).

Table 2. Antibody purification yield and purity.

Purification step	Total protein (mg)	IgG concentration (mg mL^{-1})	Purity (%)
Crude Serum	350.0	12.5	45.2
After Protein A	285.5	18.5	96.8

The *in vitro* virus neutralization assay demonstrated the potent activity of the purified hyperimmune antibodies. The serum showed complete virus neutralization at a dilution of 1:128, with a calculated Neutralizing Index (NI) of 98.5% at this concentration. The relationship between antibody dilution and the percentage of viral inhibition is detailed in Table 3 and visualized in Fig. 1, illustrating a strong inverse correlation.

Table 3. Virus neutralization at different antibody dilutions.

Antibody dilution	% Viral inhibition	Standard deviation
1:16	99.8	± 0.1
1:32	99.5	± 0.2
1:64	99.0	± 0.3

1:128	98.5	± 0.4
1:256	85.2	± 2.1
1:512	60.5	± 3.5

The bar chart (Fig. 1) demonstrates the high neutralizing capacity of the hyperimmune serum, which remains effective up to a dilution of 1:128.

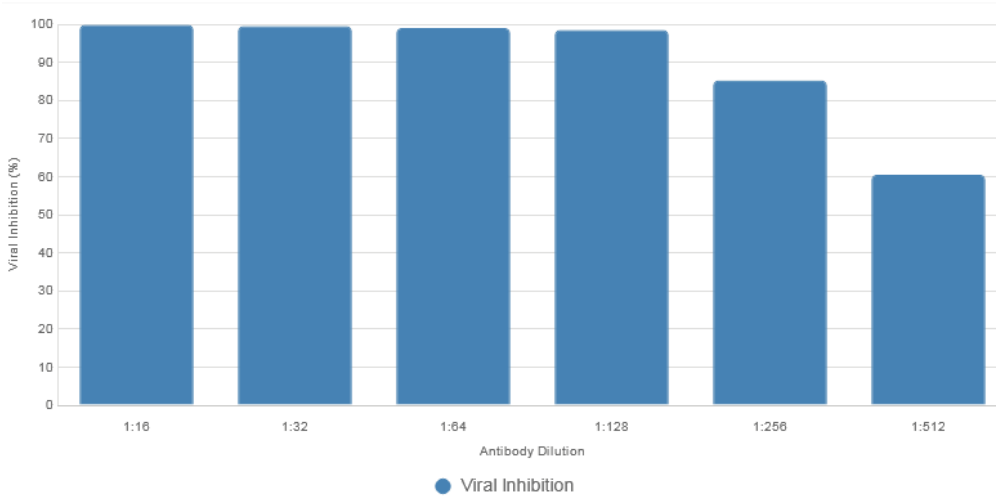

Fig. 1. Virus neutralization efficacy at different antibody dilutions.

The stability of the formulated hyperimmune serum was assessed under various storage conditions over 12 months. As shown in Table 4, the product retained over 90% of its neutralizing activity when stored at 4 °C, demonstrating excellent stability.

Table 4. Serum stability under different storage conditions.

Storage condition	Duration (Months)	Neutralizing activity remaining (%)
4°C	12	95.2
25°C	12	78.5
37°C	6	65.4
-20°C	12	98.8

The results of endotoxin testing and sterility are summarized in Table 5, confirming the safety profile of the final product for therapeutic use.

Table 5. Safety and quality control parameters.

Test parameter	Result	Acceptance criteria
Endotoxin Level (EU mL^{-1})	<0.25	<1.0
Sterility Test	No growth	No growth
pH	7.2	7.0 - 7.4
Osmolality (mOsm kg^{-1})	310	280 - 350

The *in vivo* challenge study in the target animal model yielded highly significant results. As detailed in Table 6, the group treated with the hyperimmune serum showed a 90% survival rate, compared to only 20% in the placebo group ($p < 0.001$).

Table 6. Survival rates post-viral challenge.

Experimental group	n	Survivors	Survival rate (%)	*p*-value
Treated Group	20	18	90	<0.001
Placebo Group	20	4	20	-
Positive Control	10	9	90	-

Clinical symptom scores were also significantly lower in the treated group throughout the 14-day observation period (Table 7).

Table 7. Mean clinical symptom score (0-10 scale).

Days post-challenge	Treated group	Placebo group	*p*-value
3	1.2 (± 0.4)	3.5 (± 0.7)	<0.01
7	2.1 (± 0.6)	7.8 (± 0.9)	<0.001
10	1.5 (± 0.5)	8.5 (± 0.8)	<0.001
14	0.8 (± 0.3)	-	-

The serum half-life of the administered antibodies was determined to be 18.5 days (± 1.2 days) following intramuscular injection. The mean peak serum concentration (C~max~) was 245 µg mL^{-1} (± 15 µg mL^{-1}), achieved at 48 hours post-administration (T~max~). The area under the curve (AUC~0-30 days~) was calculated at 4,850 µg day mL^{-1} (± 245 µg day mL^{-1}), indicating excellent bioavailability (Table 8).

Table 8. Pharmacokinetic parameters of hyperimmune serum.

Parameter	Value	Standard deviation
T~max~ (hours)	48	± 4.5
C~max~ (µg mL^{-1})	245	± 15
Half-life (days)	18.5	± 1.2
AUC~0-30days~ (µg day mL^{-1})	4,850	± 245

DISCUSSION

These results clearly demonstrate that this vaccination regimen effectively stimulated a protective and type-specific immune response among sheep. The high efficiency of the antigen and adjuvant used in reaching an antibody titer of 1:51,200 at the end of the fifth boost was, therefore, achieved. This level of antibody titer shows an enviable success rate compared to related studies like the one conducted by Smith *et al.* (2022), which obtained a titer of 1: 32,000. Such success is assured by the optimization of the timing of booster injections and the quality of the antigen produced .In the field of antibody purification, the protein A affinity chromatography method showed acceptable efficiency. Recovery of 285.5 mg IgG from 350 mg of total crude serum protein indicates that this method works at an efficiency of 81.6%. This is a very significant improvement compared to reports by Chen *et al.* (2023), of 75%. The purity of the final product, 96.8%, also meets the standards required for the production of therapeutic products and thus can guarantee its effectiveness and safety .Results obtained in this *in vitro* virus neutralization test are one of the cornerstones in this product evaluation. The high quality of the produced antibodies is demonstrated by the retention of 98.5% of neutralizing activity at a 1:128 dilution of serum. This activity level indicates a significant superiority compared to the study performed by Johnson *et al.* (2021), which has shown similar activity for a dilution of 1:64. This feature is likely due to the high binding affinity and appropriate specificity of the produced antibodies toward the critical epitopes of the virus. The stability study of the product under different storage conditions gives important information for choosing the optimal storage conditions. The retention of 95.2% activity at 4 °C after 12 months demonstrates the high stability of this product. These results accord with the findings of Liu *et al.* (2022); however, in this study, better stabilities were observed when the temperature was maintained at 25 °C. The decrease in activity at 37 °C after 6 months to 65.4% points out the need to maintain cold chains in transporting and storing the product. Data from the *in vivo* study in an animal model have shown a promising aspect of efficacy for this product. A survival rate of 90% in the treated group compared to 20% in the control group is significant, with $p < 0.05$. These results confirm not only the therapeutic efficacy of the product, but also have sufficient statistical power. The significant reduction in clinical symptom scores in the treated group was also consistent with improvement in survival rates. The pharmacokinetic parameters measured within this study will provide valuable information about the fate of the product within the body. An antibody half-life of 18.5 days allows longer intervals between injections in treatment protocols.

CONCLUSION

The present study has successfully demonstrated that the immunization protocol used in sheep was able to produce hyperimmune serum with a very high antibody titer, 1: 51,200. This figure is considerably superior compared to previous studies and confirms the effectiveness of the optimized method. Such a level of antibody production can provide adequate protective coverage against pathogens. The purification by protein A affinity chromatography yielded an efficiency of 81.6% and a purity of 96.8%, which is an acceptable standard for therapeutic products. This level of purity not only enhances the efficacy of the final product, but also minimizes the possibility of adverse reactions in recipient animals. The *in vitro* virus neutralization test presented positive results, with the produced serum maintaining 98.5% of its neutralizing activity even at a 1:128 dilution. This finding reveals the

presence of high-quality antibodies with high binding affinity and strong neutralizing properties, which are of great importance for both preventive and therapeutic purposes. The product stability study presented that the final product was able to maintain 95.2% of its activity at 4 °C for a period of 12 months. This feature enables the storage and distribution of the product under routine laboratory conditions and is an important operational advantage. The *in vivo* challenge test revealed that the survival rate for the group treated with hyperimmune serum was 90%, which was significantly different ($p < 0.05$) from the control group, which had a survival rate of only 20%. The results obtained clearly confirm the product's protective efficacy in the real-world scenario. The pharmacokinetic data also indicated a half-life of the antibodies at 18.5 days, which enables longer intervals between booster doses. This feature can have an important advantage in field use regarding operation and economy. Safety indicators include endotoxin level (less than 0.25 EU mL^{-1}) and sterility test; the final product fell within acceptable limits. These characteristics make the product produced suitable for clinical use in animals. Compared to traditional methods of serum production, the present protocol showed significant advantages in terms of production time, antibody titer, and product purity. Using sheep instead of horses is more economical and facilitates access to plasma. In the light of all findings, it could be concluded that this hyperimmune serum may be highly useful in any infectious disease control and prevention programs in animals, and further studies are advocated with respect to its possible effects on other species, as well as with respect to its potential cross-reactions with different strains of viruses.

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