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PREPARATION OF HYPERIMMUNE SERA FOR SARS-COV-2 VIRUS

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ABSTRACT

Hyperimmune sera are a primary component of diagnostic test systems utilising enzyme immunoassay to identify specific antibodies against infectious disease pathogens. During diagnostic assay development, hyperimmune sera function as positive controls and enable the generation of standard curves for quantitative measurement of antibody levels. Furthermore, they can be used to assess the sensitivity and specificity of established test systems and to verify novel diagnostic approaches. The objective of this study was to produce hyperimmune serum against SARS-CoV-2. Purified and concentrated virus, in conjunction with adjuvants such as aluminium hydroxide or AddaS03, was employed to immunise animals. Consequently, specific sera exhibiting activity in ELISA at dilutions of 1:1600- 1:3200 and in the diffusion precipitation reaction (DPR) at 1:8–1:32 were acquired. The research demonstrated that the use of concentrated, pure SARS-CoV-2 for animal immunisation, supplemented with adjuvants, is a viable and efficacious approach for generating highly active antibodies.

Keywords: SARS-CoV-2; immunisation; hyperimmune sera; enzyme immunoassay (ELISA); purified virus; adjuvant; AddaS03; aluminium hydroxide.

INTRODUCTION

The COVID-19 pandemic, which began in late 2019, is one of the greatest public-health hazards of the twenty-first century. Rapid responses to such pandemics require the development of effective diagnostic and therapeutic approaches [1]. Serological test methods are among the most important tools for diagnosing infectious diseases. These assays rely on specific antigens and antibodies, the latter often sourced from hyperimmune sera with high concentrations of target immunoglobulins derived from convalescent humans or immunised animals [2].

Hyperimmune sera play critical roles in both clinical and laboratory applications. They have been used therapeutically to treat severe viral infections such as COVID-19, especially when reliable antiviral medicines or vaccines are unavailable. Owing to their high titres of pathogen-specific antibodies, they may be applied for both prophylaxis and treatment, particularly in high-risk individuals [3–6].

Beyond therapeutic use, hyperimmune sera are extensively utilised in laboratory diagnostics. They are instrumental in detecting infections in which antibodies to specific antigens serve as reliable disease markers. The production and use of hyperimmune sera require strict sterility, rigorous quality control, and adherence to established biosafety regulations [5,6].

A crucial stage in developing diagnostic preparations is obtaining highly active hyperimmune sera to protein–antigen-complexes [7]. Several factors must be considered during immunisation, including dose, method, interval and multiplicity of antigen administration, the overall length of the immunisation cycle, and the use of adjuvants and immunocorrectors. Together these factors should prevent immunological tolerance in animals and ensure that immune sera with sufficiently high titres of specific antibodies are produced in a relatively short time with minimal consumption of antigenic and other materials [8].

The objective of our research was to obtain active, specific sera containing antibodies to concentrated, pure SARS-CoV-2 for subsequent application in the development of diagnostic test systems. An immunisation regimen targeting the SARS-CoV-2 strain was designed and implemented, utilising adjuvants to augment the generation of specific antibodies. The regimen, distinct in its dosing frequency, intervals and route of delivery resulted in elevated antibody titres and suggests possible involvement of cellular immunity, warranting further assessment.

MATERIALS AND METHODS

Ethical standards

All animal procedures complied with applicable international and national regulations governing the ethical care and use of laboratory animals. The study protocol was approved by the Ethics Committee of the Research Institute for Biological Safety Problems (Protocol No. 1, 24 May 2024).

Virus isolation

The SARS-CoV-2 strain SARS-CoV-2/KZ_Almaty/04.2020, originally isolated during the COVID-19 pandemic, was obtained from the microbiological collection of the Research Institute for Biological Safety Problems. Virus was propagated in Vero CCL-81 cells (ATCC, USA) and inactivated with formaldehyde (Sigma-Aldrich, USA) [9]. The inactivated material was purified by size-exclusion chromatography followed by tangential-flow ultrafiltration, and the suspension was sterilised by 0.22 µm membrane filtration. Total protein concentration was measured by the Lowrymethod using bovine serum albumin (BSA) (Sigma-Aldrich, USA) as the standard [10].

Animals

To obtain hyperimmune sera, three clinically healthy rabbits (2250- 2500 g) and two goats (6- 12 months; 18- 22 kg)

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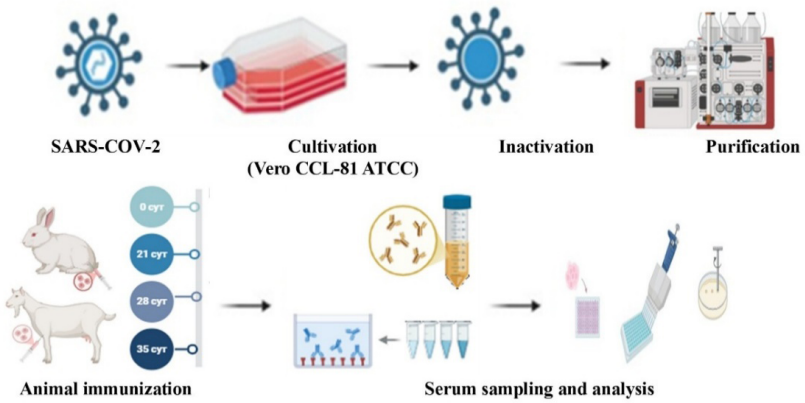

Figure 1. Study design for the production of hyperimmune sera against SARS-CoV-2.

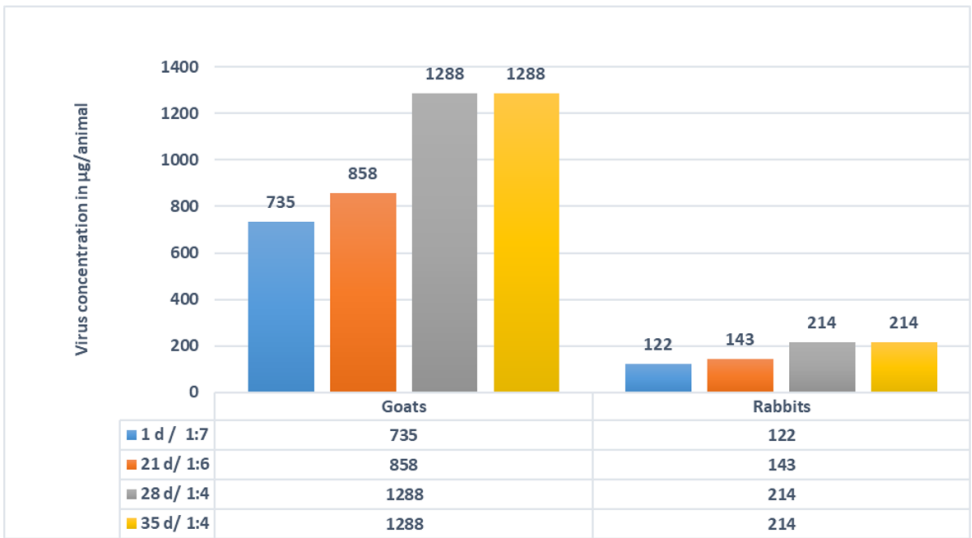

Figure 2. Schematic representation of the immunisation and hyperimmunisation protocol used in experimental animals.

of both sexes were used for each administration method. Before experimentation, all animals were screened for coronavirus antibodies by the neutralization reaction (NR) [11]. Within each species, animals were randomly allocated to two groups: antigen formulated with aluminium hydroxide or with AddaS03.

Animal immunisation

Animals received intramuscular injections of a concentrated, purified suspension of SARS-CoV-2/KZ_Almaty/04.2020 (total protein 1718 µg/mL). Doses were 0.5 mL for rabbits and 3 mL for goats, directed to prescapular and femoral regions draining to local lymph nodes. Hyperimmunisation commenced 21 days after priming and comprised three boosters at 7-day intervals (days 21, 28, and 35). Adjuvants were included in the formulation to enhance the immune response.

Sera analysis

The neutralisation reaction (NR) was performed using a standardised protocol [12] with a fixed virus dose across samples; neutralising antibody titres were calculated by the Reed-Muench method [13].

The diffusion precipitation reaction (DPR; реакция диффузной преципитации) was conducted at 24 °C in 1% agar (Difco) prepared in physiological saline with rivanol. The antigen was a concentrated, purified suspension of inactivated SARS-CoV-2. Test sera were serially diluted 1:2-1:32 and read after 24- 36 h. A positive reaction was the appearance of distinct precipitation lines between antigen wells and antibody-positive sera; negative control sera showed no lines [14]. An enzyme immunoassay (ELISA) was performed using a commercial kit for quantitative measurement of IgG specific to SARS-CoV-2 (SARS-CoV-2-IgG-IFA-BEST), following the manufacturer's instructions.

Statistical processing of data

Data were analysed in Excel and GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Variation indices included standard deviation (SD) and 95% confidence intervals (CI), with comparisons based on Student's t-test.

RESULTS

The choice of adjuvants was evaluated for generating hyperimmune sera. Aluminium hydroxide at a final concentration of 0.2% and AddaS03 at a 1:1 ratio were employed. In practice, dose-escalation regimens commonly administer increasing antigen doses at intervals of four to five days or longer, which enhances immunological reactivity during hyperimmunisation [15]. In line with these pragmatic guide-

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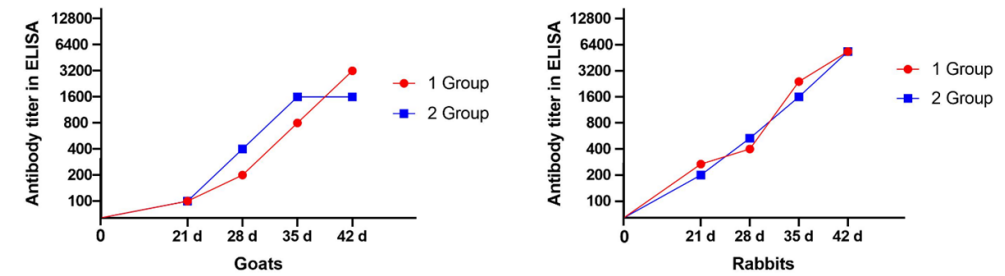

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Figure 3. ELISA-based evaluation of the activity of antigen-specific sera from goats and rabbits.

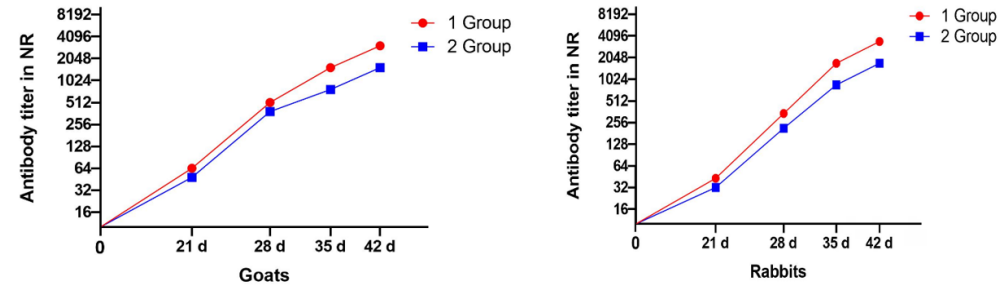

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Figure 4. Neutralising activity of antigen-specific sera from goats and rabbits.

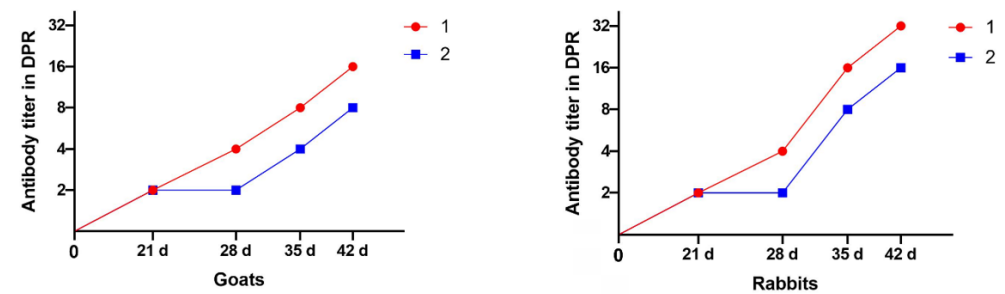

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Figure 5. Reactivity of antigen-specific goat and rabbit sera in radial DPR.

lines, animals were vaccinated with escalating antigen doses at 7- day intervals (Figure 2).

The dynamics of antibody accumulation in animal sera was assessed by DPR, NR and ELISA. The results are presented in Figures 3-5.

Figures 3–5 show that administering escalating amounts of antigen alongside adjuvants leads to an increase in the titres of specific antibodies. From 21 days after the first hyperimmunisation, antibody titres rose, indicating the onset of a humoral immune response, with peak titres reached by day 42. These findings are consistent with sustained antigen release together with adjuvant effects that augment and prolong immune activation. The use of adjuvants facilitated greater accumulation of antibodies in the sera of rabbits and goats. On day 42 after the start of hyperimmunisation, blood was collected to obtain hyperimmune sera. The results for their activity are presented in Table 1. Activity rates of hyperimmune sera to SARS-CoV-2 virus.

Animal species (immunized)	Adjuvant	Activity of specific								
		DPR			ELISA			NR		
		Antibody titer	SD	95% CI	Antibody titer	SD	95% CI	Antibody titer	SD	95% CI
Rabbits (Group 1)	aluminium hydroxide	1:32	5,33	26,12 - 34,32	1:6400	1066,67	5224,53 – 6864,36	1:3200	705,53	2302,12 – 3386,76
Goats (Group 1)		1:16	9,91	10,94 - 31,73	1:3200	826,24	1799,44 – 3533,89	1:3200	653,20	2247,73 – 3618,93

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Rabbits (Group 2)	AddaS03	1:16	7,06	14,13 - 24,98	1:6400	1411,07	4604,25 – 6773,53	1:1600	266,67	1306,13 – 1716,09
Goats (Group 2)		1:8	2,07	4.50 - 8.84	1:1600	653,20	1181,07 – 2552,27	1:1600	326,60	1123,87 – 1809,47

ELISA results were broadly comparable between adjuvants. However, in DPR and NR, and in goat ELISA, the aluminium hydroxide group achieved approximately two-fold higher titres, with all animals surpassing the titres observed with AddaS03. While the between-group differences did not reach statistical significance ($p = 0.10$), the trend was consistent across assays and species. Consistent with Table 1, rabbits receiving aluminium hydroxide generated the most active sera.

DISCUSSION

Specific sera are valuable biological preparations that expand the toolkit for diagnosing and preventing human and animal diseases. Rabbits and goats are predominantly utilised for the procurement of diagnostic sera, and the use of clinically healthy animals is essential for obtaining high-quality preparations.

Administering escalating antigen doses is an effective approach to increase antibody production to high titres. Appropriate dose selection is critical to mitigate adverse effects, including attenuation of the immune response or other side effects.

Rabbits and goats were used here for generating hyperimmune sera owing to their availability, physiological characteristics, and suitability for laboratory-scale production (rabbits) and higher-volume production (goats). A key advantage of the present material is the use of purified, concentrated virus, which strengthened the immune response and reduced the total number of immunisations. This shortened the overall schedule and reduced stress on animals. At the same time, immunisation regimens may require adjustment for different species and for larger-scale applications. Administration of purified SARS-CoV-2 elicited a robust immune response that was augmented by adjuvants; aluminium hydroxide yielded particularly enhanced sera. The use of adjuvants contributed to the development of a persistent and targeted immune response.

Aluminium hydroxide is widely used in vaccine manufacture and was included here as the predominant adjuvant employed for producing specific sera. Owing to its sorptive capacity, it functions as an antigen reservoir and elicits a Th2-biased response, enhancing recruitment and uptake of antigen by antigen-presenting cells (APCs) [16].

We also evaluated AddaS03, an oil-in-water nanoemulsion containing the biodegradable oils squalene and DL-tocopherol. Squalene-based adjuvants can enable antigen-dose sparing and elicit both cell-mediated and humoral responses (Th1/Th2) [17–18]. Reports indicate that AddaS03 can improve antigen uptake and activate innate immune cells, potentially yielding more robust T- and B-cell responses, positioning it as a candidate for enhancing vaccine immunogenicity.

Comparative analysis of the two adjuvants enables assessment of whether AddaS03 is superior to, interchangeable with, or similar in efficacy to aluminium hydroxide for hyperimmunisation to produce anti–SARS-CoV-2 hyperimmune sera [19]. Our selection of these adjuvants aimed to provide an objective evaluation of different methods to augment immune responses under hyperimmunisation conditions.

In this study, both adjuvants: aluminium hydroxide and AddaS03 enhanced production of specific antibodies, with species-dependent differences. In goats, aluminium hydroxide elicited higher ELISA titres (1:3200) than AddaS03 (1:1600), consistent with aluminium salts facilitating sustained antigen release and APC activation at the injection site. In rabbits, both adjuvants performed similarly, yielding ELISA titres of 1:6400. These observations suggest potential interspecies differences in sensitivity to particular adjuvants and their immunomodulatory effects.

Use of purified, concentrated virus together with adjuvants helped to stimulate the immune response. The resulting titres (1:1600- 1:6400) are comparable to those reported in studies of hyperimmune sera production. For example, Pakdemirli et al. (2021) reported titres up to 1:5120 after hyperimmunising horses and rabbits with inactivated SARS-CoV-2 using adjuvants on a four-dose schedule at 7- 10-day intervals [21]. Similarly, Hernan H. M. da Costa et al. (2023) showed that a three-dose DNA immunisation regimen in rabbits produced a sustained increase in IgG titre and high-affinity antibodies [22].

In DPR and NR, titres were higher with aluminium hydroxide, and animals in this group exceeded the activity of sera generated with AddaS03. Although the between-group differences were not statistically significant ($p = 0.10$), the trend was consistent across assays. This contrasts with reports such as Volosnikova et al. (2023), which observed stronger humoral responses with squalene emulsions than with aluminium adjuvants [20]. Differences may reflect species, antigen characteristics, dose, dosing frequency, and other variables. The impact of α-tocopherol in AddaS03 may also vary with immunological context. Overall, these findings underscore the need to tailor adjuvant systems to the target antigen and immunisation protocol.

The results support the use of purified SARS-CoV-2 with aluminium hydroxide to produce specific sera for diagnostic applications. The preparations obtained can be used in developing diagnostic test systems for COVID-19.

This approach contributes to building a broader collection of hyperimmune sera that can be adapted to current and emerging viral threats, which is pertinent given the mutability of coronaviruses and the potential for new variants, as well as the prospect of future pandemics caused by other highly pathogenic viruses.

CONCLUSION

Rabbits are well suited as primary producers of specific antisera against SARS-CoV-2. As part of a hyperimmunisation regimen, five consecutive injections of purified viral antigen adsorbed onto aluminium hydroxide, administered 7 days apart, should be given to achieve optimal antibody production. This regimen consistently yields high-titre antibody re-

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sponses, with titres of at least 1:6400. Owing to their higher blood yield and capacity for repeated sampling over extended periods, goats can serve as alternative producers for large-scale production when larger serum volumes are required.

FINANCING

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CONFLICT OF INTERESTS

All authors are familiarised with the content of the article and have no conflict of interest.

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SARS-COV-2 ВИРУСАНА ҚАРСЫ ГИПЕРИММУНДЫ САРЫСУЛАРДЫ АЛУ

Наханова Г.Д., Червякова О.В., Шораева К.А., Жақыпбек А.С., Молдагулова С.У., Джекебеков К.К., Абсатова Ж.С., Шаяхметов Е.А., Омуртай А.Д., Байсеит Т.И., Наханов А.К., Керимбаев А.А.

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ТҮЙІН

Гипериммундық сарысулар инфекциялық ауру қоздырғыштарына қарсы спецификалық антиденелерді анықтауға арналған иммунды ферментті талдау негізіндегі диагностикалық тест-жүйелердің негізгі компоненттерінің бірі болып табылады. Гипериммундық сарысу алу процесі бірнеше кезеңдерде маңызды рөл атқарады: ол оң бақылау үлгілері ретінде және сынамалардың антидене деңгейін сандық тұрғыда бағалауға арналған стандартты қисықтарды құрастыру үшін қолданылады. Сонымен қатар, гипериммундық сарысулар дайын диагностикалық тест-жүйелердің сезімталдығы мен ерекшелігін бағалауға, сондай-ақ жаңа диагностикалық әдістердің тиімділігін тексеруге мүмкіндік береді. Бұл зерттеудің мақсаты SARS-CoV-2 вирусына қарсы гипериммундық сарысу алу болды. Жануарларды иммундау үшін тазартылған және концентрацияланған вирустың алюминий гидроксиді немесе AddaS03 сияқты адъюванттармен біріктірілген формасы қолданылды. Нәтижесінде, ИФТ әдісімен 1:1600–3200 сұйылтуда және ДПР әдісімен 1:8–32 сұйылтуда белсенділігі бар спецификалық сарысулар алынды. Зерттеу нәтижелері көрсеткендей, жануарларды адъюванттармен бірге қолданылған концентрленген тазартылған SARS-CoV-2 вирусымен иммундау — жоғары белсенді антиденелер алудың тиімді және қолдануға жарамды әдісі болып табылады.

Түйін сөздер: SARS-CoV-2, иммундау, сарысу, иммунды ферментті талдау, тазартылған вирус, адъювант, AddaS03, алюминий гидроксиді.

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ПОЛУЧЕНИЕ ГИПЕРИММУННЫХ СЫВОРОТОК К ВИРУСУ SARS-COV-2

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АННОТАЦИЯ

Гипериммунные сыворотки являются одним из основных компонентов диагностических тест-систем на основе иммуноферментного анализа, применяемого при выявлении специфических антител к возбудителям инфекционных заболеваний Получение гипериммунных сывороток играет важную роль на различных этапах, как в качестве компонентов положительного контроля, так и для создания стандартных кривых, необходимых для количественного определения уровня антител в исследуемых образцах. Кроме того, они могут использоваться для оценки чувствительности и специфичности разработанных тест-систем, а также для валидации новых диагностических методик. Целью данной работы было получение гипериммунной сыворотки к вирусу SARS-CoV-2. Для иммунизации животных использовали очищенный и концентрированный вирус в комплексе с адъювантами – гидроксидом алюминия или AddaS03. В результате исследований были получены специфические сыворотки с активностью в ИФА и РН 1:1600-3200, в РДП 1:8-32. Исследования показали, что использование концентрированного очищенного вируса SARS-CoV-2 для иммунизации животных с добавлением адъювантов является целесообразным и эффективным методом для получения высокоактивных антител.

Ключевые слова: SARS-CoV-2, иммунизация, сыворотка, иммуноферментный анализ, очищенный вирус, адъювант, AddaS03, алюминий гидроксид.