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Immunogenic Response for the Production of Polyclonal Antibodies of Human Serum Amyloid A1 (hSAA1) in Rabbits and Mice

Saira Ahmad[1], Ukasha Tahir[1], Qurratulann Afza Gardner[1]* and Muhammad Akhtar[2]

[1]*School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*

[2]*School of Biological Sciences, University of Southampton, Southampton, SO17 1BJ, UK*

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ABSTRACT

Serum amyloid A1 (SAA1) is a high-density lipoprotein which is involved in lipid metabolism and immune regulation. It serves as a potential biomarker for the diagnosis under inflammatory pathophysiological conditions. The present study deals with the cost-effective in-house production of human SAA1 polyclonal antibodies in rabbits and mice. The immunization process consisted of 60 days, in which two booster doses were administered following 30th and 51st days of primary dose. The control and experimental animals were checked for pre-immune, test and final bleed. The pre-immune bleed of all animals showed negative response and control animals given only adjuvant did not show any cross-reactivity throughout the period of antibodies production. The titer of antibodies was analyzed by using antisera dilution ranging from 1: 100 (100X) – 1: 500, 000 (500,000X) against a constant amount of hSAA1 (200 ng). The saturated signal intensity ≥ 2.0 OD 450 nm was obtained at titer dilutions of 100X-5000X in both groups of animals. More vivid difference in titer was seen at dilution of 10,000X-200,000X where rabbit hSAA1 antisera was almost twice the strength of mice antisera which was attributed to the characteristic feature of an immune system of an animal itself. Following titer analysis, sensitivity of polyclonal antibodies raised is vital for their characterization which can be determined by investigating that how much lowest amount of antigen can be detected. The linear range of signal vs. amount of hSAA1 as an antigen was 1000-150,000 pg for mice and 10,000-150,000 pg for rabbit antisera. This gave the sensitivity of hSAA1 antisera to detect up to 1 ng of antigen. These results provide a pilot scale set-up for the production of polyclonal antibodies of clinical importance and their applications for the diagnosis of a particular antigen as a biomarker in acute and chronic disease conditions.

INTRODUCTION

Serum amyloid A (SAA) protein family belongs to high density lipoprotein (HDL) and its isoforms are either expressed constitutively or in response to stimuli such as lipid metabolism and immune response (Sack, 2018). Among the family of serum amyloid A, SAA1 and SAA2 isoforms are expressed as acute phase response which have more than 90% sequence homology (Xu *et al*., 2006). SAA1 is an important immune regulator and its serum levels are raised rapidly 1000 times under acute inflammatory pathophysiological condition (Ye and Sun, 2015). The consistent high levels of SAA1 lead to chronic inflammatory conditions such as rheumatoid arthritis, pathogenesis of tumor and amyloid A (AA) amyloidosis (Connolly *et al*., 2012, Malle *et al*., 2009, Westermark *et al*., 2015). Therefore, the therapeutic role of SAA1 is being explored to treat AA amyloidosis (Eklund *et al*., 2012) and inflammatory disorders (Scarpioni *et al*., 2016). One of the therapeutic approaches is to use natural antibodies

* Corresponding author: qagardner.sbs@pu.edu.pk; annegardner80@yahoo.co.uk
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Abbreviations used:
CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; hSAA1, human serum amyloid A1; rSAA1, rabbit serum amyloid A1; mSAA1, mice serum amyloid A1; SAA2, serum amyloid A2; pg, pictogram; ng, nanogram; IFA, incomplete Freund's adjuvant; PBS, phosphate buffer saline; PBST, phosphate buffer saline containing 0.1% Tween 20; TMB, 3,3′,5,5′-Tetramethylbenzidine

of SAA1 against chronic inflammation which can downregulate the high levels of antigen under disease conditions (Kuret *et al*., 2018).

SAA protein family is highly conserved in vertebrates throughout the evolution (Uhlar *et al*., 1994). The human SAA1 shares 82% and 74% of sequence homology with SAA1 of rabbit (*Oryctolagus cuniculus*) and mouse (*Mus musculus*), respectively.

The recombinant human SAA1 has 105 amino acids whereas rabbit and mice have 103 amino acids in SAA1. The significant difference in sequences of antigen, which is human SAA1 in this study, with host animals: rabbits and mice, is optimal for the production of SAA1 polyclonal antibodies. The choice of antibodies preparation can be polyclonal (having multiple epitopes to bind with an antigen) or monoclonal (single epitope to bind with antigen) which are immunoglobulins G (IgGs). However, the production of polyclonal antibodies is favorable due to shorter time and investment than monoclonal antibodies (Leenaars and Hendriksen, 2005). At the same time, it is challenging as the specificity and sensitivity are relatively low for polyclonal antibodies due to multiple epitope and cross-reactivity (Lipman *et al*., 2005). Being an acute reactive protein, SAA1 can pose health issues to the animals which were carefully considered by following the guidelines as described by Leenaars and Hendriksen (2005). This study aimed to analyze the production of hSAA1 polyclonal antibodies in rabbits and mice as well as the evaluation of host specific antibodies response i.e. comparison of hSAA1 antisera titer in two species under study, using indirect enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Recombinant human serum amyloid A1 (12 kDa protein in size) was cloned, expressed and purified in SBS laboratory. Directive 2010/63/EU guidelines were followed to prepare the polyclonal antibodies in these animals.

The husbandry of Swiss albino mice and rabbits was maintained in SBS Animal House. These animals were used to raise the polyclonal antibodies against recombinant human SAA1. Secondary antibodies conjugated with horseradish peroxidase enzyme i.e. HRP-conjugated rabbit anti-IgG raised in goat (catalog no. A6154) and HRP-conjugated mouse anti-IgG raised in goat (catalog no. A9044), TMB substrate were purchased from Sigma. Routine laboratory chemicals and reagents were from Thermo Scientific and Fluka.

Preparation of hSAA1 polyclonal antibodies in animals

The recombinant hSAA1 gene (Supplementary Figure 1) was expressed in *Escherichia coli* Rosetta DE3 (pLysS) and purified by affinity chromatography (Supplementary Figures 2 and 3, respectively). The protein solution of hSAA1 was prepared in 25 mM phosphate buffer saline (PBS pH 7.4, Supplementary Table I) and syringe filtered. The filtered protein solution was UV quantified at 280 nm using nanodrop (Thermo Scientific™) and actual protein amount was calculated by applying extinction coefficient of primary structure of hSAA1 (http://us.expasy.org/). The OD_{280} of 2.0 is equivalent to 1 mg/ml of hSAA1. On the basis of this calculation, 4 mg/ml solution of hSAA1 was prepared.

The Swiss albino mouse strain and local rabbit breed were used to raise polyclonal antibodies for the period of two months. The animal health was properly monitored with assistance of veterinary expert. The animals were acclimatized to the animal-house environment for a week and their body weight was recorded prior to immunization, which was 1300-1500 g of rabbits and 27 – 30 g of mice. The dose for development of polyclonal antibodies was prepared in fresh PBS with 50% of complete and incomplete Freund's adjuvant (i.e. 25% of CFA and 25% of IFA in first dose but booster doses only contained 50% of IFA). The dose preparation is described in Table 1. The prepared doses were mixed by vigorous vortexing for 3-4 hours before dose administration to animals.

Table I: Composition of primary and booster doses to raise the polyclonal antibodies against hSAA1 in mice and rabbits.

Reagents	Final concentration for primary dose	Final concentration for booster dose
Mice		
hSAA1	20 µg	10 µg
Phosphate buffer	10 mM	10 mM
NaCl	0.15 M	0.15 M
CFA	25%	–
IFA	25%	50%
Final volume	500 µl	500 µl
Rabbits		
hSAA1	200 µg	100 µg
Phosphate buffer	10 mM	10 mM
NaCl	0.15 M	0.15 M
CFA	25%	–
IFA	25%	50%
Final volume	1000 µl	1000 µl

The number of mice was 9, among which 8 were injected with dose containing antigen i.e. hSAA1 and 1 was injected with only adjuvant as a control. The number of rabbits was 4, among which 3 were experimental, injected with antigen and 1 was a negative control as described above. Zero bleed or pre-immune bleed was obtained from rabbits and mice before giving any dose. In the case of rabbits, blood was withdrawn from the marginal vein of ear and in case of mice, blood was obtained by cardiac puncture. Sera was prepared and aliquots of sera were stored at – 80 °C till further use.

The dose was injected subcutaneously at the dorsal side of thorax or abdominal region of an animal body. The first day of immunization process was considered as a 0 day when primary dose was administered. At 30th day of experiment, first booster dose containing 10 µg and 100 µg of hSAA1 in mice and rabbits were administered respectively, and same formulation of doses were given on 51st day of experiment as a secondary booster. In the case of rabbits, on the 40th day i.e. after 10 days of first booster, test bleed was obtained from the marginal vein of ear and analyzed for antibodies production by ELISA. Whereas in case of mouse, test bleed was not collected because the required amount of serum would be obtained by cardiac puncture which requires sacrificing the animal. The final bleed was collected at 61st day by cardiac puncture by administering a cocktail of ketamine hydrochloride (35 mg/kg of animal) and xylazine (7 mg/kg of animal) in the peritoneal cavity of animal, serum was prepared and stored at -20 °C.

Preparation of antisera dilutions

Antisera dilutions were prepared in blocking buffer (5% skimmed milk prepared in 1X saline phosphate buffer pH 7.4 containing 0.1% v/v of Tween-20) in the following manner: For 100 times diluted anti-serum i.e. 1:100, 1 µl of prepared serum was diluted with 99 µl of blocking buffer and it was labelled as 100X dilution. Similarly, remaining dilutions required were prepared and written as 1000X, 2000X etc. which were actually 1:1000 or 1:2000 respectively.

Screening of polyclonal hSAA1 by in-direct ELISA

The presence of polyclonal antibodies was analyzed by indirect ELISA. Three sets of 100X diluted anti-sera; zero, test and final bleed, were initially screened for the presence of hSAA1 polyclonal antibodies titer by indirect ELISA method.

Each set of antisera from respective animals was analysed in duplicate. Microtiter plate was coated with 200 ng/100 µl/well of antigenic protein (hSAA1) diluted in 50 mM KCl borate buffer pH 8.0 and incubated overnight at 4 °C under humid conditions, by covering a plate with wet tissue towel. Next day, coated wells were washed three to four times with 1X PBST buffer pH 7.4 containing 0.1% v/v of Tween-20 (phosphate buffer saline containing tween 20) and blocked by adding 100 µl of blocking buffer (5% skimmed milk prepared in 1X phosphate buffer saline pH 7.4 containing 0.1% v/v of Tween-20) followed by incubation at 37 °C for 2 hours. The plate was washed with 1X PBST three to four times. 100 µl of diluted antisera of mice and rabbit prepared in fresh blocking buffer (as mentioned above), containing primary antibody against hSAA1, was added in each well, and incubated at 37 °C for 1 hour. Wells were again washed for four times and incubated with the respective 10000 times diluted HRP conjugated secondary antibody (i.e. anti-mice IgG in goat and anti-rabbit IgG in goat) for 1 hour 37 °C. Wells were washed six times with 1X PBST buffer and 100 µl of freshly prepared TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution was added in each well and incubated at room temperature for 10-15 minutes (TMB working solution was prepared by mixing 10 ml of 0.1 M sodium acetate buffer (pH 6.0), 6.2 µl of 30% (v/v) H_2O_2 with 100 µl of TMB stock of 10 mg/ ml in DMSO). Blue color developed as the TMB was oxidized after the reaction with HRP-conjugated IgG. Reaction was quenched with 100 µl of 2 M H_2SO_4 giving the yellow color which was analyzed by measuring OD_{450} nm on ELISA reader (HumaReader).

Antibody titer and sensitivity analysis

Antibody titer is a measurement of antibodies level in blood or serum and antibody sensitivity is defined as minimum amount of antigen to be detected by specific dilution of antibodies.

The polyclonal antibodies titer in final bleed was analysed by indirect ELISA where amount of antigen was kept constant i.e. 200 ng/well and various dilutions of antisera (both mice and rabbits) were prepared ranging from 100-500,000 times diluted in fresh blocking solution, in duplicates for each dilution.

After analyzing the results for titer by plotting a graph in Microsoft Excel programme, sensitivity assays were performed using various amounts of hSAA1 in KCl borate buffer pH 8.0 ranging from 50-400,000 pg/ well (in duplicates) whereas 40,000 and 20,000 dilutions of hSAA1 antisera raised in rabbits and mice were used respectively as these gave the best titer result. The results for sensitivity analysis were analyzed using online software for dose-response curves “IC50 toolkit (ic50.tk)” under these following links: http://www.ic50.tk/multikm.html, and http://www.ic50.tk/multiic.html. The graphs were plotted between average of signal intensity which was an absorbance at 450 nm and amount of antigen (pg),

using "IC50 toolkit (ic50.tk)" to provide the best fit curve.

RESULTS

Figure 1 shows sequence alignment of rSAA1, hSAA1 and mSAA1.

hSAA1 polyclonal antibodies

In initial screening, 100X (1: 100) dilution of hSAA1 antisera raised in rabbits and mice were used. The immunogenic response against hSAA1 was analyzed on: 0 day before injecting any antigen to animals (Zero bleed), 41st (Test bleed) and 61st day (Final bleed) as shown in Figure 2. The zero bleed was to evaluate pre-immune conditions of animal as host antibodies could be present against an antigen under study, which could cross-react to hSAA1. In zero bleed there was no signal detected which confirmed that there was no immune activation against the antigen. Figure 2A shows approximately 30% titer development of hSAA1 antibodies in the test bleed of experimental rabbits (R1, R2 and R3) as compared to final bleed, whereas there was no signal in control rabbit presenting no antibodies development.

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hSAA1          MRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGVWAAEAIS
rSAA1          --RWFSFIGEATQGAWDMWRAYSDMREANYINADKYFHARGNYDAAQRGPGGVWAAKVIS
mSAA1          --GFFSFVHEAFQGAGDMWRAYTDMKEANWKNSDKYFHARGNYDAAQRGPGGVWAAEKIS
                  :***: ** :** ******:**:***: .:**************:********* **

hSAA1          DARENIQRFFGHGAEDSLADQAANEWGRSGKDPNHFRPAGLPEKY
rSAA1          DAREDLQRLMGHGAEDSMADQAANEWGRSGKDPNHFRPKGLPDKY
mSAA1          DGREAFQEFFGRGHEDTIADQEANRHGRSGKDPNYYRPPGLPDKY
               *.** :*.::*:* **::*** **. ********::** ***:**
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Fig. 1. Sequence alignment of SAA1 of human (hSAA1), rabbit (rSAA1) and mouse (mSAA1). Asterisk (*) is for identical amino acids, colon (:) for similar nature of amino acids in three species, single dot (.) shows conservation of amino acids in two species and space below the amino acids represent the completely different amino acids in three species.

The Figure 2A also highlights that adjuvant did not stimulate any significant immune response and hence did not incorporate any false effect in the production of antibodies. Similarly, hSAA1 antisera in mice were analyzed as mentioned earlier in methods. There was no test bleed in case of mice, as animals would have been sacrificed to get enough serum of for analysis. It was ensured to get the optimal immune response in final bleed. The antigen: antibody complex response obtained with 1: 100 times dilution of hSAA1 anti-serum in mice is shown in Figure 2B.

Substantial titer of hSAA1 antibodies was observed in all of the experimental mice and it was more or less similar to each other as shown in Figure 2B. The hSAA1 antibodies response showed significant production of hSAA1 polyclonal antibodies.

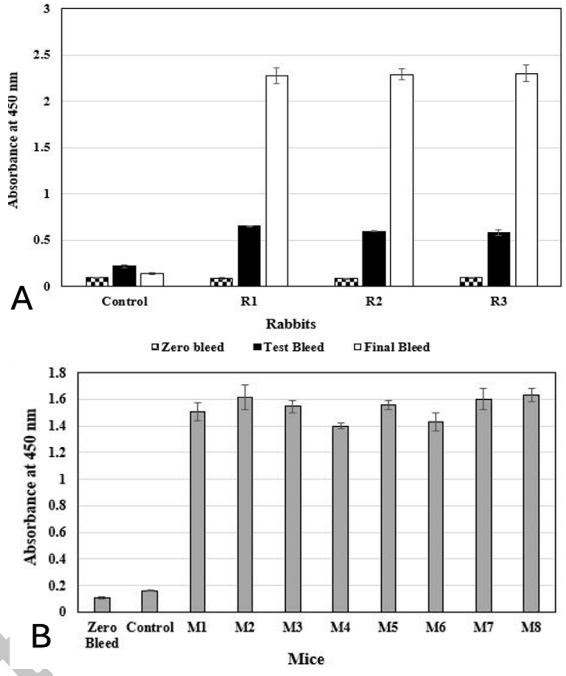

Fig. 2. Representation of immunogenic response of 100X dilution hSAA1 anti-sera, (A) Rabbits (B) Mice.

Figures 2A and 2B showed significant saturated signal at 100X dilutions for hSAA1 antisera from both the species therefore, for further experiments, much lower dilutions than 100X, were selected to analyze the titer.

Titer and sensitivity analysis of hSAA1 polyclonal antibodies

Titer of hSAA1 polyclonal antibodies was analyzed with a fixed amount of coated antigen i.e. 200 ng/well, using antisera dilutions ranging from 1: 100-1: 500, 000 or 100X to 500, 000X (materials and methods).

In case of rabbit, the saturated signal observed at OD_{450} was greater than 2.0 at 100-10,000 times diluted antisera and for 20,000-60,000 times dilution, the signal range was OD_{450} 1.0- 2.0. The titer signal gradually decreased with an increase of dilution factor till 500,000 however the significant signal was monitored at 80,000-200,000 times dilution where OD_{450} was 1.0 – 0.5. Whereas, in case of mice hSAA1 antisera OD_{450} greater than 2.0 was detected at 100-5000 times dilution and at 10,000-60,000X dilution, OD_{450} signal was 1.0-2.0 (Fig. 3).

This range of signal intensity obtained with respect to dilution factor of antisera in both species is tabulated in Supplementary Table II.

On the basis of Figure 3, the efficacy of titer in rabbit

and mice hSAA1 antisera, was comparable at lower titer range 10, 000-250, 000, when signal was not saturated as given in Table II. The results also exhibit that rabbit antisera had twice the signal strength as compared to signal obtained in case of mice anti-sera.

Following titer analysis, hSAA1 antisera produced in rabbit and mice were characterized by plotting a standard curve for sensitivity assay, i.e. how much lower amount of antigen can be detected. For this purpose, the most suitable titer dilution was selected which was 40,000X and 20,000X dilution of rabbit and mouse hSAA1 anti-sera, respectively using 50-400,000 pg/well of hSAA1 antigen (Fig. 4).

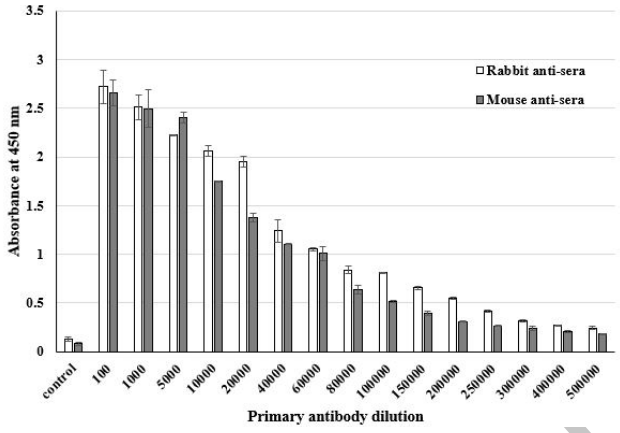

Fig. 3. Titer analysis of hSAA1 polyclonal antibodies raised in rabbits and mice against 200 ng hSAA1.

Table II: Signal intensities comparison of rabbit antisera and mouse antisera.

Dilution Factor	Signal intensity of rabbit antisera (OD 450 nm)	Signal intensity of mouse antisera (OD 450 nm)	Signal strength ratio of rabbit antisera to mouse antisera
10,000	2.1	1.7	1.23 : 1
20,000	2.0	1.4	1.43 : 1
100,000	0.8	0.51	1.6 : 1
150,000	0.65	0.39	1.7 : 1
200,000	0.55	0.31	1.8 : 1
250,000	0.42	0.26	1.6 : 1

For the complete range of antigen amount used, the graph presented a hyperbola picture (Fig. 4A). To detect the linear antigen-antibody complex response, the inset of Figure 4B, showing a gradual increase in signal for a range of 1000-150,000 pg/well of hSAA1 antigen which reached plateau afterwards in both mice and rabbits. The effective sensitivity of hSAA1 polyclonal antibodies derived from these results for selected dilutions of antisera was up to 1 ng (1000 pg).

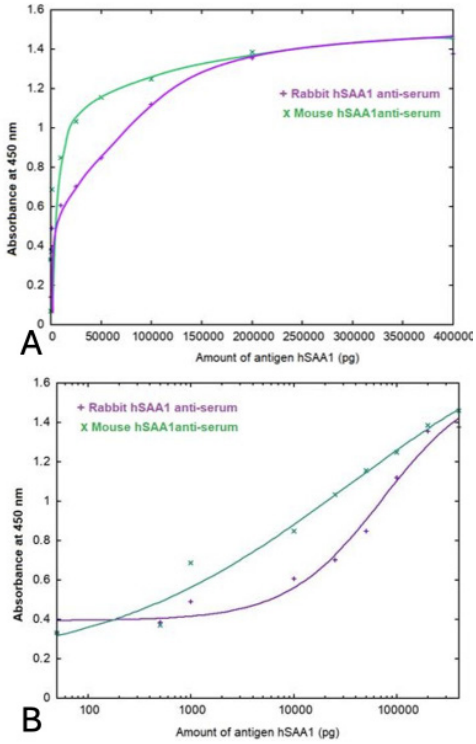

Fig. 4: hSAA1 antigen sensitivity analysis against 40,000- and 20,000 times diluted rabbit and mice hSAA1 antisera, respectively. A, Hyperbolic curve representation of 0-500,000 pg of hSAA1 antigen analyzed by selected dilution of rabbit and mice antisera; B, In-set of curves in (A) using 50-150, 000 pg of hSAA1, showing the sigmoidal curves for analyzing the linear range of antigen: antibody complex response.

DISCUSSION

Serum amyloid A1 is an acute phase high-density lipoprotein (Sack, 2018) which also acts as a biomarker in progression of viral infections such as COVID-19 (Abbas *et al*., 2022), inflammatory diseases such as urticaria (Carvalla *et al*., 2024), autoinflammatory disorders and rheumatoid arthritis. (Sorić Hosman *et al*., 2021, Almusalami *et al*., 2023). The quick approach to evaluate SAA1 as a biomarker, is the detection of SAA1 in serum or plasma by using its antibodies. The nature of antibodies can be either monoclonal or polyclonal which depends on their pros and cons as described by Leenaars and Hendriksen, (2005) such as less time, cost and issue of

cross-reactivity.

This study highlights production of hSAA1 polyclonal antibodies in animal models. The first challenge of production of hSAA1 polyclonal antibodies is its sequence conservancy in vertebrates (Uhlar *et al*., 1994). The primary structure similarity >90% would hinder in producing the potential polyclonal antibodies. Therefore, two different animal species were selected after performing sequence alignment with rabbit and mice. Also, the husbandry of these animals was manageable under in-house environment. Human SAA1 showed 82% sequence homology with rabbit SAA1 and 74% with mice SAA1 which were promising to raise polyclonal antibodies in these hosts (Fig. 1). The second challenge was to maintain animal health during the period of two months because SAA1 is involved in inflammatory response (Eklund *et al*., 2012). This issue is important to address because immune response is also activated by using Freund's adjuvant as an immuno booster, which is an important constituent of dose formulation in raising polyclonal antibodies (Fishback *et al*., 2016). Therefore, animals were monitored for their feeding behavior and weight measurement. It was observed after the administration of first booster, rabbits' weight was reduced about 200-300 g and mice reduced 5-7 g than their weight at the start of experiment (1300-1500 g and 27-30 g, respectively). At the end of experiment, rabbits lost 500-600 g and in the case if the mice lost 10-12 g of their body weight although they were regularly administered supplements prescribed by veterinary expert, as it was an expected phenomenon to occur in production of hSAA1 polyclonal antibodies (Report of the Federation of European Laboratory Animal Science Associations, FELASA, 1994).

Analysis of polyclonal antibodies was carried by indirect ELISA which shows the direct response of antibodies against immobilized antigen and amplified signal due to multiple epitopes availability for host specific labelled secondary antibodies (Kohl and Ascoli, 2017). The pre-immune response analysis satisfied two basic requirements: firstly, that there should be no active immune response in animals and secondly the antisera raised should not show cross-reactivity against hSAA1 antigen with the animal's self-antigen present in hosts. In this experiment, the control animals which were only administered with adjuvant (immuno booster), were decisive for validity of specificity of polyclonal antibodies production and titer analysis as it gave insignificant signal during the course of experiment i.e. pre-immune, test and final bleed (Fig. 2). Whereas the titer obtained in test and final bleed of experimental animals showed the activation of hosts' immune response to produce antibodies against hSAA1 antigen (Fig. 2). The significant titer signal was observed in the linear range of 10, 000 - 250,000X in both rabbit and mice antisera. The exact titer of antibodies is important to evaluate prognosis and diagnosis of diseases because it indicates the levels of antibodies (Daschner, 1976). The high titers of antibodies produced in animal models show effectiveness of antigen to provoke immune response and presenting its epitopes for antibodies production (Chan *et al*., 1982). Our results showed almost twice titer of hSAA1 antisera in rabbits than mice as shown in Table II showing potential production of hSAA1 polyclonal antibodies. This titer difference was mainly due to recognition ability of rabbit for epitopes and antigen than mice (Spieker-Polet *et al*., 1995) and making rabbit a more suitable host for the production of polyclonal antibodies against immunogenic proteins.

The sensitivity of hSAA1 polyclonal antibodies was analyzed using appropriate antisera dilutions 40,000X and 20,000X of rabbit and mice respectively to analyze 50-400,000 pg of hSAA1 antigen. These antisera dilutions were chosen based upon titer analysis and both of these dilutions gave enough saturated signal (1-1.5 OD_{450}) against supersaturated antigen amount 200 ng of hSAA1. The sigmoidal curve for sensitivity analysis showed a better picture of linearity for antigen: antibody complex response in the range of 1000-150,000 pg (Fig. 4B). Hence, this data provided the sensitivity value of 1 ng for the selected dilutions of in-house produced polyclonal antibodies from both the species. This sensitivity results are significantly comparable to commercially available monoclonal hSAA1 antibodies using sandwich ELISA, showing the detection limit of 1-10 ng of antigen in patients' samples (Metwalli *et al*., 2021, Wu *et al*., 2022). In view of these findings, hSAA1 polyclonal antibodies prepared can be applied for the detection of serum hSAA1 levels in various autoimmune and inflammatory pathophysiological conditions.

CONCLUSION

This study highlighted the effective production of polyclonal antibodies of an immunogenic antigen such as hSAA1 in rabbits and mice. There was no pre-immune response and antibody cross-reactivity in both animals. However, the strength of hSAA1 rabbit antisera was twice as that of mice titer, which can be attributed to the behavior of hosts' immune system. The sensitivity of hSAA1 polyclonal antibodies was approximately up to 1 ng of both animals' antisera. Based upon its sensitivity at specific antisera dilution, these antibodies can be used for sero-diagnosis of hSAA1 in acute and chronic disease conditions.

DECLARATIONS

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Ethics statement

The present study was ethically approved by the University of the Punjab, Lahore and Directive 2010/63/EU guidelines were followed to prepare the polyclonal antibodies in these animals.

IRB approval

IRB committee approved the study under the reference number Bioethics/050, notification no. 8194/Punjab/ NRPU/R&D/HEC/ 2017.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20240519104210

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Supplementary Material

Immunogenic Response for the Production of Polyclonal Antibodies of Human Serum Amyloid A1 (hSAA1) in Rabbits and Mice

Saira Ahmad[1], Ukasha Tahir[1], Qurratulann Afza Gardner[1]* and Muhammad Akhtar[2]

[1]School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan
[2]School of Biological Sciences, University of Southampton, Southampton, SO17 1BJ, UK

Supplementary Table I: Posphate-buffered saline (PBS) 1 liter according to cold spring Harbor Laboratory*.

Reagent	For 1X working solution	Final concentration 1X working solution	For 10X working solution	Final concentration 10X working solution
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM

* https://cshprotocols.cshlp.org/

Supplementary Table II: Comparison of titer analysis of hSAA1 polyclonal antibodies raised in rabbit and mice using fixed amount of hSAA1 antigen (200 ng).

Mice antisera dilution	Titer analysis by OD_{450} nm	Rabbit antisera dilution
100-5000	>2.0	100-10,000
10,000-60,000	2-1.0	20,000-60,000
80,000-100,000	1.0-0.5	80,000-200,000
150,000-500,000	<0.5	250,000-500,000

* Corresponding author: qagardner.sbs@pu.edu.pk; annegardner80@yahoo.co.uk
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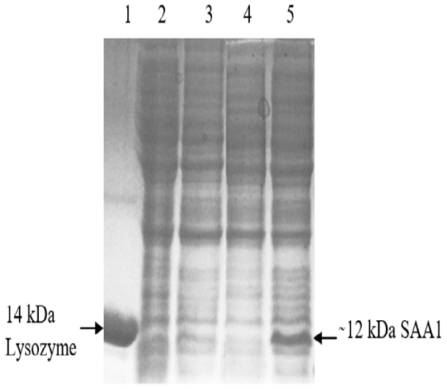
     M  K  L  L  T  G  L  V  F  C  S  L  V  L  G  V  S  S  R  S
Native      CAT ATG AAG CTT CTC ACG GGC CTG GTT TTC TGC TCC TTG GTC CTG GGT GTC AGC AGC CGA AGC  63
Synthetic   --- ATG AAA CTG CTG ACC GGT CTG GTT TTC TGC TCT CTG GTT CTG GGT GTT TCT TCT CGT TCT  63
            *** ** ** ** ** ** *** *** *** *** ** ** ** *** *** **          **

     F  F  S  F  L  G  E  A  F  D  G  A  R  D  M  W  R  A  Y  S
Native      TTC TTT TCG TTC CTT GGC GAG GCT TTT GAT GGG GCT CGG GAC ATG TGG AGA GCC TAC TCT  123
Synthetic   TTC TTC TCT TTC CTG GGT GAA GCA TTC GAC GGT GCG CGT GAC ATG TGG CGT GCT TAC TCT  123
            *** ** ** *** ** ** ** ** ** ** ** ** ** *** *** *** * ** *** ***

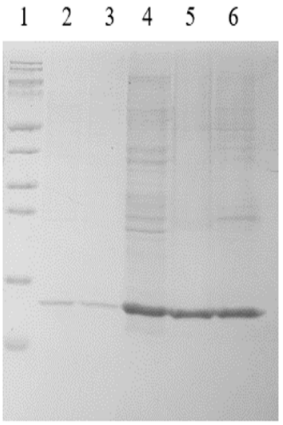
     D  M  R  E  A  N  Y  I  G  S  D  K  Y  F  H  A  R  G  N  Y
Native      GAC ATG AGA GAA GCC AAT TAC ATC GGC TCA GAC AAA TAC TTC CAT GCT CGG GGG AAC TAT  183
Synthetic   GAT ATG CGT GAA GCT AAC TAC ATT GGT TCT GAT AAA TAC TTC CAC GCT CGT GGT AAC TAT  183
            ** *** * *** ** ** *** ** ** ** ** *** *** *** ** *** ** ** *** ***

     D  A  A  K  R  G  P  G  G  V  W  A  A  E  A  I  S  D  A  R
Native      GAT GCT GCC AAA AGG GGA CCT GGG GGT GCC TGG GCT GCA GAA GTG ATC ACC GAT GCC AGA  243
Synthetic   GAC GCT GCT AAA CGT GGT CCG GGT GGT GTT TGG GCT GCT GAA GCT ATC TCT GAT GCT CGT  243
            ** *** ** *** * ** ** ** *** * *** *** ** *** * *** * *** ** *
     E  N  I  Q  R  F  F  G  H  G  A  E  D  S  L  A  D  Q  A  A
Native      GAG AAT ATC CAG AGA TTC TTT GGC CAT GGT GCG GAG GAC TCG CTG GCT GAT CAG GCT GCC  303
Synthetic   GAA AAC ATC CAG CGT TTC TTC GGT CAC GGT GCT GAA GAT TCT CTG GCT GAT CAG GCT GCT  303
            ** ** *** *** * *** ** ** ** *** ** ** ** ** *** *** *** *** *** **
     N  E  W  G  R  S  G  K  D  P  N  H  F  R  P  A  G  L  P  E
Native      AAT GAA TGG GGC AGG AGT GGC AAA GAC CCC AAT CAC TTC CGA CCT GCT GGC CTG CCT GAG  363
Synthetic   AAC GAA TGG GGT CGT TCT GGT AAA GAT CCG AAC CAC TTC CGT CCG GCT GGT CTG CCG GAA  363
            ** *** *** ** * *** *** ** ** ** *** *** ** ** *** ** *** ** **
     K  Y
Native      AAA TAC TGA --- --- 378
Synthetic   AAA TAC TAA AAG CTT 378
            *** *** * *
```

Supplementary Fig. 1. Sequence alignment of synthetic human *SAA1* gene having *Escherichia coli* preferred codons with native *SAA1*. The highlighted sequences show alteration in genetic codon. Emboldened and underlined sequences show signal peptide.



Supplementary Fig. 2. 15% Tricine PAGE for expression analysis of recombinant human SAA1
Lane 1: Lysozyme as a protein marker
Lane 2: Uninduced pET-21a vector
Lane 3: pET-21a vector induced with 0.2mM IPTG
Lane 4: Uninduced *hSAA1-pET21a*
Lane 5: *hSAA1-pET21a* induced with 0.2mM IPTG



Supplementary Fig. 3. 15 % Tricine-PAGE of purified recombinant human SAA1
Lane 1: Protein marker (26616) PageRuler™
Lane 2: Reductive sample of combined fraction of Octyl-FF (reverse phase hydrophobic column)
Lane 3: Non-reductive sample of combined fraction of Octyl-FF
Lane 4: Dialysed SAA1 protein sample before purification
Lane 5: Non-reductive sample of combined fraction of Q-FF (anion exchange column)
Lane 6: Reductive sample of combined fraction of Q-FF