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Production of anti-idiotype immunoglobulin Y against African swine fever virus using purified porcine immunoglobulin G

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Abstract

Background African swine fever (ASF) is a lethal viral disease threatening the global swine industry. Anti-idiotype antibodies (Ab2) mimicking viral epitopes serve as promising surrogate antigens for vaccine and diagnostic development.

Objective This study aimed to produce anti-idiotype immunoglobulin Y (IgY) against African swine fever virus (ASFV) by immunizing laying hens with purified ASFV-specific porcine immunoglobulin G (IgG).

Methods ASFV-specific IgG (Ab1) was purified from ASF-vaccinated pigs serum and convalescent plasma of naturally infected pigs by stepwise ammonium sulfate precipitation (25% and 50% saturation) and dialysis. Purified IgG was characterized using Bradford assay, indirect ELISA, and SDS-PAGE. Two Hy-Line Brown laying hens were immunized with serum-derived (A1) or plasma-derived (A2) IgG, using Freund's complete adjuvant for primary immunization, followed by three booster injections with Freund's incomplete adjuvant at two-week intervals. Serum and egg yolk samples collected over 13 weeks were analyzed using agar gel precipitation test (AGPT).

Results Purification increased total protein while retaining ASFV-specific antibody reactivity. SDS-PAGE confirmed porcine IgG heavy (~53–55 kDa) and light (~24–25 kDa) chains, alongside additional bands indicating partial purification. Serum antibodies reactive to porcine IgG were first detected at weeks 2 (A2) and 4 (A1) post-primary immunization, with egg yolk antibodies appearing one week later. Both serum and egg yolk achieved a maximum AGPT titer of 3 $\log_2$ (1:8 dilution).

Conclusion Immunization with ASFV-specific porcine IgG successfully elicited reactive IgY in laying hens. Further validation of anti-idiotype specificity and antigen-mimicking properties is required before considering these antibodies as surrogate antigens for ASF vaccine and diagnostic development.

Keywords African swine fever virus | anti-idiotype antibody | immunoglobulin G | immunoglobulin Y | surrogate antigen

Introduction

African swine fever (ASF) is a highly infectious hemorrhagic disease that affects domestic and wild pigs, and is characterized by high morbidity and mortality. Its causative agent, the African swine fever virus (ASFV), is a large, icosahedral, double-stranded DNA virus with nucleocytoplasmic replication belonging to the genus Asfivirus within the family Asfarviridae (Li *et al.*, 2022; Ciputra *et al.*, 2023). Clinical presentations range from peracute and acute to chronic forms, with severe disease characterized by sudden death, high fever, and extensive hemorrhages in the skin and inter-

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nal organs. In acute cases, death can occur within three to ten days, with mortality frequently exceeding 90% (Chenais *et al.*, 2019). Following its initial identification in Kenya in 1920, ASF has spread internationally. Genotype I was introduced into Europe from West Africa in 1960, whereas genotype II spread from East Africa to Georgia in 2007 and subsequently through Europe and into China in 2018 (Li *et al.*, 2022). The continuing global burden is reflected in WOAH surveillance data: between January 2022 and 28 February 2026, ASF was reported in 74 countries and territories, with 1,152,369 cases in domestic pigs and 48,613 cases in wild boars recorded through the World Animal Health Information System (WOAH, 2026). In Indonesia, ASF was first detected in North Sumatra in 2019 and subsequently spread to other regions, including Bali and East Nusa Tenggara, causing substantial losses to the pig production sector (Tenaya *et al.*, 2023). By December 2024, the disease had been reported in 32 provinces (SVC, 2024). Continued occurrence was documented in March 2026, when national surveillance recorded five reported events involving 32 cases in East Manggarai Regency, East Nusa Tenggara, and Dumai City, Riau (DGLS, 2026).

Transmission of ASF occurs through direct contact with infected animals, contaminated pork products, fomites, and soft tick vectors of the genus Ornithodoros (Njau *et al.*, 2021). Controlling the virus remains a significant challenge because ASFV can effectively evade both innate and adaptive immune responses. Research indicates that the virus inhibits Major Histocompatibility Complex (MHC) Class II processing, suppresses macrophage activation, and evades effector cells, such as cytotoxic T-cells (Zhu *et al.*, 2019). Consequently, prevention still relies heavily on early detection and strict biosecurity (Primatika *et al.*, 2021). While Vietnam authorized the first live-attenuated vaccine (LAV) for local use in 2022, global adoption remains hindered by concerns regarding safety and stability (Fan *et al.*, 2024).

Given the lack of conventional vaccines, advanced immunological approaches are urgently required. Specific antibodies against ASFV play a crucial role in the detection and neutralization of the virus. Convalescent plasma and serum from pigs that have recovered from infection are primary sources of these specific antibodies, containing Immunoglobulin G (IgG) that can bind to ASFV antigens (Hornbeck 2015). Neutralizing antibodies in these serums can inhibit ASFV replication in macrophages and monocytes, the virus's primary target cells (Canter *et al.*, 2022). This passive immunity approach mirrors successful therapies used in other viral outbreaks, such as COVID-19 and H1N1, where convalescent plasma helped reduce viral loads and mortality (Lokat *et al.*, 2023; Mubarok *et al.*, 2023).

One promising development in ASFV immunotherapy is the production of anti-idiotype antibodies (Ab2). A primary antibody (Ab1) that recognizes a viral antigen can act as a new antigen to trigger the production of a secondary antibody (Ab2) that mimics the structure of the original viral epitope, a concept known as the "internal image" (Paryati *et al.*, 2006). These anti-idiotype antibodies have been shown to stimulate the formation of Ab3, which recognizes the actual virus. To develop such a system for ASF, highly purified ASFV-specific IgG is required as a starting antigen. Furthermore, producing anti-idiotype antibodies using Immunoglobulin Y (IgY) from chicken egg yolks offers an ethical, economical, and efficient alternative. Layer chickens, particularly the Hy-Line Brown strain, are ideal biological models because of their high egg productivity and consistent immune response, allowing specific antibodies to be harvested non-invasively from yolks a few weeks after immunization (Saputri *et al.*, 2024; Azis *et al.*, 2013).

Despite the potential of this technology, research on the production of chicken IgY-based anti-idiotype antibodies against ASF in Indonesia remains limited. Addressing this gap is critical for improving early detection and developing effective immune responses against the virus. Therefore, this study aimed to produce anti-idiotype immunoglobulin Y (IgY) against ASFV by immunizing Hy-Line Brown laying hens with purified ASFV-specific porcine IgG. Purification of ASFV-specific IgG (Ab1) from convalescent plasma and serum by stepwise ammonium sulfate precipitation was performedas a preliminary step to obtain a suitable immunogen.

Methods

Purification of ASFV-Specific IgG)

Ammonium sulfate precipitation. ASFV-specific IgG was purified from two sources: (1) serum from ASF-vaccinated pigs (Ab1 serum) and (2) convalescent plasma obtained from PUSVETMA, Surabaya (Ab1 plasma). Purification was performed using a graduated ammonium sulfate precipitation method (Fishman & Berg, 2018). Initial centrifugation was performed at 3,000 × g for 30 minutes at 4°C. Non-target proteins were first precipitated by adding saturated ammonium sulfate to a 25% saturation level, followed by 5–15 hours of incubation at 4°C. The supernatant was then collected and adjusted to 50% saturation to precipitate the target IgG. After a second incubation and centrifugation, the resulting pellet was resuspended in PBS buffer at a 1:4 (v/v) ratio. The solution was dialyzed using a 10–25 kDa MWCO membrane in PBS for 24–48 h to remove residual salts.

Protein quantification and characterization. The protein concentration of the purified IgG was measured using the Bradford method (Bradford, 1976; Stoscheck, 1990) with Bovine Serum Albumin (BSA) as the standard. Absorbance was measured at 520 nm using a microplate reader. To evaluate purity, SDS-PAGE (4–12% Bis-Tris gels) was performed. Protein samples were mixed with the sample buffer and heated under three conditions (60°C for 3 minutes, 95°C for 5 minutes, and 95°C for 10 minutes) before being loaded into the gel. Electrophoresis was run at 200 V and 500 mA for 100 minutes, followed by staining with Coomassie Brilliant Blue and destaining to visualize protein bands (Rachmawan *et al.*, 2025).

Specificity and acctivity testing (ELISA). The activity of the purified IgG was validated using the ID Screen® African Swine Fever Indirect ELISA kit. Samples, along with positive and negative controls, were incubated in microtiter plates. After washing and adding the conjugate, the reaction was visualized with a substrate solution and stopped with a stop solution. The results were read at 450 nm. Samples were considered positive if the S/P% value was ≥ 40%.

Production and evaluation of ASFV anti-idiotype antibodies in laying hens

Experimental animals. Two 28-week-old Hy-Line Brown laying hens were used to the produce ASFV anti-idiotype antibodies. Before immunization, the hens were acclimatized for two weeks in battery cages and provided with commercial layer feed and water ad libitum. Their health was monitored twice daily for signs of stress or illness (Saputri *et al.*, 2024). All animal procedures were conducted in accordance with animal welfare guidelines and approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University (approval No. 056/KEH/SKE/VI/2026).

Immunization protocol. Two chickens were immunized with different antigens: Chicken 1 (A1) received Ab1 serum, and Chicken 2 (A2) received Ab1 plasma. Each hen received a fixed antigen dose of 5 mg per immunization, without adjustment for body weight. The antigen was emulsified with an adjuvant to form a water-in-oil suspension (Amanu *et al.*, 2016). Primary immunization was performed at week 0 via the subcutaneous (SC) route using Freund's Complete Adjuvant (FCA) at a 7:3 ratio, followed by booster injections administered intramuscularly (IM) at weeks 1, 3, and 5 using Freund's Incomplete Adjuvant (FIA) at a 7:3 ratio.

Collection and preparation of serum and egg yolk samples. Blood and egg samples were collected weekly from week 1 to week 13 post-primary immunization. Blood (1.5–2 mL) was drawn from the brachialis vein, and serum was separated and stored at −20°C. Egg yolks were separated and pooled weekly for each chicken. The yolks were diluted in PBS (1:1), homogenized, and centrifuged at 15,000 rpm for 15 minutes at 4°C. The resulting supernatant (water-soluble fraction) was stored at −20°C for subsequent IgY detection (Saputri *et al.*, 2024).

Detection of specific IgY (ASF anti-idiotype antibodies/Ab2). The presence of Ab2 in chicken serum and egg yolk against ASFV-specific porcine IgG (Ab1) was assessed using the agar gel precipitation test (AGPT). A 1% agarose gel containing 0.01% sodium azide was prepared on glass slides, and the wells were arranged in a hexagonal pattern. The central well contained purified porcine IgG, while the surrounding wells contained chicken serum or egg yolk samples (25 µL per well). The slides were incubated at room temperature and examined after 24, 48, and 72 h. A distinct white precipitate line between the central and sample wells was considered a positive reaction. For semi-quantitative assessment, samples were tested at serial two-fold dilutions ranging from 1:2 to 1:64. The endpoint titer was defined as the highest sample dilution that produced a visible precipitation line and was expressed as the base-2 logarithm of the reciprocal dilution. Accordingly, endpoint dilutions of 1:2, 1:4, and 1:8 corresponded to titers of 1, 2, and 3 $\log_2$, respectively. Samples showing no detectable precipitation line at any tested dilution were recorded as 0 for tabulation and graphical presentations.

Data analysis

The total protein concentrations and ELISA S/P% values before and after IgG purification were summarized descriptively. Weekly AGPT endpoint titers in serum and egg yolk were expressed as $\log_2$ reciprocal dilutions and are presented in tables and line graphs to describe the onset, peak, persistence, and subsequent changes in detectable antibody reactivity. Each immunization condition was represented by one hen (n = 1), and weekly measurements were repeated observations from the same individual. No inferential statistical tests were performed, and no p-values were calculated for comparisons between immunization conditions or sampling weeks.

Results

Purification and characterization of ASFV-specific IgG

Total protein concentration in purified serum and plasma fractions was determined using a simple linear regression standard curve (y=0.0379x+0.5013). Post-purification measurements indicated a clear increase in both absorbance values and total protein concentrations for both convalescent plasma and serum samples. The specific results are summarized in **Table 1.**

The activity and specificity of the purified IgG before and after IgG purification were verified via an ASF-specific indirect ELISA. ASFV-specific antibodies were detected in all tested samples, with S/P% values consistently falling within the positive category, as presented in **Table 2.**

Figure 1 shows the SDS-PAGE profiles of serum and convalescent plasma before and after IgG purification, with samples heated under three conditions. The molecular weight of

Table 1 Total protein concentration of convalescent plasma and serum samples before and after purification (Bradford Assay)

Sample type	Pre-Purification Abs (520 nm)	Pre-Purification Conc (mg/mL)	Post-Purification Abs (520 nm)	Post-Purification Conc (mg/mL)
Convalescent plasma	1.13	16.48	1.26	20.04
Serum	1.33	21.76	1.40	20.04

Abs: absorbance; Conc: concentration

Table 2 ELISA test results for convalescent plasma and serum samples before and after purification

Sample type	Pre-purification S/P%	Interpretation	Post-purification S/P%	Interpretation
Convalescent plasma	45.08	Positive	67.21	Positive
Serum	76.42	Positive	55.43	Positive

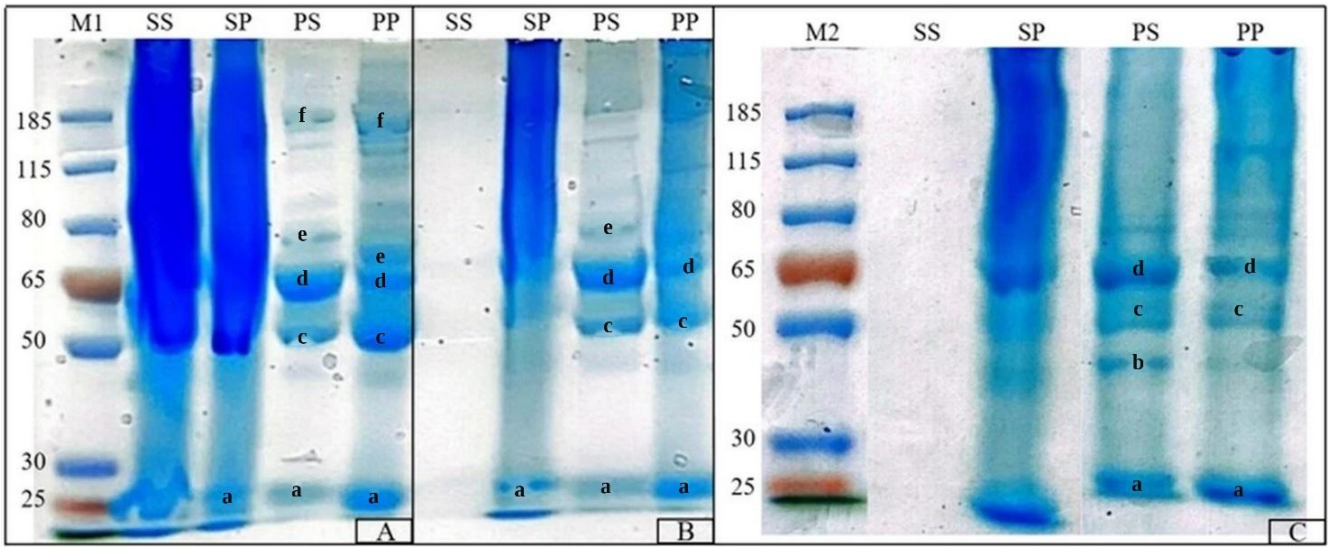


Figure 1 SDS-PAGE profiles of porcine serum and convalescent plasma before and after IgG purification under different heating conditions. Samples were heated at (A) 60°C for 3 min, (B) 95°C for 5 min, and (C) 95°C for 10 min. M1 and M2: molecular weight markers (kDa); SS: serum before purification; SP: serum after purification; PS: convalescent plasma before purification; PP: convalescent plasma after purification. Lettered bands: a, putative IgG light chain (~25 kDa); b, putative heavy-chain fragment (~43–45 kDa; panel C only); c, putative IgG heavy chain (~50–55 kDa); d, albumin-like protein (~65 kDa); e, putative transferrin-like protein (~75–80 kDa); f, high-molecular-weight proteins/aggregates (~185 kDa; panel A only).

each band was estimated using a linear regression based on the relative mobility of the marker proteins. After heating at 60°C for 3 min (**Figure 1A**), the plasma samples (PS and PP) showed three IgG-related bands: intact IgG (band f, ~150–155 kDa), heavy chain (band c, ~53–54 kDa), and light chain (band a, ~24–25 kDa). In contrast, the purified serum sample (SP) mainly showed the light-chain band (a). After heating at 95°C for 5 min (**Figure 1B**), the intact IgG band disappeared, leaving only distinct heavy-chain (c) and light-chain (a) bands, which indicates that IgG had separated into its chains. After heating at 95°C for 10 min (**Figure 1C**), the lanes showed an albumin-like non-IgG band (d) and a lower-molecular-weight band (b, ~43–45 kDa) consistent with IgG fragments, suggesting further protein degradation.

ASFV anti-idiotype antibody production in layer chickens

Serum antibody reactivity with porcine IgG preparations was detected by the formation of white precipitation lines in the agar gel precipitation test (AGPT). A representative positive reaction obtained from A2 serum collected four weeks after primary immunization with plasma-derived IgG is shown in **Figure 2**. In A1, antibodies were first detected at week 4 at 2 $\log_2$ (1:4), followed by maximum titers of 3 $\log_2$ (1:8) during weeks 5–9. Titers subsequently decreased to 2 $\log_2$ (1:4) and remained at this level during weeks 10–13. In A2, antibodies were first detected at week 2 at 2 $\log_2$ (1:4), the maximum observed serum titer for this hen, which persisted through week 9. Titers then decreased to 1 $\log_2$ (1:2) during Weeks 10–13. These observations showed an earlier onset of detectable serum antibody reactivity in A2 and higher peak titers in A1 (**Table 3** and **Figure 3A**).

Detectable antibody reactivity in egg yolk appeared one week later than in serum in both hens. In A1, antibodies were first detected at week 5 at 2 $\log_2$ (1:4), followed by maximum titers of 3 $\log_2$ (1:8) during weeks 6–8 and again at week 10. The titer decreased to 2 $\log_2$ at Week 9, became undetectable at weeks 11–12, and returned to 1 $\log_2$ at week 13. In A2, antibodies were first detected at week 3 at the maximum observed titer of 3 $\log_2$ (1:8), which persisted through week 4 and recurred during Weeks 6–8. Titers were 2 $\log_2$ at Week 5 and during weeks 9–11, became undetectable at week 12, and returned to 2 $\log_2$ at week 13. Therefore, weeks 6–8 represented a shared collection window during which both hens consistently exhibited the maximum observed egg yolk AGPT titer (**Table 4** and **Figure 3B**).

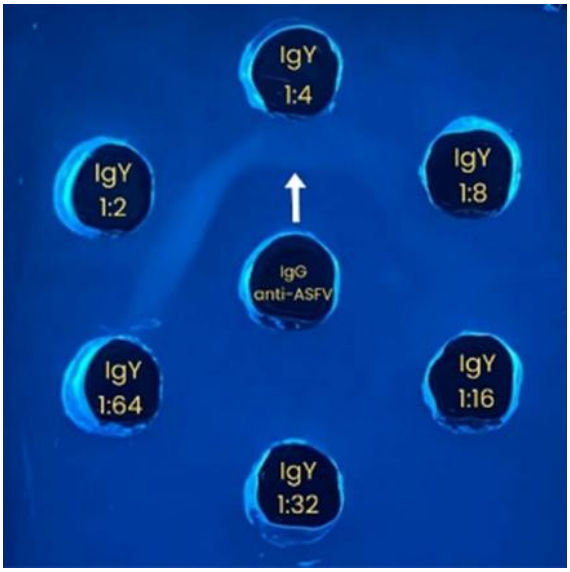


Figure 2 Representative positive agar gel precipitation test (AGPT) of chicken serum collected four weeks after primary immunization with IgG purified from porcine convalescent plasma (A2). The central well contained ASFV-specific porcine IgG, while the surrounding wells contained serial two-fold dilutions of chicken serum (1:2–1:64). The arrow indicates a visible precipitation line demonstrating serum antibody reactivity with porcine IgG preparation.

Table 3 Agar gel precipitation test (AGPT) titers (log_2) of chicken serum from Ab1 serum-immunized and Ab1 plasma-immunized chickens

Chicken code	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13
A1	0	0	0	2	3	3	3	3	3	2	2	2	2
A2	0	2	2	2	2	2	2	2	2	1	1	1	1

A1: Immunized with Ab1 serum; A2: Immunized with Ab1 plasma.

Table 4 Agar gel precipitation test (AGPT) titers (log_2) of chicken egg yolk from Ab1 serum-immunized and Ab1 plasma-immunized chickens

Chicken code	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13
A1	0	0	0	0	2	3	3	3	2	3	0	0	1
A2	0	0	3	3	2	3	3	3	2	2	2	0	2

A1: Immunized with Ab1 serum; A2: Immunized with Ab1 plasma.

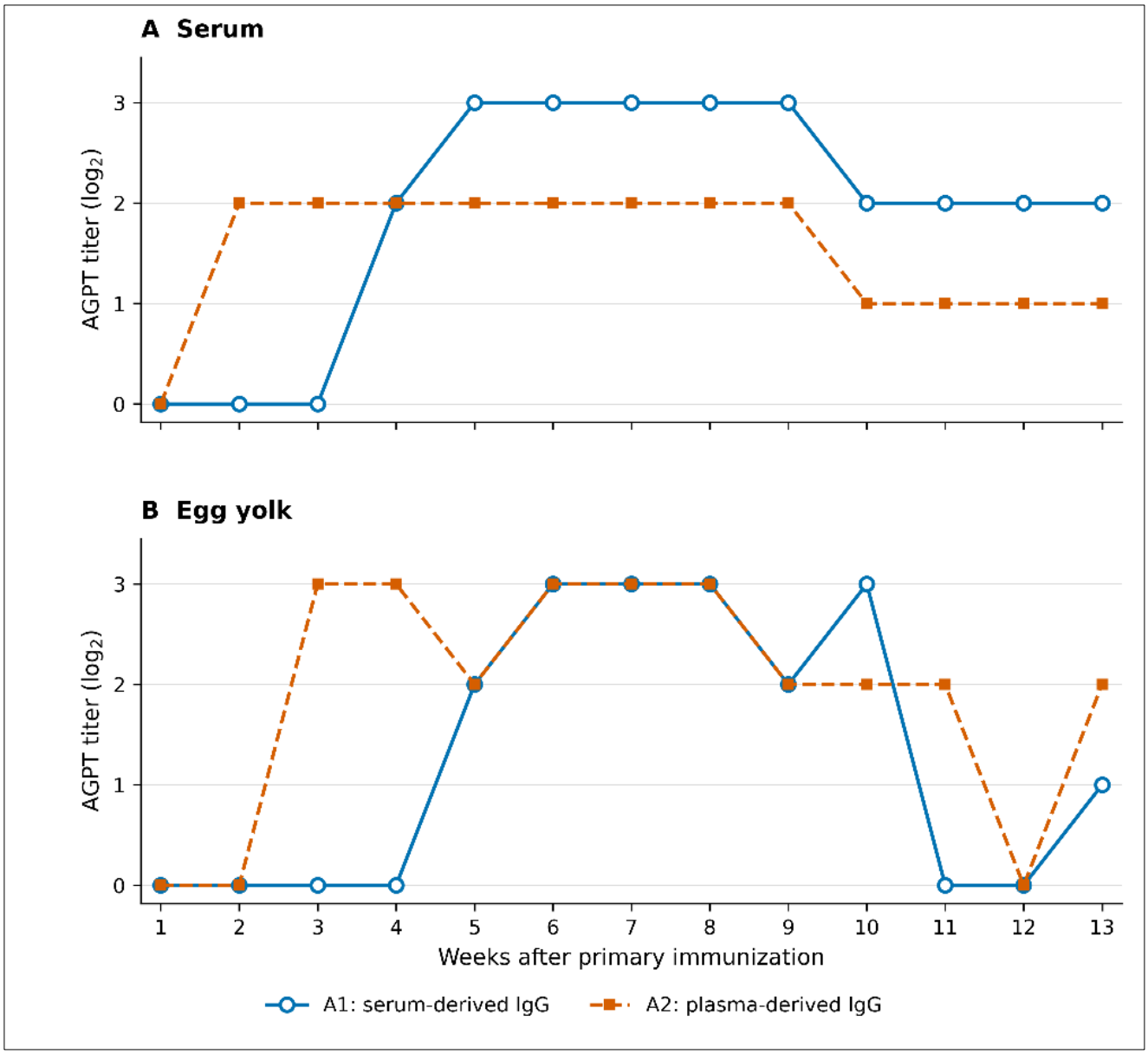


Figure 3 Temporal patterns of AGPT antibody titers in chicken serum and egg yolk following immunization with porcine IgG. (A) Serum and (B) egg yolk titers measured weekly for 13 weeks after primary immunization. A1 received IgG purified from porcine serum, whereas A2 received IgG purified from porcine convalescent plasma. Titers are expressed as $\log_2$ reciprocal endpoint dilutions. Values of 0 indicate no detectable precipitation reaction under the assay conditions. Each series represents one hen; egg yolk samples were pooled weekly for each hen. Lines connect observed values to facilitate visualization and do not represent fitted curves.

Discussion

The results in **Table 1** demonstrate a clear increase in protein concentration for both convalescent plasma (from 16.48 mg/mL to 20.04 mg/mL) and serum (from 21.76 mg/mL to 23.77 mg/mL). This increase is a direct result of the "salting out" mechanism during ammonium sulfate precipitation, where protein solubility decreases, causing it to precipitate from the solution (Ha *et al.*, 2021). However, the Bradford assay is non-specific, as it measures total protein via Coomassie blue G-250 interactions; therefore, non-target proteins also contribute to the final concentration (Hueso *et al.*, 2022; Mamaeva *et al.*, 2022).

This non-specificity explains why total protein increases do not always correlate with ELISA S/P% values. While plasma S/P% increased to 67.21%, serum S/P% decreased from 76.42% to 53.43% (**Table 2**). This variation depends on the proportion of functional IgG remaining after fractionation (Fishman & Berg, 2018). The decrease in serum activity may be attributed to "matrix effects" or changes in pH and ionic strength during dialysis, which can trigger antibody aggregation and diminish binding capacity in ELISA tests (Moussa *et al.*, 2016; Heads *et al.*, 2019; Finocchiaro *et al.*, 2026).

Characterization via SDS-PAGE under reducing conditions successfully dissociated IgG into light chains (~25 kDa) and heavy chains (~50–55 kDa) (Kirley & Norman, 2018; Zheng *et al.*, 2021). Optimal denaturation was achieved at 95°C for 5 minutes, where the intact IgG band (~150 kDa) completely disappeared. Conversely, prolonged heating (10 minutes) led to protein degradation and the formation of IgG fragments at 42.81 kDa (Song *et al.*, 2022; Pamukcu & Atik, 2025). The presence of non-IgG bands, such as albumin (~66 kDa), transferrin (~80 kDa), and fibrinogen (~66–69 kDa), indicates that ammonium sulfate precipitation alone is insufficient to produce purely isolated IgG (Akram *et al.*, 2017; Nencini *et al.*, 2025).

The formation of specific precipitation lines in the AGPT results confirms that immunization with ASFV-specific antibodies (Ab1) successfully induced the production of anti-idiotype antibodies (Ab2) in both serum and egg yolks. This supports Jerne's Idiotypic Network Theory, which suggests that the hypervariable region of an immunoglobulin can act as an antigen, triggering the production of secondary antibodies (Ab2) that can competitively inhibit original antigen binding (Murphy & Weaver, 2017; Paryati *et al.*, 2020). The temporal dynamics revealed a "lag phase" in the first few weeks, typical for initial immune cell activation (Chalghoumi *et al.*, 2009; Capota *et al.*, 2025). The subsequent rise in titers following boosters reflects a secondary immune response mediated by memory B cells (Saputri *et al.*, 2024). Variations between the two chickens (A1 and A2) suggest that the molecular composition of the antigen source serum versus plasma influences immunogenicity (Poetri & Soejoedono, 2006).

The detection of antibodies in egg yolk occurred one week later than in serum in both hens. This delay is consistent with the physiological transfer of IgY from the bloodstream to developing egg yolks over approximately 3–6 days (Chalghoumi *et al.*, 2009; Okamoto *et al.*, 2024). The observed fluctuations and temporary absence of detectable precipitation reactions in the later weeks may reflect variations in antibody deposition or the detection limits of AGPT (Oladele *et al.*, 2004). Based on the observed AGPT titers, weeks 6–8 after primary immunization represented a suitable egg collection window, during which both hens consistently exhibited the maximum observed titer of 3 $\log_2$ (1:8). However, this period should be considered a preliminary collection window rather than an established optimal harvest time, because IgY concentration and total yield were not quantified.

Several limitations should be acknowledged. First, ammonium sulfate precipitation did not completely remove non-target proteins from the porcine IgG preparations. These residual proteins may have elicited additional antibody responses, complicating the interpretation of the AGPT reactions. Second, AGPT demonstrated antibody reactivity with porcine IgG preparations but did not establish that this reactivity was directed specifically against idiotypic determinants or that the resulting antibodies mimicked ASFV epitopes. The absence of detectable precipitation lines at some time points may also reflect antibody concentrations below the assay's detection threshold. Third, the use of one hen per antigen source precluded the estimation of between-animal variability within each immunization condition. Because the antigen source was confounded with individual animal identity, differences between A1 and A2 could not be attributed specifically to serum-derived versus plasma-derived IgG. Repeated weekly measurements described each hen's response trajectory but did not provide independent biological replication. These findings should therefore be considered preliminary descriptive observations. Future studies should include additional purification steps, assays that distinguish anti-idiotype reactivity from responses to other IgG determinants or residual proteins, multiple hens per condition, and measurements of IgY concentration and total yield.

Conclusion

This study demonstrated that IgG-containing fractions retaining ASFV-specific antibody reactivity could be obtained from porcine convalescent plasma and serum by ammonium sulfate precipitation. These preparations served as immunogens that elicited antibodies reactive with porcine IgG in both chicken serum and egg yolk. Weeks 6–8 after primary immunization represented a shared egg collection window at the maximum observed AGPT titer of 3 $\log_2$ (1:8). However, the absence of biological replication prevented the attribution of differences between the hens to antigen source, and anti-idiotype specificity remains to be established. These preliminary findings provide a foundation for further characterization of candidate anti-idiotype IgY and evaluation of its potential applications in ASFV research.

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Author contribution ARR: Investigation, formal analysis, data curation, writing – original draft, review, and editing; IKMP: Investigation, formal analysis, data curation, project administration, and writing – original draft; IWTW: Supervision, writing – review and editing; ONP: Conceptualization, resources, methodology, supervision, writing – original draft, review, and editing.

Declaration of generative AI in writing process During the preparation of this work, the author(s) used ChatGPT (OpenAI) and Claude (Anthropic) to check grammar, improve the clarity and language of the manuscript, and assist in revising the figure (relabeling of band annotations). After using these tools, the author(s) carefully reviewed and edited all AI-assisted content and take full responsibility for the content of the published article.

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