

Characterization and Immunogenicity of Thyroid Stimulating Hormone (TSH) Isolated from Neonatal Umbilical Cord Serum

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Abstract

Congenital hypothyroidism (CH) is a leading cause of intellectual disability in newborns. However, early detection through thyroid stimulating hormone (TSH) screening can reduce its adverse effects. In Indonesia, production of anti-TSH antibodies is very limited, which hinders screening coverage. This study aimed to isolate the TSH fraction from umbilical cord serum and evaluate its immunogenicity. Protein was isolated from serum and subjected to SDS-PAGE analysis. The 28 kDa was identified by Western blot using an anti-TSH β monoclonal antibody, with recombinant TSH β as a control, and was isolated by electroelution. Silver staining of the electroeluted confirmed 28 kDa, with ATR-FTIR and LC-HRMS profiles consistent with a glycoprotein hormone. The fraction was used to immunize rabbits, producing polyclonal antibodies with peak reactivity at a dilution of 1:200 in an indirect ELISA and optimal binding at a dilution of 1:10 in a dot blot. Western blot confirmed that the anti-TSH antibodies recognized both recombinant TSH β and the 28 kDa antigen. Histopathology of the spleen showed a significant increase in the induction group 110.25 ± 11.67 compared to the negative control $88.47 \pm 6.67 \mu\text{m}$. Immunohistochemistry revealed increased CD4⁺ expression 48.26 compared to the negative control 45.53, indicating T-helper cell activation, with specific antibody reactivity against thyrotropic cells in the rat anterior pituitary. Reactivity to neonatal serum is consistent with the results of the commercial TSH ELISA kit. These results provide an initial basis for the further development of antibodies for neonatal CH screening.

Keywords

Thyroid Stimulating Hormone, Umbilical Cord Serum, Congenital Hypothyroidism, Polyclonal Antibodies

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1. INTRODUCTION

Congenital hypothyroidism (CH) is a metabolic disorder resulting from insufficient thyroid hormone synthesis or action at birth, representing one of the most prevalent and preventable causes of permanent intellectual and neurodevelopmental disability worldwide (Aulanniam, 2023; Toktaş et al., 2023; Zhang et al., 2024). Both permanent and transient forms of CH necessitate early identification to avert long-term neurological sequelae ((Asena et al., 2020)). Current neonatal screening programs rely on the measurement of TSH levels, ideally performed between 48 and 72 hours postpartum to minimize false-positive results attributable to the physiological TSH surge and the relative immaturity of the hypothalamic-pituitary-thyroid axis at birth (Alameer et al., 2022; Jansen et al., 2022; Salim et al.,

2022).

In Indonesia, the widespread practice of discharging neonates within 24 hours of delivery renders timely heel-prick sample collection within the recommended window logistically challenging, contributing to persistently low national screening coverage (Pulungan et al., 2019, 2024). Compounding this challenge are the invasive nature of heel-prick sampling, parental refusal, restricted access to standardized dried blood spot materials, and dependence on centralized laboratory infrastructure. Most critically, domestic availability of affordable, locally manufactured anti-TSH antibodies remains severely limited, necessitating reliance on costly imported immunoreagents and thereby constraining program scalability. Collectively, these factors delay CH diagnosis and compromise the

timely initiation of hormonal replacement therapy, underscoring the urgent need for cost-effective alternative strategies compatible with routine neonatal care (Khairana et al., 2025; Lu and Shi, 2024).

Neonatal umbilical cord serum represents a promising alternative biological source for TSH isolation and antibody development. Cord blood offers several practical advantages: it can be collected non-invasively at the time of delivery without requiring postnatal recall visits; it reflects intrauterine thyroid hormone status, thereby circumventing post-birth TSH fluctuations; and it provides sufficient volume for repeated analytical procedures—attributes of particular value in resource-constrained healthcare settings (Zulkarnain et al., 2019; Zhu et al., 2024). TSH derived from cord blood retains its native glycoprotein structure and physiological epitopes, rendering it suitable as an immunogen for polyclonal antibody production. Building upon previous work from our research group on TSH monoclonal antibody development (Aulanniam, 2023; Wang et al., 2024) the present study advances these efforts by: (1) employing TSH isolated directly from neonatal umbilical cord serum as a biologically native antigen; and (2) adopting a polyclonal antibody approach that offers broader epitope coverage and greater production feasibility in low-resource settings.

The present study was therefore designed to isolate and characterize TSH from neonatal cord serum, evaluate its immunogenic potential in rabbit models, and assess the functional performance of the resulting polyclonal antibodies through multiple complementary immunoassay formats. The ultimate objective is to provide a scientific foundation for the development of locally produced immunoreagents capable of supporting accessible neonatal CH screening in Indonesia.

2. EXPERIMENTAL SECTION

2.1 Materials

Neonatal umbilical cord blood serum was used as the primary biological source for TSH isolation. The chemical used for serum protein isolation includes phosphate buffer saline (PBS; Biogear; Jakarta; Indonesia), Tween-20 (Biorad; California; USA), phenylmethylsulfonyl fluoride (PMSF; Himedia; India), dimethyl sulfoxide (DMSO; Emsure; Darmstadt; German), absolute ethanol (EtOH; supelco; Darmstadt; German), tris-HCl buffers pH 6.8 (Sigma-Aldrich; Buchs; Swiss). Protein concentration was measured using an Implen NanoPhotometerTM Pearl (Model 3242; Munich; German). Protein identification was used as the characterization includes sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Biorad; California; USA), acrylamide (Sigma-Aldrich; Buchs; Swiss), sodium dodecyl sulfate (SDS; Promega; Wisconsin; USA), ammonium persulfate (APS; Biorad; California; USA), TEMED (Merck; Darmstadt; German), and reducing sample buffer (RSB; Visual Protein; Taipei; Taiwan), Molecular Weight Marker using Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific; Vilnius; Lithuania).

To identify the TSH contained in neonatal umbilical cord serum samples, the Western blot method was performed using

immunodetection reagents comprised a mouse monoclonal anti-TSH β antibody (D-6; Santa Cruz Biotechnology; Cat. No. SC-36580), alkaline phosphatase-conjugated goat anti-mouse IgG (Invitrogen; LOT ZE4333455A), Membran polyvinyliden fluoride (PVDF; Biorad; California; USA), bovine serum albumin (BSA; Himedia; India), chromogenic substrates NBT-BCIP (Thermo Scientific; Rockford; USA), methanol (Supelco; Pennsylvania; USA). Electroelution was performed using cellophane membrane (SERVA; Heidelberg; German), sodium dihydrogen phosphate (NaH₂PO₄; Nacalai Tesque; Kyoto; Japan), sodium hydrogen phosphate (Na₂HPO₄; Nacalai Tesque; Kyoto; Japan). ATR-FTIR spectra were acquired using a Shimadzu FTIR-8400S spectrometer. Amino acid analysis was conducted with an LCMS MS system (Shimadzu LC-MS/MS 8060NX) equipped with an Intrada Amino Acid column, including chemical reagent hydrochloric acid (HCl; Smart-Lab; Tangerang; Indonesia), sodium hydroxide (NaOH; Merck; Darmstadt; German).

For SDS-PAGE silver staining, the materials used included ethanol (EtOH; Supelco; Pennsylvania; USA), glacial acetic acid (GAA; Supelco; Pennsylvania; USA), silver nitrate (AgNO₃; Serva; Heidelberg; German), sodium thiosulfate (Nacalai Tesque; Kyoto; Japan), sodium carbonate (Na₂CO₃; Nacalai Technique; Kyoto; Japan), formaldehyde (Nacala Technique; Kyoto; Japan), Coomassie Brilliant Blue (Biorad; California; USA) and distilled water.

TSH antibody polyclonal production from three male New Zealand White rabbits (*Orytolagus cuniculus*) was use complete Freund's Adjuvant (CFA; Sigma-Aldrich; Missouri; USA), incomplete Freund's Adjuvant (IFA; Sigma-Aldrich; Missouri; USA). Blood collection was performed using sterile syringes and 24G needles (OneMed; Gresik; Indonesia) and vacutainer tubes (Vaculab[®]; Mojokerto; Indonesia). Immunoglobulin G (IgG) isolation used 50% saturated ammonium sulfate (SAS; Merck, Darmstadt; German), sodium dihydrogen phosphate (NaH₂PO₄; Nacalai Tesque; Kyoto; Japan), sodium hydrogen phosphate (Na₂HPO₄; Nacalai Tesque; Kyoto; Japan), and Tris-HCl buffer (Sigma-Aldrich; Buchs; German)

For the indirect ELISA, the materials used included electroeluted TSH antigen as the coating antigen, carbonate-bicarbonate coating buffer (pH 9.6), phosphate buffer saline (PBS; Biogear; Jakarta; Indonesia), Tween 20 (Biorad; California; USA), and bovine serum albumin (BSA; Himedia; India) for coating, washing, and blocking steps. Antisera obtained from immunized rabbits were used as the primary antibody, while AP-conjugated goat anti-rabbit IgG (rockland; LOT #26283) was used as the secondary antibody. The enzymatic reaction was visualized using p-nitrophenyl phosphate (pNPP; Thermo Scientific; Darmstadt; German) substrate prepared in diethanolamine (DEA; Sigma-Aldrich; Missouri; USA) buffer and terminated with 3 M NaOH (Merck; Darmstadt; German). The assay was performed using microtiter plates and a microplate reader. For TSH concentration measurement in neonatal cord serum, a Thyroid Stimulating Hormone ELISA Kit (Cat. No. MBS590037; MyBioSource; San Diego; USA)

was used. The kit consisted of microtiter plates pre-coated with anti-TSH antibody, TSH calibration standards, sample diluent, wash buffer, HRP-conjugated monoclonal anti-TSH detection antibody, TMB substrate (Thermo Scientific; Rockford; USA), and stop solution. Absorbance was measured using a microplate reader. For the dot blot assay, nitrocellulose (NC) membranes were used as the medium for antigen immobilization. The antigen used was electroeluted TSH, while neonatal serum samples were used to evaluate antibody reactivity in a biological matrix. phosphate buffer saline (PBS; Biogear; Jakarta; Indonesia) and Tween 20 (Biorad; California; USA) were used as washing solutions, and bovine serum albumin (BSA; Himedia) was used as the blocking agent. The generated polyclonal anti-TSH antibody was used as the primary antibody, followed by an AP-conjugated secondary antibody for detection. Signal visualization was performed using chromogenic substrates NBT-BCIP (Thermo Scientific; Rockford; USA). Dot intensities were analyzed using ImageJ software.

For splenic histopathological examination, spleen tissues obtained from immunized and control rabbits were used. The tissues were fixed in 10% neutral buffered formalin (NBF) and subsequently processed using graded ethanol solutions (70%, 80%, 96%, and 100%), a clearing agent, and paraffin as the embedding medium. Tissue sections were stained with hematoxylin and eosin (H&E) and permanently mounted using Entellan® mounting medium. Tissue processing and examination were performed using a rotary microtome, water bath, microscope slides, and a light microscope. For immunohistochemical analysis using staining kit IHC (UltraTek HRP Anti-Polyvalent (DAB) staining system scytek; Logan; USA), paraffin-embedded spleen tissue sections from immunized and control rabbits and anterior pituitary gland sections from Wistar rats were used. The materials included xylene, graded ethanol, distilled water, citrate buffer (pH 6.0), 3% H₂O₂, and a protein-based blocking solution. Mouse monoclonal anti-CD4⁺ (Santa Cruz) antibody was used as the primary antibody for spleen tissue, while the generated polyclonal anti-TSH antibody was used for anterior pituitary tissue. Biotinylated anti-rabbit secondary antibody and streptavidin-HRP were used for signal detection and amplification. Immunoreactivity was visualized using DAB chromogen, followed by counterstaining with Mayer's hematoxylin. Entellan® mounting medium was used for permanent mounting, while non-immune rabbit serum was used as the negative control.

2.2 Methods

2.2.1 Serum Protein Isolation and Identification of the TSH Protein Fraction

Umbilical cord blood samples were obtained from neonates at Melati Husada Mother and Child Hospital, Malang, Indonesia, using purposive sampling. Ethical approval for human sample collection was obtained from the Health Research Ethics Committee Brawijaya University (No. 36/EC/KEPK/01/2024). More than 30 neonatal umbilical cord serum samples were successfully collected and purified. From these, nine samples

were randomly selected for the initial SDS-PAGE analysis. The number of samples analyzed in a single experiment corresponded to the capacity of one electrophoresis gel (10 wells), with one well reserved for the molecular weight marker. Samples were not pooled prior to protein isolation. Protein was isolated from serum with 0.4 mM PBST-PMSF at a ratio of 1:5. The sample was sonicated at a frequency of 400 Hz for 10 minutes. The sample was then centrifuged at 6000 rpm for 15 minute, 4°C to separate the soluble fraction. Protein was mixed with ethanol at a ratio of 1:1, incubated at 20°C overnight and the sample was then centrifuged at 10000 rpm for 15 minute 4°C. Resuspended sample in Tris-HCl buffer pH 6.8. The protein concentration of the sample was determined using a NanoPhotometer at a wavelength of 280 nm. The sample concentration was expressed in µg/mL (n=3) (Fauziah et al., 2026).

To identify the various protein bands contained in the sample, the SDS-PAGE method was performed. Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad mini-gel system according to the manufacturer's instructions. Discontinuous gels were prepared using a 12% resolving (separating) gel and a 5% stacking gel. The resolving gel was prepared by combining 1,700 µL distilled water, 2,000 µL 30% acrylamide/bis-acrylamide, 1,250 µL 1.5 M Tris-HCl (pH 8.8), 50 µL 10% SDS, 50 µL 10% ammonium persulfate (APS), and 5 µL TEMED. The stacking gel was prepared by combining 1,240 µL distilled water, 260 µL 30% acrylamide/bis-acrylamide, 500 µL 0.5 M Tris-HCl (pH 6.8), 30 µL 10% SDS, 30 µL 10% APS, and 5 µL TEMED. APS and TEMED were added last and the mixture was gently homogenized prior to casting. The resolving gel was poured first into the assembled glass plate cassette and allowed to polymerize for approximately 30 min at room temperature. The stacking gel was then overlaid and a comb was inserted to form sample wells. Once polymerized, the gel cassette was mounted in the electrophoresis tank and filled with 1× Tris-glycine-SDS running buffer. Protein samples were prepared by mixing with reducing sample buffer (RSB) containing 0.5 M Tris-HCl (pH 6.8) and denatured by heating at 94°C for 5 min. Denatured samples and a prestained molecular weight marker were loaded into the wells. Electrophoresis was conducted at a constant voltage of 150 V until the dye front reached the bottom of the resolving gel. The gel was stained with Coomassie Brilliant Blue, and the molecular weight of the target protein was determined by comparing its migration distance with the protein ladder (Panjaitan et al., 2025).

To identify the TSH contained in neonatal umbilical cord serum samples, the Western blot method was performed with the following steps. Polyvinylidene fluoride (PVDF) membrane, were pre-wetted in methanol for 30 seconds, then equilibrated in transfer buffer prior to use. Proteins separated by SDS-PAGE were transferred onto the PVDF membrane using a wet transfer system at 100 V for 90 minutes. The membrane-gel assembly was arranged in a sandwich configuration (filter pa-

per-filter paper-membrane-gel-filter paper-filter paper) and placed in the transfer chamber filled with transfer buffer to the indicated mark. Following transfer, membranes were blocked with PBST-1% BSA for 1 hour at room temperature with shaking to prevent non-specific binding. After washing three times with PBST (5 minutes each), membranes were incubated with a mouse monoclonal anti-TSH β primary antibody (D-6, Santa Cruz Biotechnology; Cat. No. SC-365801) diluted 1:500 or 1:1000 in PBST-1% BSA. Incubation was carried out for 2 hours with shaking, followed by overnight incubation at 4°C without shaking. Membranes were then washed three times with PBST (5 minutes each) and incubated for 1 hour at room temperature with an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG secondary antibody (Lot No. ZE4333455A) diluted 1:2500 in PBST. After three additional PBST washes (5 minutes each), membranes were equilibrated in AP buffer for 1 minute. Immunoreactive bands were visualized by addition of BCIP-NBT substrate (1:50 in AP buffer) in the dark. The reaction was terminated by washing with distilled water or 0.5 M EDTA solution. The presence of TSH was confirmed by the appearance of a protein band at approximately 28 kDa (Zulkarnain et al., 2019).

To identify TSH 28 kDa by silver staining procedure. First, the gel was prepared for silver staining and immersed in the fixer solution for 45 minutes. The gel was then washed with distilled water for 30 minutes, followed by sensitization with 0.02% sodium thiosulfate solution for 15 minutes. After sensitization, the gel was washed three times with distilled water for 20 second. The gel was subsequently immersed in 0.1% silver nitrate (AgNO₃) solution at 4°C for 15 minute and then rinsed with distilled water for 1 minutes. The gel was developed using a developer solution containing 3% sodium carbonate (Na₂CO₃) until the protein bands became visible. The staining reaction was then stopped by adding glacial acetic acid (Kim et al., 2016).

2.2.2 Electroelution of the 28 kDa TSH

Following SDS-PAGE separation and Western blot confirmation, bands at approximately 28 kDa were excised from 16 preparative SDS-PAGE gels. Excised gel bands were placed in cellophane tubing filled with 0.2 M phosphate buffer and immersed in an electrophoresis chamber containing 0.1 M phosphate buffer. Electroelution was performed at 30 V and 4°C for 18 hours. The electroeluted protein was precipitated with cold absolute ethanol (1:1, v/v), resuspended in Tris-HCl buffer (pH 6.8), and the protein concentration determined by NanoPhotometer prior to immunization (Fauziah et al., 2026).

2.2.3 ATR-FTIR Spectroscopic Characterization

The electroeluted TSH and neonatal umbilical cord serum were characterized using Fourier Transform Infrared (FTIR) spectroscopy over the spectral range of 400–4000 cm⁻¹ to identify functional groups and glycoprotein-associated signals. To further elucidate the biochemical composition of the purified protein, amino acid analysis was performed using Liquid

Chromatography High Resolution Mass Spectrometry (LC-HRMS) following acid hydrolysis (Madaniyah et al., 2025).

2.2.4 LC-HRMS Amino Acid Analysis

Amino acid profiling of neonatal cord serum and the electroeluted TSH fraction was performed by LC-HRMS following acid hydrolysis. Samples (0.5 g) were hydrolyzed using a Microwave Digestion System (195°C, 30 min) in 10 mL of 6 N HCl and 10 mL of 4 N NaOH, then neutralized to pH 7.0 and diluted 25-fold with 0.1 N HCl. Hydrolyzed samples were filtered through 0.22 μ m syringe filters prior to injection. Chromatographic separation was performed on an Intrada Amino Acid column (50 mm $\times$ 3 mm) operated in LPGE mode at 40°C, with an injection volume of 1 μ L. The mobile phase consisted of Phase A (0.3% formic acid in acetonitrile) and Phase B (acetonitrile:100 mM ammonium formate, 20:80, v/v), delivered at a flow rate of 0.6 mL/min under the following gradient program: 86% A/14% B held from 0 to 3.13 min; linearly ramped to 0% A/100% B at 10.13 min; held until 11.13 min; then returned to initial conditions at 11.15 min. Mass spectrometric detection was carried out in positive ionization mode under electrospray ionization (ESI) conditions using the following interface parameters: nebulizing gas flow 3 L/min, heating gas flow 15 L/min, interface temperature 400°C, desolvation temperature 650°C, DL temperature 250°C, heat block temperature 400°C, and drying gas flow 3 L/min. Amino acids were identified based on chromatographic retention times and accurate masses referenced against an authenticated external standard mixture (2.5 μ mol/mL stock; calibration range 0.1–50 nmol/mL) (Hayati et al., 2024).

2.2.5 Polyclonal Antibody Production

All animal procedures related to polyclonal antibody production were conducted in accordance with institutional guidelines and approved by the Animal Care and Use Committee (No. 179-KEP-UB-2024). Three male New Zealand White rabbits (3 months old) were immunized with the electroeluted 28 kDa TSH fraction to generate polyclonal anti-TSH antibodies; one rabbit served as an untreated control. Immunogen was emulsified 1:1 (v/v) with CFA for primary immunization and with IFA for subsequent booster immunizations, yielding a total injection volume of 500 μ L per dose. Immunizations were administered via intramuscular injection into the semimembranosus muscle at multiple sites. Blood samples (3 mL) were collected weekly from the auricular vein, commencing prior to immunization (week 0) and continuing through 10 collection time points across the immunization schedule. Collected blood was allowed to clot for 1 hour at room temperature and centrifuged at 3000 rpm for 15 minutes to obtain serum.

For IgG enrichment, serum was mixed with an equal volume of 50% saturated ammonium sulfate (SAS), vortexed, and centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet was washed once under identical conditions, resuspended in 0.2 M phosphate buffer, and dialyzed overnight at 4°C against 0.1 M phosphate buffer. The dialyzate was mixed with absolute

ethanol (1:1, v/v), refrigerated overnight, and centrifuged. The resulting pellet was air-dried and reconstituted in Tris-HCl buffer (pH 6.8) (Fauziah et al., 2026).

2.2.6 Indirect ELISA

Antibody titers in immunized rabbit antisera were determined by indirect enzyme-linked immunosorbent assay (ELISA) performed in duplicate. Microtiter wells were coated with the electroeluted TSH antigen diluted 1:1 in carbonate-bicarbonate coating buffer (pH 9.6) at 50 μ L/well, and incubated overnight at 4°C. Wells were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST; 200 μ L/well, 3 min per wash), then blocked with 1% BSA in PBS (100 μ L/well) for 2 hours at room temperature. Following three additional washes with PBST, serial dilutions of rabbit antisera (1:50 to 1:6400 in PBST) were added at 100 μ L/well and incubated for 2 hours at room temperature. Wells were washed three times with PBST as described above, after which alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:2000 in PBST) was added at 100 μ L/well and incubated for 2 hours at room temperature. After a final three-cycle wash with PBST, p-nitrophenyl phosphate (pNPP) substrate dissolved in 10% diethanolamine buffer was added at 50 μ L/well and incubated for 30 minutes at room temperature in the dark. The enzymatic reaction was terminated by the addition of 3 M NaOH (50 μ L/well), and absorbance was measured at 405 nm using a microplate reader (Aulanniam, 2023; Fauziah et al., 2026).

TSH concentrations in ten neonatal cord serum samples were independently quantified using an ultra-sensitive sandwich ELISA kit (Thyroid Stimulating Hormone ELISA Kit, Cat. No. MBS590037, MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. The kit employs a quantitative sandwich immunoassay format in which the microtiter plate wells are pre-coated with a monoclonal anti-TSH capture antibody. All reagents were equilibrated to room temperature for at least 30 minutes prior to use. A set of six TSH calibration standards was reconstituted and serially diluted to generate a standard curve ranging from 0 to 12 μ IU/mL. Serum samples were used undiluted or diluted as appropriate in the sample diluent provided. Standards and samples were added to the pre-coated wells (100 μ L/well) in duplicate and incubated for 2 hours at 37°C. Wells were washed four times with 1 $\times$ wash buffer (300 μ L/well per wash) to remove unbound material. A horseradish peroxidase (HRP)-conjugated monoclonal anti-TSH detection antibody (100 μ L/well) was then added to each well and incubated for 1 hour at 37°C. Following four additional wash cycles, tetramethylbenzidine (TMB) substrate solution (90 μ L/well) was added and the plate was incubated for 15–30 minutes at 37°C in the dark, allowing color development proportional to the amount of TSH bound. The enzymatic reaction was terminated by the addition of stop solution (50 μ L/well of 0.5 M H₂SO₄), and the optical density (OD) was measured at 450 nm using a microplate reader within 10 minutes of adding the stop solution. TSH concentrations in

unknown samples were interpolated from the four-parameter logistic (4-PL) standard curve generated from the calibration standards. The minimal detectable concentration of this assay is 0.05 μ IU/mL, with no detectable cross-reactivity with FSH, LH, or prolactin.

2.2.7 Dot Blot Assay

Nitrocellulose (NC) membranes were cut to 8 $\times$ 10 cm, equilibrated in phosphate-buffered saline (PBS), air-dried, and mounted onto a dot blot apparatus. TSH antigen was diluted serially (1:10, 1:20, 1:40, and 1:80) in sodium azide solution to a total volume of 5 μ L per well (2.5 μ L sample + 2.5 μ L sodium azide) and spotted onto the membrane. Following spotting, membranes were washed three times with PBST (10 μ L per wash, 3 minutes each), then blocked with 5% BSA for 1 hour at room temperature. After three additional PBST washes, membranes were incubated for 2 hours with the generated polyclonal antibody at serial dilutions of 1:10, 1:20, 1:40, 1:80, 1:100, and 1:120. Membranes were washed again three times with PBST, followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibody diluted in PBS. After a final round of PBST washes, 5 μ L of Western Blue substrate was applied and membranes were incubated for 30 minutes in the dark. The reaction was stopped by addition of 5 μ L distilled water. Membranes were air-dried and signal intensities were quantified using ImageJ software ($n = 2$). Dot blot analysis was additionally performed on ten neonatal serum samples at an antibody dilution of 1:20 to evaluate antibody reactivity in a complex biological matrix (Ramadani et al., 2024).

Dot blot analysis was subsequently performed on ten neonatal serum samples to evaluate antibody reactivity in a complex biological matrix. NC membranes were prepared as previously described. Each serum sample (2.5 μ L) was mixed with an equal volume of sodium azide solution (2.5 μ L) and spotted onto designated wells of the dot blot apparatus. Membranes were washed three times with PBST (10 μ L per wash, 3 minutes each), blocked with 5% BSA for 1 hour at room temperature, and washed again three times with PBST. Membranes were then incubated for 2 hours with the polyclonal antibody at a dilution of 1:20, followed by three PBST washes. AP-conjugated secondary antibody diluted in PBS was applied and membranes were incubated for 1 hour, after which membranes were washed three times with PBST. Western Blue substrate (5 μ L) was added and membranes were incubated for 30 minutes in the dark. The reaction was terminated by addition of 5 μ L distilled water. Membranes were air-dried and dot intensities were quantified using ImageJ software and compared against the standard curve established from the antigen dilution series (Nur et al., 2026; Ramadani et al., 2024).

2.2.8 Splenic Histopathology

Spleen tissues were harvested immediately following humane euthanasia of immunized and control rabbits. Tissue samples were fixed in 10% neutral buffered formalin (NBF) for a minimum of 24 hours to arrest cellular activity and prevent au-

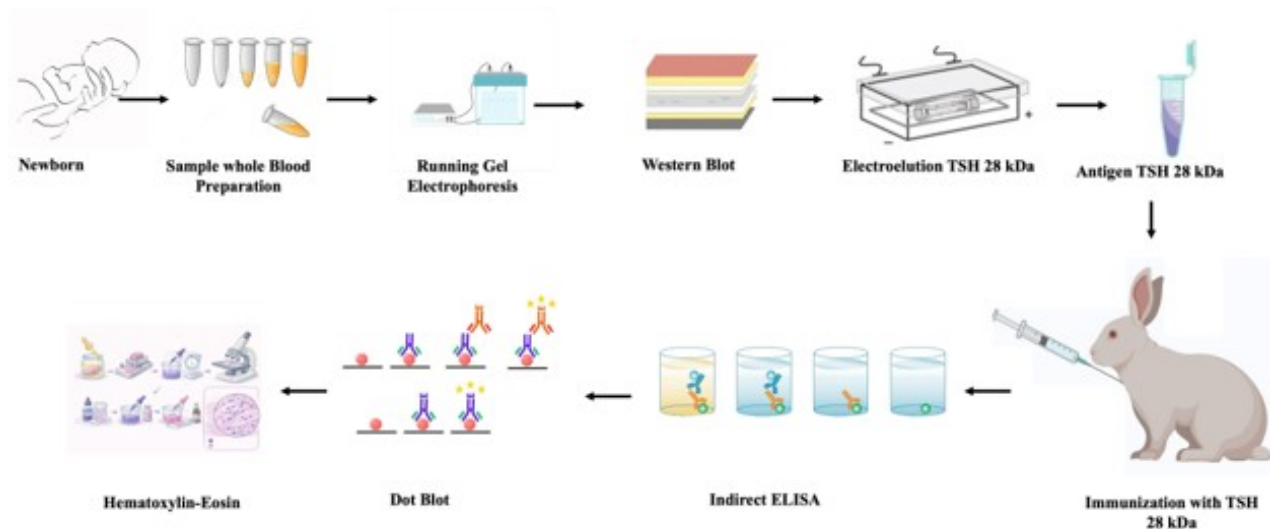


Figure 1. Systematic Workflow was Implemented for the Isolation, Identification, and Immunogenic Evaluation of TSH from Neonatal Umbilical Cord Serum

tolytic degradation. Fixed tissues were subsequently processed through a graded ethanol dehydration series (70%, 80%, 96%, and 100% absolute ethanol), followed by clearing and paraffin impregnation using standard histological processing protocols. Tissues were then embedded in paraffin blocks using appropriate molds and allowed to solidify completely. Sections of 4 μm thickness were cut using a rotary microtome and floated on a water bath at 49°C to allow section flattening and adhesion to glass slides. Slides were transferred to an incubator and dried for 2–3 hours prior to staining. Sections were stained with hematoxylin and eosin according to standard procedures, and permanently mounted using Entellan® mounting medium. Stained sections were examined by light microscopy. White pulp diameters were measured from five independent fields per animal and compared between immunized and control groups using an independent-samples t-test; statistical significance was set at $P < 0.05$ (Aulanniam, 2023).

2.2.9 Immunohistochemistry (IHC)

Immunohistochemical analysis was performed on paraffin embedded spleen tissue sections from immunized and control rabbits, as well as on anterior pituitary gland sections from normal Wistar rat models to evaluate TSH immunoreactivity. Sections were deparaffinized by sequential immersion in xylene (three changes) followed by rehydration through a graded ethanol series (100%, 90%, 80%, 70%, and 30%) and rinsing in distilled water for 5 minutes. Antigen retrieval was performed by heating sections in citrate buffer (pH 6.0) at 95°C for 20 minutes. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 for the appropriate duration, followed by a blocking step using a protein-based blocking solution for 1 hour at room temperature. Slides were then rinsed three times in distilled water (2 minutes per wash) (Nur et al., 2026; Tuska et al., 2025).

Sections were incubated with the primary antibody Mouse Monoclonal Anti CD4+ (1:1000, 20 μL per section) for spleen tissue, or the generated polyclonal anti-TSH antibody for pituitary sections for 1 hour at room temperature, followed by three washes in distilled water (2 minutes each). Biotinylated anti-rabbit secondary antibody (Anti-Rabbit Biotin Conjugate, 1:2500, 20 μL per section) was applied for 1 hour at room temperature and washed as described above. Signal amplification was achieved by incubation with streptavidin-horseradish peroxidase (SA-HRP) for 40 minutes, followed by three washes in distilled water (2 minutes each). Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) chromogen, applied until a brown signal was evident. Sections were rinsed twice in distilled water (2 minutes each) and stored overnight at 4°C. The following day, sections were counterstained with Mayer's hematoxylin (30 μL per section, 3–4 minutes), rinsed in distilled water, briefly immersed in alkaline water (pH > 7.0) for 1 minute to facilitate bluing, and rinsed again. Sections were air-dried and permanently mounted with Entellan® mounting medium. Negative control sections were processed identically, with the primary antibody replaced by non-immune rabbit serum (Nur et al., 2026; Tuska et al., 2025).

3. RESULTS AND DISCUSSION

3.1 Serum Protein Isolation and Quantification

A systematic workflow was implemented for the isolation, identification, and immunogenic evaluation of TSH from neonatal umbilical cord serum (Figure 1). The initial extraction step employed PBST-PMSF buffer to maintain physiological pH (7.4) and prevent protein denaturation, while Tween-20 facilitated disruption of lipid-protein complexes to enhance protein recovery (Quinn et al., 2022). PMSF served as a potent, irreversible serine protease inhibitor, covalently modifying the

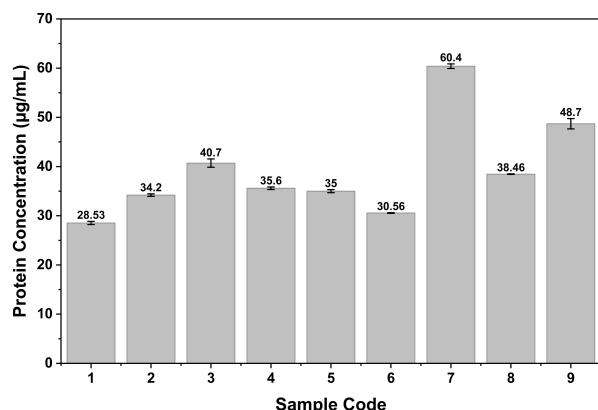


Figure 2. Protein Concentration Results From Neonatal Umbilical Cord Serum Samples Quantified by NanoPhotometer at 280 nm

active-site serine residue and preventing endogenous enzymatic degradation of the target protein (Pavani et al., 2021; James and Tawfik, 2001). Subsequent cold ethanol precipitation selectively reduced protein solubility by disrupting hydrophobic interactions and hydrogen bonding, precipitating proteins while leaving low-molecular-weight contaminants in solution (Andreadis and Moschakis, 2023; Boxi et al., 2020).

Protein concentration analysis of neonatal umbilical cord serum samples revealed inter-sample variability, with the highest concentration recorded in sample number 7 (60.4 µg/mL) (Figure 2). This variability is consistent with reported physiological differences in cord blood biochemical parameters among individual neonates and does not reflect inadequate extraction efficiency (Sefa Sag and Elemen, 2023; Vander Elst et al., 2024; Cocucci et al., 2022).

3.2 SDS-PAGE and Western Blot Confirmation of the 28 kDa TSH Protein

SDS-PAGE analysis of neonatal umbilical cord serum was protein isolated using PBST-PMSF 0.4 mM (Figure 3a) revealed multiple protein bands spanning a broad range of molecular weights, consistent with the intrinsic complexity of serum protein composition. Following electrophoresis and Coomassie Brilliant Blue staining, a prominent band migrating at approximately 28 kDa was identified as a candidate TSH-containing fraction (Grünert et al., 2011; Zabczyńska et al., 2018; Das et al., 2020).

Western blot analysis using a mouse monoclonal anti-TSHβ primary antibody confirmed the immunoreactivity of the 28 kDa protein, showing a single specific immunoreactive band with no additional nonspecific cross-reactive signals (Figure 3b). This result supports the selectivity of the anti TSHβ antibody under the applied experimental conditions. It is important to note, however, that Western blot immunoreactivity with an anti TSHβ antibody provides supportive, but not sequence-specific, confirmation of TSH identity (Ha et al., 2025; Pillai-Kastoori

et al., 2020). As peptide-level molecular identification by LC-MS/MS was not performed in the present study, the isolated fraction is consistently referred to throughout this report as the TSH 28 kDa rather than purified or confirmed TSH. The 28 kDa bands were excised from 16 preparative SDS-PAGE gels; electroelution of the pooled excised bands yielded a total volume of 2 mL with a protein concentration of 0.305 mg/mL, providing sufficient immunogen for subsequent rabbit immunization.

Figure 4. Detection of the 28 kDa TSH fraction. Following SDS-PAGE separation, the most prominent protein band observed at approximately 28 kDa was selected and subsequently subjected to Western blot analysis to further verify its immunoreactivity and confirm its identity as TSH. Western blot analysis was performed using a mouse monoclonal anti-TSHβ primary antibody (D-6, Santa Cruz Biotechnology). A single immunoreactive band was detected at approximately ±28 kDa, indicating the presence of an TSH in the purified neonatal serum. The specificity of the signal, without the presence of additional non-specific cross-reactive bands, supports the selectivity of the anti TSHβ antibody under the applied conditions. Western blot analysis consistently detected a single specific band at 28 kDa, which has previously been reported as the TSH (Aulanniam, 2023). Based on the combined SDS-PAGE and Western blot results, the 28 kDa band was confirmed as the TSH and selected for subsequent electroelution to obtain antigen for polyclonal antibody production. Based on the combined SDS-PAGE and Western blot results, the 28 kDa band was identified as a TSH containing and selected for subsequent electroelution for polyclonal antibody production.

To support the identification of the isolated 28 kDa protein, several complementary analytical approaches were employed. Following purification, SDS-PAGE analysis consistently demonstrated the presence of a protein band at approximately 28 kDa. The identity of this band was further evaluated by Western blot analysis using a mouse monoclonal anti-TSHβ primary antibody (D-6, Santa Cruz Biotechnology), which detected a distinct immunoreactive band at the same molecular weight in the purified neonatal umbilical cord serum sample. Recombinant human TSHβ protein was included as a positive control and exhibited a corresponding immunoreactive band at approximately 28 kDa, further supporting the specificity of antibody recognition. In contrast, a blank purification buffer processed without serum sample showed no detectable immunoreactive bands, indicating that the purification reagents did not contribute contaminating proteins or nonspecific immunoreactive signals (Sule et al., 2023). Purified human serum albumin was also included as a negative protein control and showed no immunoreactive band at approximately 28 kDa, with protein detection observed only within its expected molecular weight range of approximately 60–70 kDa, indicating the absence of detectable cross-reactivity with the anti-TSHβ antibody under the experimental conditions (Mustelin et al., 2025; Hutapea et al., 2023).

Furthermore, LCMS MS analysis was performed on the

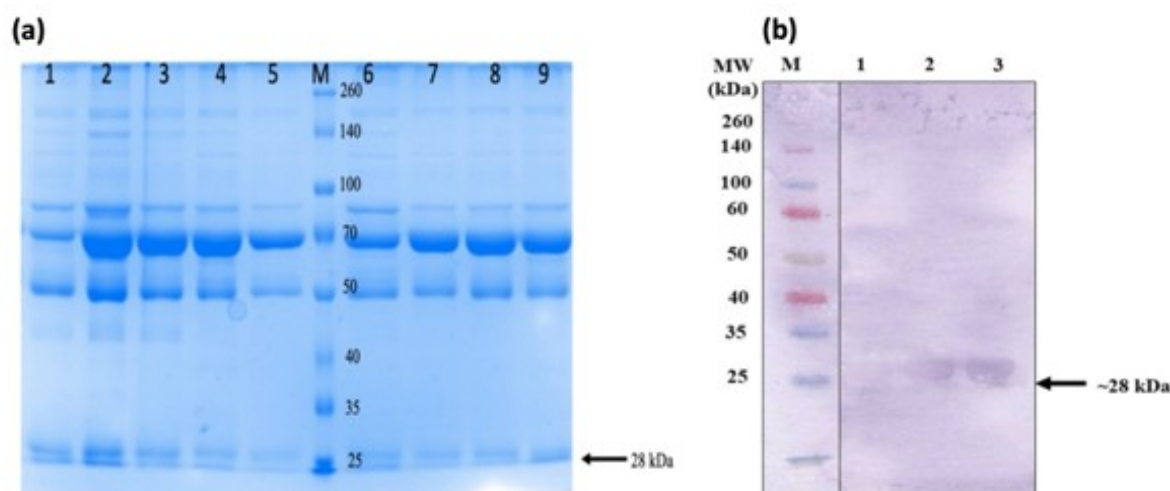


Figure 3. Biochemical and Immunochemical Detection of a Serum-Derived TSH-Enriched Antigen (28 kDa) by SDS-PAGE (a) and Western Blot Analysis (b)

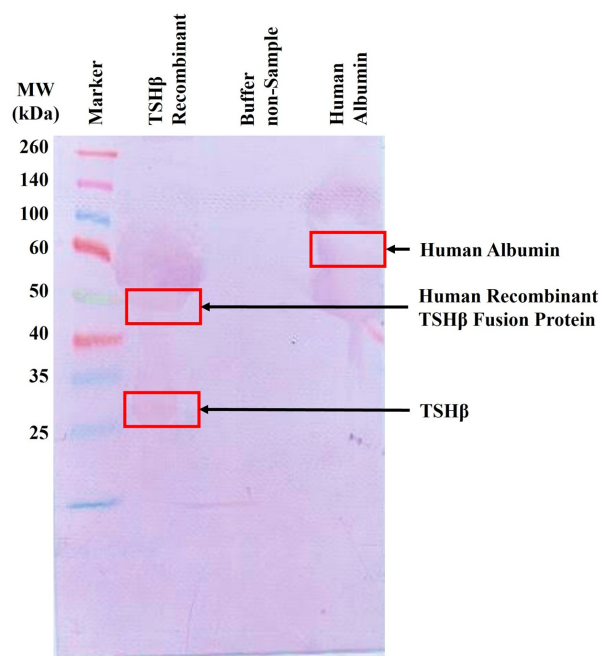


Figure 4. Western Blot Analysis Demonstrating the Immunoreactivity of TSH β Recombinant, Blank Buffer (Negative Control, Non-Sample), and Human Serum Albumin (HSA).

electroeluted protein following acid hydrolysis to determine its amino acid composition. It should be noted that this analysis was intended to characterize the amino acid constituents of the isolated protein rather than to perform peptide sequencing or determine the primary amino acid sequence. Therefore, although the LCMS MS results confirmed the presence of amino acid constituents in the electroeluted protein, they do not provide definitive sequence-based protein identification (Zhao et al., 2023). Collectively, the concordant findings obtained from SDS-PAGE, immunoreactivity with the monoclonal anti-TSH β antibody, the recombinant human TSH β positive control, the absence of immunoreactivity in the blank purification buffer and human serum albumin negative controls, and the LC-HRMS amino acid composition analysis provide supportive evidence that the isolated 28 kDa protein corresponds to TSH. Nevertheless, definitive confirmation of protein identity would require peptide sequencing by LC-MS/MS proteomics, which was beyond the scope of the present study because of limitations in instrument availability, research budget, and project timeframe. This limitation should be considered when interpreting the findings, and future studies incorporating peptide sequencing are warranted to further validate the identity of the isolated protein (Yang and Hui, 2025; Li et al., 2013).

Western blot analysis using a mouse monoclonal anti-TSH β antibody (clone D-6, Santa Cruz Biotechnology) identified a single immunoreactive protein band at approximately 28 kDa in neonatal umbilical cord serum. Based on this immunological confirmation, only the corresponding 28 kDa gel band was excised from the preparative native PAGE gel and subjected to electroelution to recover the TSH-immunoreactive protein fraction. The recovered protein concentration, determined using NanoDrop spectrophotometry, was 0.305 mg/mL. To verify that the electroeluted sample corresponded to the excised 28 kDa gel slice, the recovered protein fraction was an-

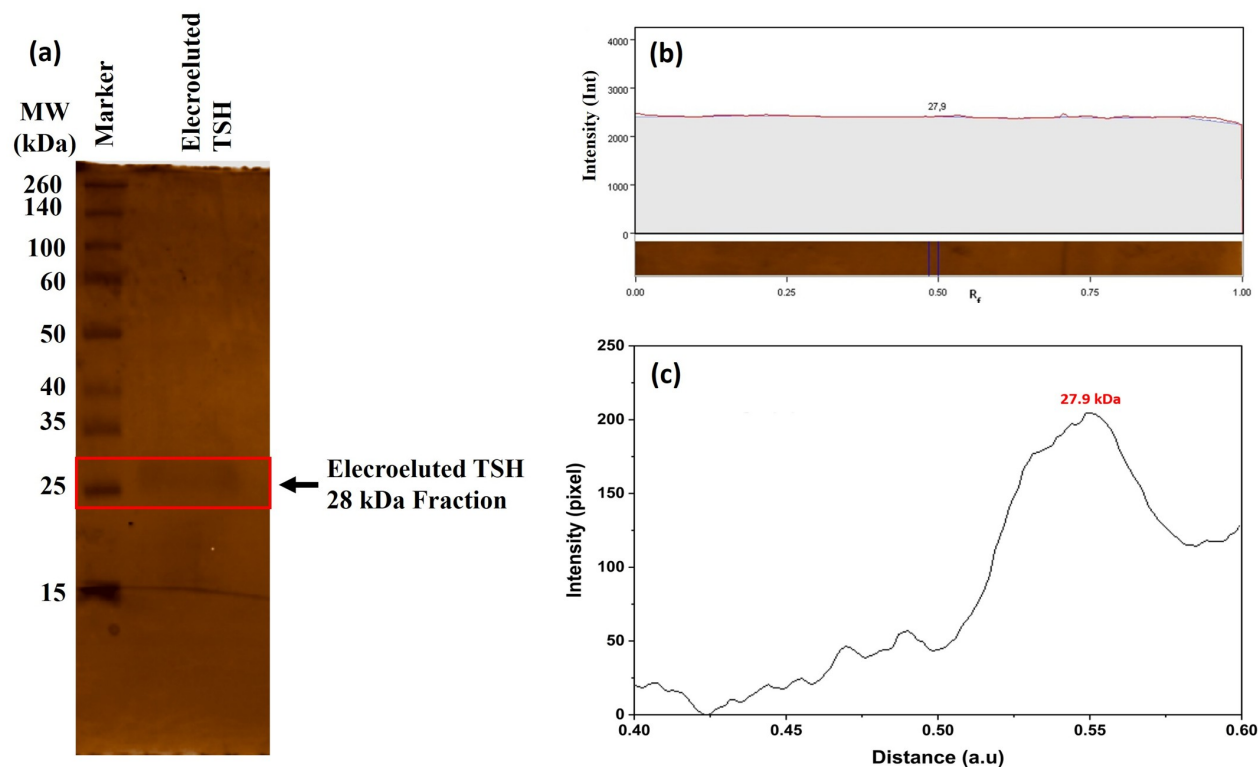


Figure 5. (a) SDS-PAGE of the 28 kDa TSH Antigen With Silver Staining, (b) Lane and Band Analysis, and (c) Densitometric Intensity Profile Generated Using ImageJ

alyzed by SDS-PAGE followed by silver staining (Figure 5a). The silver-stained gel showed a single detectable protein band with a relative front (R_f) value of 0.511, corresponding to the migration position of the previously identified 28 kDa TSH immunoreactive band (Figure 5b). Densitometric analysis using ImageJ yielded a single intensity profile (a single peak) with an intensity of 201 pixels, confirming that the analyzed sample originated from the electroelution of an excised 28 kDa protein band (Figure 5c).

It should be noted, however, that while Western blot immunoreactivity with an anti TSH β antibody provides supportive evidence, sequence-specific molecular confirmation such as LC-MS/MS peptide identification was not performed in the present study (Shukla et al., 2023; Vázquez-Iglesias et al., 2017). Therefore, the isolated fraction is consistently referred to herein as a "TSH 28 kDa" rather than purified TSH. To obtain sufficient antigen for immunization, the 28 kDa bands were excised from 16 SDS-PAGE gels and subjected to electroelution. The pooled electroeluted fraction yielded a total volume of 2 mL with a protein concentration of 0.305 mg/mL. This recovery reflects the cumulative quantity of the TSH obtained from pooling multiple gel-excised bands and served as the basis for subsequent rabbit immunization for polyclonal antibody production.

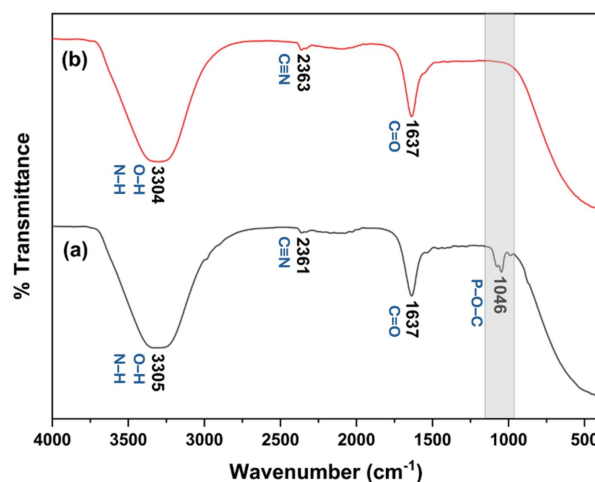


Figure 6. FTIR Spectra of Electroeluted TSH Antigen (Black) and Neonatal Serum (Red)

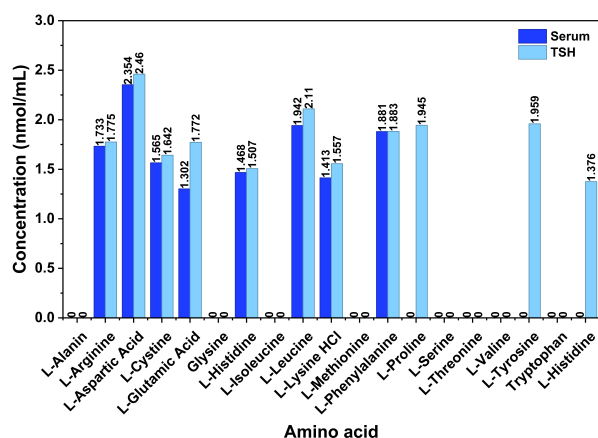


Figure 7. LC-HRMS Amino Acid Profiling of Neonatal Cord Serum and the Electroeluted TSH

3.3 Structural Characterization by ATR-FTIR

ATR-FTIR spectroscopy (Figure 6) was applied to characterize functional group composition in neonatal cord serum and the electroeluted TSH. Both spectra displayed a broad O–H/N–H stretching absorption at 3304–3305 cm^{-1} , attributable to hydrogen-bonded water molecules and protein backbone amide groups. The reduced band intensity in the electroeluted fraction is consistent with partial removal of non-target biomolecules and progressive enrichment of specific protein components. A band at 2361–2363 cm^{-1} , attributable to C $\equiv$ N stretching vibrations or dissolved CO₂, remained largely unchanged, indicating that the purification process exerted minimal influence on these molecular species (Zhang et al., 2021; Shukla et al., 2023).

A prominent absorption at 1637 cm^{-1} corresponds to the Amide I band (C=O stretching of the protein backbone), confirming the presence of protein secondary structures in both samples. Variations in band intensity following electroelution reflect changes in protein concentration and compositional complexity (Tiernan et al., 2020). Notably, a distinct absorption at 1046 cm^{-1} was observed in the electroeluted fraction, assignable to P–O–C stretching or carbohydrate-associated C–O vibrations characteristic of glycoprotein structures. Given the well-established glycoprotein nature of TSH a heterodimeric glycoprotein hormone bearing complex N-linked oligosaccharide chains this observation qualitatively supports enrichment of TSH-associated glycoprotein content in the electroeluted fraction after removal of interfering serum components. Nevertheless, this finding is considered supportive rather than definitive evidence of TSH identity (Waeytens et al., 2023).

3.4 LC-HRMS Amino Acid Profiling

LC-HRMS amino acid profiling of neonatal cord serum and the electroeluted TSH fraction revealed differences in amino acid composition, consistent with enrichment of the target protein during the electroelution process (Figure 7). Amino acids

including L-Arginine, L-Aspartic Acid, L-Cystine, L-Glutamic Acid, L-Histidine, L-Leucine, L-Lysine, and L-Phenylalanine were detected in both samples, with slightly higher concentrations in the electroeluted fraction. For example, L-Aspartic Acid increased from 2.354 to 2.46 nmol mL^{-1} , and L-Leucine increased from 1.94 to 2.11 nmol mL^{-1} following electroelution. Additionally, L-Proline, L-Tyrosine, and L-Histidine were detected exclusively in the electroeluted fraction, suggesting their origin from successfully isolated TSH rather than from the serum matrix. L-Tyrosine (1.959 nmol/mL) is of particular interest as it serves as a biosynthetic precursor for thyroxine (T4) and triiodothyronine (T3). Amino acids including Glycine, L-Isoleucine, L-Methionine, L-Serine, L-Threonine, L-Valine, and Tryptophan were below detection limits in both samples (Xu et al., 2023).

Comparison of the detected amino acid profile with the reported human TSH sequence (PDB: 7T9I) demonstrated similarities in residue composition, including arginine, aspartic acid, glutamic acid, leucine, lysine, phenylalanine, and cystine/cysteine, which are known constituents of the TSH α - and β -subunits (Faust et al., 2022). The presence of cystine is particularly informative, as TSH is a cysteine-rich glycoprotein that requires intramolecular disulfide bonds for structural stability and biological activity. The collective ATR-FTIR and LC-HRMS data support compositional enrichment of the isolated fraction with characteristics consistent with TSH, although definitive sequence-level confirmation by LC-MS/MS peptide identification was not performed and is recommended in future studies (Feldt-Rasmussen et al., 2021).

The LC-HRMS analysis performed in this study was based on acid hydrolysis followed by amino acid profiling (Nunes et al., 2024). Consequently, the obtained data describe the amino acid composition of the isolated protein rather than its primary amino acid sequence. Because different proteins may exhibit overlapping amino acid compositions, these results should be interpreted as supportive biochemical characterization rather than definitive evidence of TSH identity (Thompson and Pickard, 2024). Sequence-level confirmation would require intact protein mass analysis or peptide mapping by LC-HRMS proteomics.

The assignment of the isolated 28 kDa fraction as a TSH enriched protein was based on convergent evidence rather than a single analytical method. First, SDS-PAGE consistently showed a protein migrating at approximately 28 kDa. Second, Western blot using a monoclonal anti-TSH β antibody detected a single immunoreactive band at the corresponding molecular weight. Third, recombinant human TSH β produced the expected immunoreactive signal, whereas purification blanks and albumin negative controls showed no immunoreactivity. Furthermore, ATR-FTIR and LCHRMS amino acid profiling demonstrated biochemical characteristics consistent with glycoprotein hormones. Collectively, these findings support, but do not definitively establish, the identity of the isolated protein.

Although the isolated 28 kDa protein exhibited immunoreactivity toward a monoclonal anti-TSH β antibody and was

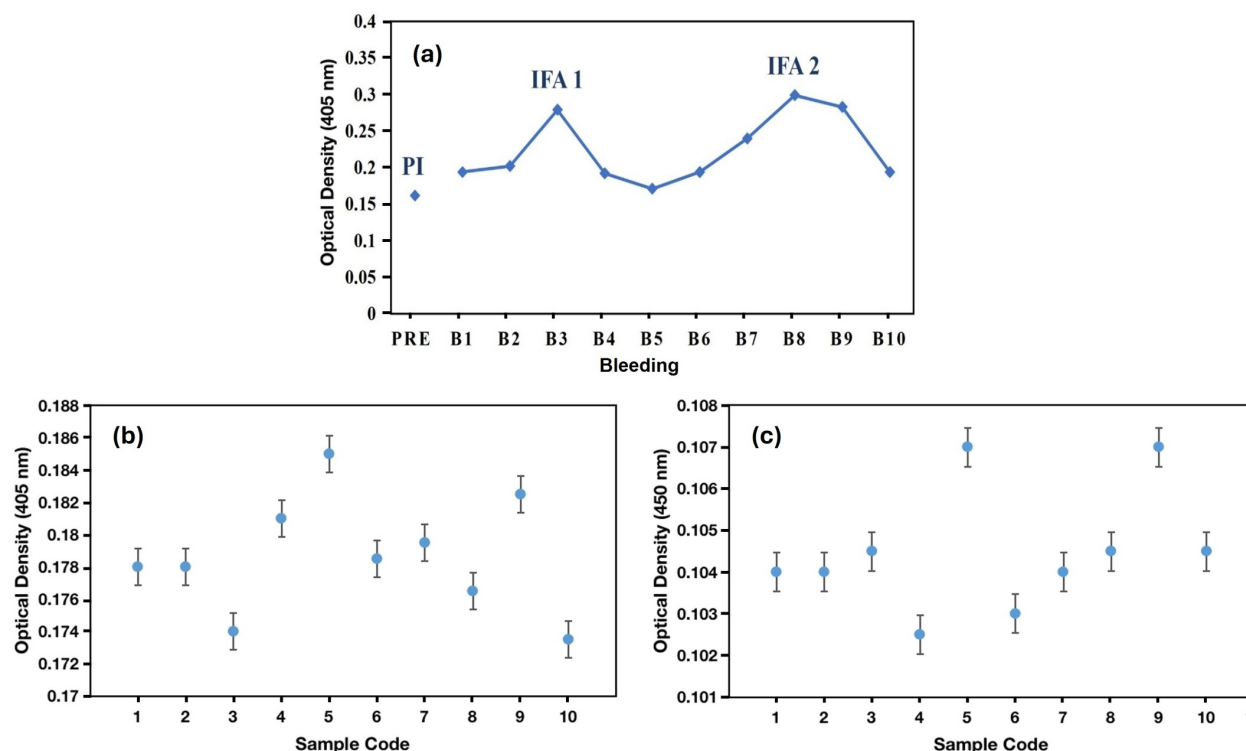


Figure 8. Indirect ELISA Antibody Titer Profile Across Immunization Weeks at Serial Serum Dilutions (a). Comparative ELISA Absorbance Responses of Ten Neonatal Serum Samples Using AP/pNPP (b), and HRP/TMB (c)

supported by SDS-PAGE, positive and negative immunological controls, ATR-FTIR glycoprotein characterization, and LCHRMS amino acid profiling, definitive protein identification was not established because peptide sequencing by LCHRMS proteomics was not performed. Consequently, the isolated protein should be interpreted as a TSH-enriched 28 kDa fraction rather than sequence-confirmed TSH. Future studies employing bottom up LCHRMS proteomic analysis will be necessary to verify protein identity and evaluate sample purity.

3.5 Indirect ELISA: Polyclonal Antibody Titer and Immunoreactivity in Neonatal Serum

Following immunization, polyclonal antibody titers were determined by indirect ELISA using the electroeluted TSH fraction as the coating antigen (Figure 8a). A progressive increase in antibody titer was observed from Week 1 to Week 5 post-second booster, with peak reactivity at a 1:200 serum dilution, yielding absorbance values substantially exceeding those of the negative control. This result confirms successful induction of TSH-specific IgG antibodies and effective enrichment of the immunoglobulin fraction following SAS precipitation (Duong et al., 2021; Yaghoobizadeh et al., 2023; Najam et al., 2024).

To evaluate preliminary diagnostic applicability, the generated polyclonal antibody was assessed in two ELISA detection formats using ten neonatal cord serum samples of unclassified clinical status (Figures 8b and 8c). In the AP/pNPP system, absorbance values ranged from 0.1735 to 0.1850 (mean:

0.1787 ± 0.0036). In the HRP/TMB system, absorbance values ranged from 0.1025 to 0.1070 (mean: 0.1045 ± 0.0015). The AP/pNPP system produced higher endpoint absorbance values than HRP/TMB, which may offer a practical advantage in resource-limited settings given the greater thermostability of alkaline phosphatase conjugates and the pNPP substrate. However, direct comparison of raw OD values between the two systems is not appropriate due to differences in enzyme kinetics and detection wavelengths (Boelen et al., 2023; Nagasaki et al., 2023).

The commercial sandwich ELISA kit (MBS590037) showed that TSH concentrations varied across the ten neonatal serum samples, with the lowest concentration detected in Sample number 10 (0.155 ± 0.126 $\mu\text{IU/mL}$) and the highest concentration detected in Sample number 4 (17.453 ± 0.484 $\mu\text{IU/mL}$) (Figure 9b). A standard curve (Figure 9a) of high linearity was obtained ($y = 0.0731x + 0.0762$; $R^2 = 0.999$). Clinically, cord blood TSH in healthy neonates generally falls within 1–17 $\mu\text{IU/mL}$; values exceeding 20 $\mu\text{IU/mL}$ are indicative of suspected CH. The majority of samples (1, 5, 6, 7, 8, 9) fell within the normal range. Samples 2, 3, and 4 approached the upper normal threshold, warranting clinical follow-up. Sample 10 exhibited an unexpectedly low TSH level, potentially indicative of central hypothyroidism, although clinical confirmation is necessary (Sadeghi et al., 2024).

A directional concordance in signal patterns was observed

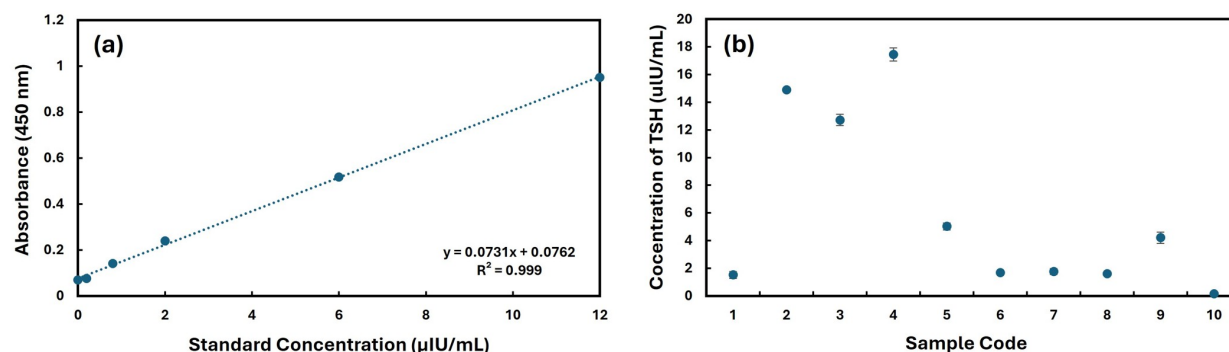


Figure 9. (a) TSH Standard Curve and (b) Serum TSH Levels Were Measured Using a TSH ELISA Kit (MyBiosource, MBS590037) Based on the Quantitative Sandwich ELISA Method

between the commercial TSH ELISA and the in-house AP/pNPP and HRP/TMB systems: samples with the highest reference TSH concentrations (2, 3, 4) consistently produced higher absorbance signals across all three formats, while Sample 10 generated consistently low signals (Borges et al., 2022). This concordance provides initial evidence that the generated polyclonal anti-TSH antibody may be capable of reflecting TSH concentration differences in a neonatal serum matrix (Calidonio and Hamad-Schifferli, 2023). However, given the narrow absorbance range of the in-house systems and the absence of a validated calibration curve, the current data should be interpreted as preliminary analytical reactivity rather than diagnostic performance (Zhao et al., 2021).

Quantitative TSH values from the commercial kit ELISA (MBS590037) served as reference data to evaluate the concordance of immunoreactive signal patterns generated by the in-house polyclonal anti-TSH antibody-based ELISA systems (AP/pNPP and HRP/TMB) across the same ten samples. A general directional concordance was observed among all three systems: Samples 2, 3, and 4, which yielded the highest TSH concentrations by gold standard, also produced relatively higher absorbance signals in both in-house detection systems, while Sample 10, with the lowest TSH level, generated consistently low signals across all three assay formats (Wauthier et al., 2022).

Samples number 5 and number 9, with moderate TSH concentrations (5.025 and 4.204 µIU/mL, respectively), also showed consistent responses in both the AP/pNPP and HRP/TMB systems. Nevertheless, the narrow absorbance range observed in both in-house systems relative to the wide TSH concentration range revealed by the gold standard reflects the inherent limitation of semi-quantitative formats when applied to complex biological matrices without validated calibration. The AP/pNPP system produced higher endpoint absorbance values than the HRP/TMB system, which may offer a practical advantage in resource-limited settings given the greater stability of alkaline phosphatase conjugates and the pNPP substrate at ambient temperature. However, direct OD comparison between the two systems is not appropriate due to differences

in enzyme reaction kinetics and detection wavelengths (Liu et al., 2023).

3.6 Dot Blot Analysis

The dot blot analysis across varying dilutions of antibody and antigen reveals a clear trend in antigen-antibody interactions. Based on the Figure 10, color intensity measurements using ImageJ, it is evident that higher concentrations of both antibody and antigen yield stronger signal intensities, indicating a higher degree of antigen-antibody complex formation on the membrane surface (Janes, 2015).

The combination of antibody shown in Figure 10a, the dilution 1:10 and antigen dilution 1:10 produced the highest intensity value of 61462.66, representing optimal binding conditions. At this concentration, sufficient antibody molecules are available to recognize and bind the epitopes present on the antigen, resulting in a strong and visible signal. Conversely, at the highest dilution (antibody 1:120 and antigen 1:80), the signal intensity dropped markedly to 760.6, indicating reduced complex formation due to the limited availability of both binding components. This overall pattern demonstrates a positive correlation between antibody/antigen concentration and signal intensity.

Interestingly, intermediate dilutions such as antibody 1:20 with antigen 1:10 (59859.9) or antibody 1:40 with antigen 1:10 (48245.8) still resulted in relatively high signal intensities. This suggests that the antibody retains effective binding capacity even at lower concentrations, up to a certain threshold. These findings provide valuable insight into the selection of optimal working dilutions for immunodetection assays. While the strongest signal was observed at the highest concentrations, dilutions such as 1:20 or 1:40 for the antibody combined with 1:10 or 1:20 antigen still offer robust detection while being more economical in reagent usage. Dot blot analysis was also performed using a 1:20 antibody dilution, selected based on the previous antigen-antibody optimization results. The application of this dilution to ten neonatal serum samples provided additional qualitative evidence that the antibody retained immunoreactivity in a serum-based format. However,

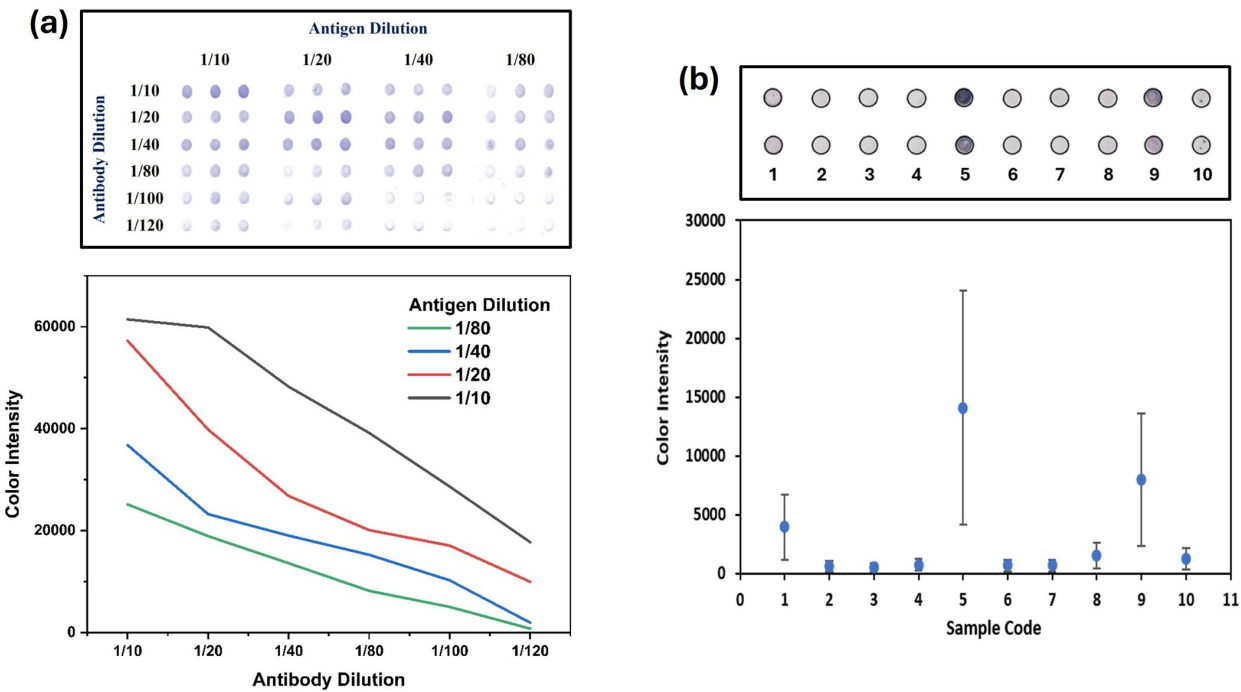


Figure 10. (a) Dot Blot Analysis of TSH Antigen and Polyclonal Anti-TSH Antibody at Varying Dilutions; Color Intensity Quantified by ImageJ. (b) Dot Blot Reactivity of Ten Neonatal Serum Samples at a 1:20 Antibody Dilution.

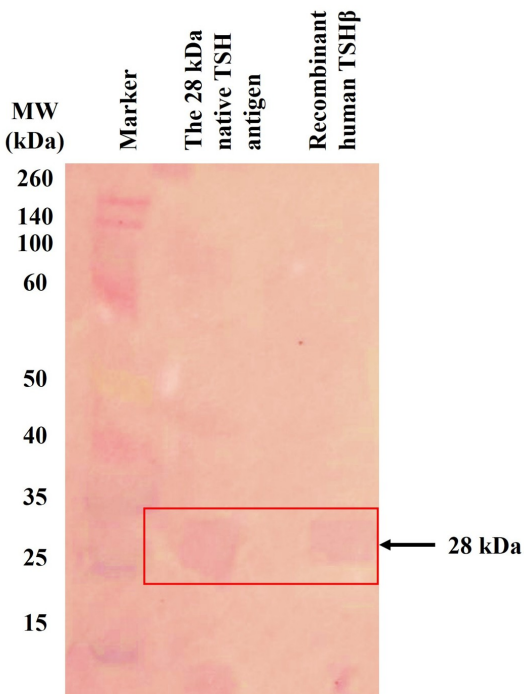


Figure 11. Western Blot Analysis Demonstrating the Immunoreactivity of the 28 kDa Native TSH Antigen and the Recombinant Human TSH β Using the Generated Polyclonal Anti-TSH Antibody.

because the samples were not clinically classified as congenital hypothyroidism-positive or -negative, the present ELISA and dot blot results should be interpreted as preliminary evidence of analytical reactivity rather than diagnostic validation.

Dot blot analysis was further performed on ten neonatal serum samples using the polyclonal anti-TSH antibody at a 1:20 dilution, which was selected based on the previous antigen-antibody optimization Figure 10b. This dilution was chosen because it still produced a detectable signal while allowing more efficient antibody use. The dot blot results showed variable signal intensities among the neonatal serum samples. Several samples produced relatively low signals, whereas stronger immunoreactive responses were observed in selected samples, particularly sample 5 and sample 9. Sample 1 also showed a detectable signal, although greater variability was observed between duplicate measurements. These findings indicate that the generated polyclonal anti-TSH antibody retained immunoreactivity when applied to neonatal serum, a more complex biological matrix than the electroeluted antigen used during optimization. However, the observed differences in dot blot intensity cannot yet be interpreted as differences in serum TSH concentration or congenital hypothyroidism status, because the clinical classification of the neonatal samples was unknown. Therefore, these results should be regarded as preliminary evidence of analytical reactivity rather than diagnostic validation (Li et al., 2025; Andreasson et al., 2015).

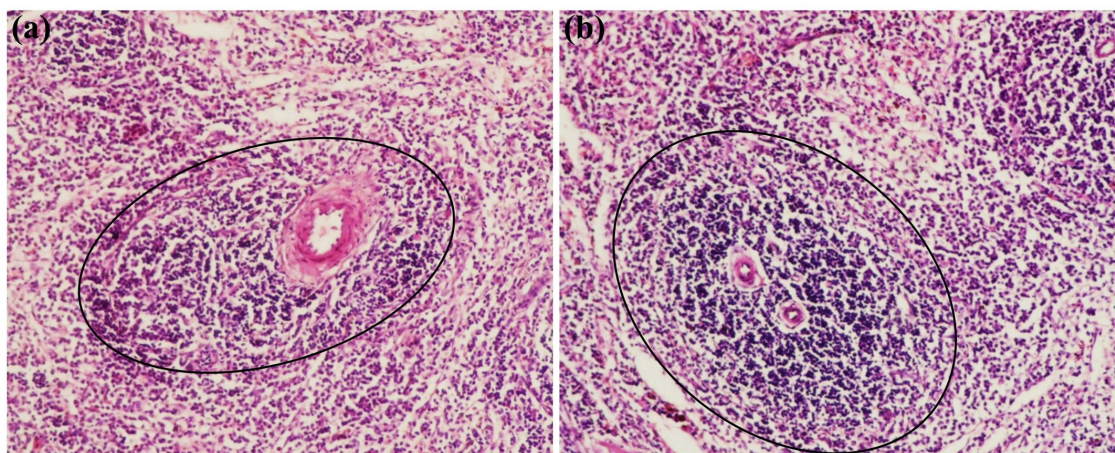


Figure 12. Histopathological Appearance of Rabbit Spleen, (a) Control Group, (b) TSH 28 kDa Induced Group.

3.7 Western Blot Analysis Confirming the Immunoreactivity of the 28 kDa Native TSH Protein Using a Polyclonal Anti-TSH Antibody

To further validate the immunoreactivity of the generated polyclonal anti-TSH antibody, Western blot analysis was performed using recombinant human TSH β and the purified 28 kDa native TSH antigen as target proteins (Figure 11). Recombinant human TSH β was included as a reference protein because of its well-characterized identity and immunological relevance, whereas the purified 28 kDa antigen represented the native serum-derived protein used as the immunogen for antibody production (Wang et al., 2025).

The generated polyclonal anti-TSH antibody successfully detected immunoreactive bands corresponding to both recombinant human TSH β and the purified 28 kDa native TSH antigen. The presence of immunoreactive bands in both samples indicates that the generated antibody retained its ability to recognize the target antigen under the denaturing conditions of SDS-PAGE and Western blotting (Mahmood and Yang, 2012). Importantly, the recognition of recombinant human TSH β demonstrates that the antibody binds to an authentic TSH protein, while the detection of the purified 28 kDa antigen confirms that the antibody also recognizes the native serum-derived antigen used during immunization.

These findings provide additional evidence supporting the successful production of the polyclonal anti-TSH antibody. The comparable immunoreactivity observed for both recombinant human TSH β and the purified 28 kDa native antigen suggests that the antigenic determinants recognized by the generated antibody are preserved in both protein preparations despite differences in their origin. This observation further supports the conclusion that the purified 28 kDa protein contains TSH-specific immunoreactive epitopes and validates its suitability as an immunogen for polyclonal antibody production. Furthermore, the ability of the generated antibody to recognize both recombinant and native forms of TSH demonstrates its potential utility as an immunological reagent for subsequent an-

alytical applications. The Western blot results complement the ELISA findings by confirming that the antibody recognizes the target protein in an independent immunodetection platform. Collectively, these results strengthen the evidence that the generated polyclonal antibody possesses specific immunoreactivity toward the 28 kDa TSH antigen and recombinant human TSH β (Datta et al., 2026).

3.8 Histopathological Features of Rabbit (*Oryctolagus cuniculus*) Spleen

Histopathological examination of rabbit spleen tissues was performed as a supportive assessment of systemic immune activation following immunization with the TSH 28 kDa. In the immunized group (Figure 12a), significant enlargement of the white pulp was observed, with a mean value of $110.25 \mu\text{m} \pm 11.67$, compared with the negative control group (Figure 12b), which showed a mean value of $88.47 \mu\text{m} \pm 6.67$ ($P < 0.05$). This enlargement was accompanied by focal sinusoidal congestion and mild inflammatory cell infiltration. These findings are consistent with lymphoid compartment activation in response to antigen exposure, reflecting the engagement of the adaptive immune system during polyclonal antibody production (Stebegg et al., 2018; Zheng et al., 2022). It should be noted, however, that splenic histopathological changes represent indirect and supportive evidence of immune activation and do not constitute direct proof of antibody specificity or immunoassay performance (Pulendran et al., 2021). The primary evidence for antibody specificity and reactivity is provided by the indirect ELISA and dot blot results described in the preceding sections. The histopathological data are therefore presented as complementary findings that support the overall immunogenic response elicited by the antigen, rather than as standalone evidence of diagnostic utility (Uhlen et al., 2016; Allam et al., 2024).

The enlargement of the white pulp may reflect lymphocyte proliferation, particularly within lymphoid follicles and germinal centers, as part of the adaptive immune response to the

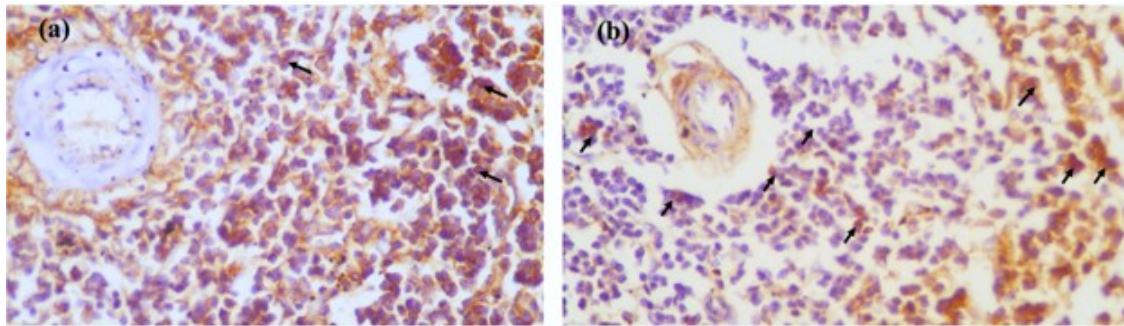


Figure 13. Immunohistochemical Expression of CD4⁺ in the Rabbit (*Oryctolagus Cuniculus*) Spleen (400× Magnification). (a) Control Group; (b) Rabbits Immunized With the Purified 28 kDa TSH Protein. Black Arrows Indicate CD4⁺ Cells (Brown Staining) in the Peripheral Zone

administered TSH antigen. The spleen is a major secondary lymphoid organ involved in antigen recognition, lymphocyte activation, and antibody production. Therefore, the increased white pulp diameter observed in the induced group supports the immunogenic potential of the 28 kDa TSH antigen and is consistent with the production of polyclonal antibodies in immunized rabbits (Cyster and Allen, 2019; Victora and Nussenzweig, 2026).

However, the vascular changes, including focal sinusoidal congestion and mild hemorrhagic areas, should be interpreted as supportive histological findings rather than primary evidence of antibody production. Overall, these results indicate that induction with the 28 kDa TSH antigen stimulated splenic lymphoid activation, as demonstrated by significant white pulp enlargement and the associated inflammatory responses. This histological evidence supports the systemic immune response generated during the production of polyclonal antibodies (Sulistyaningsih et al., 2024; Lewis et al., 2019).

3.9 Immunohistochemical Analysis of CD4 Expression in the Spleen of Rabbits (*Oryctolagus cuniculus*)

Immunohistochemical analysis of CD4⁺ expression in the spleen revealed a significant increase in the immunized group compared with the control group ($P < 0.05$). CD4⁺ expression increased in the rabbits immunized with the purified 28 kDa TSH protein Figure 13b, with a value of 48.26 ± 0.06 , compared with 45.53 ± 0.12 in the negative control group Figure 13a. This represents a 5.99% increase in CD4⁺ expression compared with the negative control group. In addition to the quantitative increase, CD4⁺ positive cells exhibited a broader distribution throughout the splenic tissue of the immunized group. These findings indicate that immunization with the purified 28 kDa TSH protein successfully stimulated the adaptive immune response, particularly through the activation of CD4⁺ T helper cells.

The increased expression of CD4⁺ observed in this study indicates that immunization with the purified 28 kDa TSH protein effectively activated CD4⁺ T helper cells in the spleen. As a major secondary lymphoid organ, the spleen is the primary site

where circulating antigens are captured by antigen-presenting cells (APCs), processed, and presented to native CD4⁺ T cells through major histocompatibility complex class II (MHC II) molecules (Miller et al., 2015; Liu et al., 2005). Antigen recognition, together with co-stimulatory signals, initiates the activation, proliferation, and differentiation of CD4⁺ T helper cells, which subsequently orchestrate the adaptive immune response through the secretion of various cytokines (?).

Activated CD4⁺ T helper cells play a pivotal role in the development of humoral immunity by providing essential signals for B-cell activation, proliferation, germinal center formation, immunoglobulin class switching, and differentiation into antibody-producing plasma cells. Therefore, the significantly higher CD4⁺ expression observed in rabbits immunized with the purified 28 kDa TSH protein reflects the successful induction of antigen-specific adaptive immunity and demonstrates that the immunization strategy effectively stimulated the cellular events required to support antibody production Eisenbarth et al. (2021); Glaros et al. (2026). These findings are consistent with the successful generation of anti-TSH polyclonal antibodies obtained in the present study, indicating that immunization with the purified 28 kDa TSH protein elicited a coordinated adaptive immune response involving T helper cell activation and the subsequent development of humoral immunity (Luckheeram et al., 2012).

3.10 Immunohistochemistry of TSH Expression in Rat Pituitary Gland

The immunohistochemistry (IHC) assay conducted on the brain tissues of rat in this study played an important role in confirming that the generated polyclonal antibodies were capable of specifically recognizing the TSH hormone within a complex biological environment (Webster et al., 2021). IHC enables the visualization of the distribution of the target antigen (TSH) directly within the tissue (in situ), thereby providing contextual information regarding the precise sites of hormone expression (Hewitt et al., 2014).

From Figure 14 micrographs demonstrate the progressive localization of TSH-positive cells within the anterior pituitary.

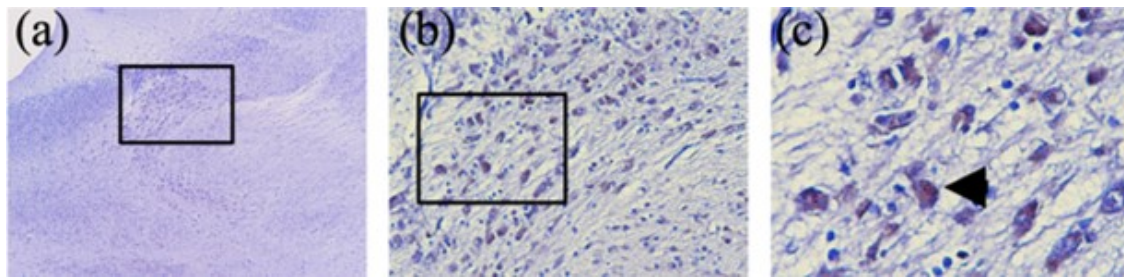


Figure 14. TSH Expression in the Rat Pituitary Gland by Immunohistochemical Staining in 100× (a); 400× (b) and 1000× (c) Magnifications

The boxed regions in the left and middle panels indicate areas of higher magnification. At high magnification (right), brownish-violet cytoplasmic staining (arrowhead) identifies thyrotroph cells for TSH, confirming antibody specificity and effective antigen recognition *in situ* (Asa et al., 2022; Fleseriu et al., 2025).

The present study provides compelling evidence that the polyclonal antibody raised against the 28 kDa antigenic fraction of neonatal serum corresponding to the thyroid stimulating hormone (TSH) subunit exhibits high immunoreactivity and cellular specificity within the rat pituitary gland. The immunohistochemical staining revealed a distinct cytoplasmic localization of TSH in cells distributed throughout the anterior pituitary, consistent with the known topography of thyrotrophs (Sánchez-Jaramillo et al., 2025; Doi et al., 2021). These immunopositive cells, characterized by an intense brownish-violet signal, confirm that the generated antibody successfully recognizes endogenous TSH within its native tissue microenvironment. The efficacy of this polyclonal antibody reflects the antigenic integrity of the 28 kDa TSH epitope and the strong immunogenic response elicited in the host animal. By targeting multiple epitopes, polyclonal antibodies often provide enhanced sensitivity over monoclonal counterparts an advantage evident in this study. The reactivity confined to pituitary thyrotrophs confirms high specificity with minimal cross-reactivity. This pattern aligns with previous anti-TSH immunolabeling reports in rodent and primate pituitaries, validating the biological fidelity of the antibody developed herein (Dutta et al., 2021; Nwokoro et al., 2023). The IHC results demonstrated cytoplasmic DAB-positive staining localized to cells morphologically consistent with thyrotrophs in the anterior pituitary, supporting the immunoreactivity of the generated polyclonal anti-TSH antibody in a tissue-based format. Negative control sections showed no detectable background staining under the applied conditions, providing procedural validity for the assay. It should be noted, however, that these IHC findings should be interpreted as preliminary and supportive evidence of antibody reactivity rather than definitive proof of high specificity or minimal cross-reactivity. Formal cross-reactivity testing against structurally related glycoprotein hormones, including LH, FSH, and hCG, was not performed in the present study and is recommended for future validation (Altay et al., 2021).

4. CONCLUSIONS

This study produced and characterized a polyclonal antibody against a putative TSH-enriched 28 kDa protein fraction isolated from neonatal umbilical cord serum. ATR-FTIR and LC-HRMS amino acid analyses revealed compositional characteristics consistent with glycoprotein hormones; however, because sequence-level confirmation by LCHRMS peptide sequencing was not performed, the isolated protein should be interpreted as a TSH-enriched fraction rather than sequence-confirmed TSH. The generated antibody exhibited consistent immunoreactivity toward the target antigen in indirect ELISA, dot blot, and Western blot analyses, recognized endogenous TSH in rat anterior pituitary thyrotrophs by immunohistochemistry, and produced measurable reactivity toward neonatal serum samples in AP/pNPP and HRP/TMB ELISA formats, with trends comparable to a commercial TSH ELISA. Histopathological findings further indicated that immunization successfully elicited an adaptive humoral immune response. Collectively, these results provide preliminary, proof-of-concept evidence that neonatal cord serum-derived TSH can serve as an affordable local antigen source for polyclonal antibody production in Indonesia. Nevertheless, the antibody should currently be regarded as a reagent with promising immunoreactivity toward TSH and a potential candidate for further development of neonatal congenital hypothyroidism (CH) screening assays, rather than a validated diagnostic reagent. Comprehensive analytical validation (sensitivity, limit of detection and quantification, precision, repeatability, and reproducibility), formal cross-reactivity assessment against structurally related glycoprotein hormones (LH, FSH, and hCG), interference testing, affinity measurement, stability studies, and clinical validation using clinically confirmed CH-positive and CH-negative neonatal samples, including evaluation of diagnostic sensitivity, specificity, receiver operating characteristic (ROC) analysis, positive predictive value, and negative predictive value, are essential before the antibody can be considered for routine application in neonatal CH screening programs.

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