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ELISA Based hTSH Assessment Using Two Sensitive and Specific Anti-hTSH Polyclonal Antibodies

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***Abstract*—**Production of specific antibody responses against hTSH is a cumbersome process due to the high identity between the hTSH and the other members of the glycoprotein hormone family (FSH, LH and HCG) and the high identity between the human hTSH and host animals for antibody production. Therefore, two polyclonal antibodies were purified against two recombinant proteins. Four possible ELISA tests were designed based on these antibodies. These ELISA tests were checked against hTSH and other glycoprotein hormones, and their sensitivity and specificity were assessed. Bioinformatics tools were used to analyze the immunological properties. After the immunogen region selection from hTSH protein, c terminal of B hTSH was selected and applied. Two recombinant genes, with these cut pieces (first: two repeats of C terminal of B hTSH, second: tetanous toxin+B hTSH C terminal), were designed and sub-cloned into the pET32a expression vector. Standard methods were used for protein expression, purification, and verification. Thereafter, immunizations of the white New Zealand rabbits were performed and the serums of them were used for antibody titration, purification and characterization. Then, four ELISA tests based on two antibodies were employed to assess the hTSH and other glycoprotein hormones. The results of these assessments were compared with standard amounts. The obtained results indicated that the desired antigens were successfully designed, sub-cloned, expressed, confirmed and used for *in vivo* immunization. The raised antibodies were capable of specific and sensitive hTSH detection, while the cross reactivity with the other members of the glycoprotein hormone family was minimum. Among the four designed tests, the test in which the antibody against first protein was used as capture antibody, and the antibody against second protein was used as detector antibody did not show any hook effect up to 50 miu/l. Both proteins have the ability to induce highly sensitive and specific antibody responses against the hTSH. One of the antibody combinations of these antibodies has the highest sensitivity and specificity in hTSH detection.

***Keywords*—**hTSH, bioinformatics, protein expression, cross reactivity.

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I. Introduction

ASSESSING thyroid stimulating hormone (hTSH) or thyrotropin levels is the first measure to diagnose hyperthyroidism and hypothyroidism. This hormone is a heterodimeric glycoprotein composed of two α and β subunits which is synthesized and secreted as a 28–30 kDa hormone by thyrotrope cells. The investigations are still ongoing to circumvent the existing obstacles ahead of developing diagnostic approaches [1], [2]. Immunoassays are among the most commonly used platforms for hTSH assessment. However, due to high homology between hTSH and other members of the glycoprotein hormone family like FSH, LH and HCG, production of anti-hTSH antibodies remains to be an outstanding issue [3]. Glycoprotein hormones share a common alpha chain and a beta chain with high homology (40%) [4]. The 30 amino acids from C terminal region of Beta chain is the most heterologous region [5]. Moreover, there is an over 94% of identity between the human and rabbit or mouse hTSH sequences which makes issue of eliciting specific antibodies even more serious [6]. The existing natural variants of hTSH immune epitopes could lead to false results from monoclonal antibodies [3], [7]. Given the aforementioned challenges, recombinant proteins designed to include hTSH regions with the least identity to the other hormones of the family could bring about amenable results [4]. The aforementioned 31 amino acids region from C terminal region of Beta chain meets the criteria to be used as an immunogen. However, this antigen would suffer from the low molecular weight problem to trigger a strong immune response. It has been shown that encompassing a protein fusion would be an appealing strategy to circumvent this problem [8], [9]. Precise design of a fusion protein would be key to have a highly immunogenic recombinant antigen [10]. In this regard, the employed fusion peptide should be capable of alleviating the induction of humoral immunity, compensating for the low molecular weight of target antigen and rising the least amount of immunity while resulting in specific immune response against the target antigen. Bioinformatics approaches are contemporary deemed as suitable tools to design such immunogenic antigens [11], [12] and even understand the biological mechanisms [13], [14] and interactions [15], [16] of proteins. Among the common fusion proteins exploited to enhance the immune responses against short antigens, the PII and P30 universal T cell epitopes from the C fragment of the tetanus toxin are reported to be highly efficient [17]. The inclusion of these epitopes would help to elicit T helper branch of the immune response and consequently would overcome the humoral tolerance issue

[18].

In the present study, we have employed bioinformatics tool to design two immunogenes capable of eliciting specific polyclonal anti-hTSH antibodies. One of the antigens included two repeats of C terminal region from Beta chain, while the second one contained the PII and P30 universal T cell epitopes along with the C terminal region from Beta chain. The obtained antibodies were used to design four possible ELISA platforms for hTSH detection. The quality of hTSH detection was assessed in the context of all ELISA platforms.

II. Methods

A. Antigen Design

The sequences of 31 amino acids region from C terminal region of Beta chain along with the sequence of PII and P30 universal T cell epitopes from the C fragment of the tetanus toxin were obtained from UniProt Database at http://uniprot.org/. The first protein was designed connecting two repeats of the C terminal region to each other (here after called as FP1). The second antigen was designed adding the PII and P30 universal T cell epitopes to N terminus of the sequence from C terminal region of Beta chain (here after called as FP2). The sequences were connected using the flexible GGGGS linker. The 3D structures of the antigens were predicted using I-TASSER and LOMETS servers at http://zhanglab.ccmb.med.umich.edu/I-TASSER and http://zhanglab.ccmb.med.umich.edu/LOMETS/ which are based on threading method. The quality of the final models was assessed by QMEAN, Prosa and RamPage servers at https://swissmodel.expasy.org/qmean/, https://prosa.services.came.sbg.ac.at/prosa.php and http://mordred.bioc.cam.ac.uk/~rapper/rampage.php, respectively. The predictions for the antigenicity were done by VaxiJen server at http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html. The surface accessibility of the antigens was predicted by the NetSurfP at http://www.cbs.dtu.dk/services/NetSurfP/. The DNA sequence for the antigens was codon optimized according to the *E. coli* codon preference using Jcat tool at http://www.jcat.de/. The gene sequences were synthesized by Generay Biotech Co, Ltd. (China).

B. Gene Cloning and Protein Expression and Purification

The cloning of the genes for the FP1 and FP2 into pET32a plasmid and the protein expression, purification and verification was done according to the method employed by Mard-soltani et al. [19]. Briefly, gene cloning for both antigens was done by the conventional method, the protein expression was done by pET32a plasmid, and the expression was induce using IPTG and the purification was done using conventional Ni-NTA affinity chromatography column and the six histidine tags added to N terminus of the fusion proteins.

C. In vivo Immunization

The immunization of the New Zealand white female rabbits (two rabbits for each test group of FP1 and FP2, a control rabbit with PBS injections a control rabbit with no injections) was performed adapting a method performed in our lab by Mard-soltani et al. Briefly, the rabbits were immunized with recombinant fusion proteins mixed with Freund's adjuvant (intra-muscularly into the large muscle of the rear legs). The complete Freund's adjuvant with 500 µg of FP1 and FP2 was used for the first injection and the incomplete Freund's adjuvant with FP1 and FP2 was used for following injections. The total of six injections was performed with 14 days intervals. The first bleeding was done after the third injection.

D. Antibody Titration

The obtained blood samples were used to prepare the serum. An indirect ELISA method was employed to assess the antibody titers against FP1 (here after called as pAB1) and FP2 (here after called as pAB2) within the obtained serum samples. The purified FP1 and FP2 antigens were diluted to 2 µg/ml in 100 mM carbonate–bicarbonate coating buffer (28.6 mM Na_2CO_3, 71.4 mM NaHCO3, pH 9.6), and then 100 µl/well of each antigen was coated on a 96-well immunoplate at 4 °C overnight. The washing steps were done by PBS-T buffer (PBS containing 0.05% Tween 20), and the blocking was done with 200 µl of 5% skim milk for 2 hr at 25 °C. The conventional ELISA was done using several dilutions of the serum samples (1:500, 1:1000, 1:2000, 1:4000, 1:8000 1:16000 and 1:32000 in PBS) as the primary antibody and 1:2000 diluted HRP-conjugated mouse anti-rabbit IgG (Thermo Scientific, MA, USA) as the secondary antibody. 50 µl of tetramethyl benzidine substrate (TMB) reagent (BD Biosciences Pharmingen, CA, USA) was added for color development, while the Color development was stopped by adding 50 µl of 2.5 M of H_2SO_4.

E. Antibody Purification

The serum was applied on the Protein A affinity chromatography column (according to the manufacturer's instructions) to purify the raised polyclonal antibodies (IgGs). The protein A binding step and the elution step were performed consecutively in 0.02M sodium phosphate (pH 7.0) and 0.1 M citric acid (pH3.0), respectively. The eluted pAB1 and pAB2 were immediately neutralized to physiological pH adding 1M Tris-HCl buffer (pH9.0). Then, the pAB1 and pAB2 were concentrated to 1 mg/ml using 50 kDa-molecular mass cutoff concentrators.

F. Biotinylation of Antibodies

The concentrated pAB1 and pAB2 antibodies were dialyzed in B Reaction Buffer (100 mM carbonate, pH 8.4). Then, 10 mg of biotin was dissolved in 1 ml anhydrous DMSO immediately before use. The biotin solution was mixed with the antibody at the ratio of 80 µg per mg of antibody. Then, the mixture was incubated at room temperature for 4 hours with rotation. Ultimately the unreacted biotin was removed by dialysis in Storage Buffer (10 mM Tris, 150 mM NaCl, 0.1% NaN_3, pH 8.2).

G.ELISA Test Design and Assessment of Their Sensitivity and Specificity

The obtained pAB1 and pAB2 were used as capture and detection agents to design four possible sandwich ELISA platforms. The first platform used pAb1 as both capture and detection (EP1), the second platform used pAb2 as both capture and detection (EP2), the third platform used pAb1 as capture and pAb2 as detection (EP3) and the fourth platform used pAb2 as capture and pAB1 as detection (EP4) antibody. Conventional sandwich ELISA was performed using 5 µg/well of each capture (without biotinylation) and detection antibodies (biotinylated). The blocking was done with 200 µl of 5% skim milk for 2 hr at 25 °C. The washing steps were done by PBST. HRP conjugated streptavidin was added to each well for color development by TMB. Color development was stopped by adding 50 µl of 2.5 M of H_2SO_4. To determine the specificity of each ELISA platform, 5 µIU/ml of hTSH, LH, FHS and HCG was added to each well (in duplicate) to assess the cross reactivity according to the following formula:

Cross Reaction= (OD of the Glycoprotein Hormone - OD of the PBS) / (OD of the hTSH – OD of the PBS) x 100

The sensitivity of the ELISA platforms was assessed by performing similar sandwich ELISA tests (in the context of 4 possible ELISA platforms) using 0.0, 3.125, 6.25, 12.5, 25, and 50 µIU/ml of hTSH, PBS and the serum containing hTSH to each designed ELISA platform. The sensitivity of platforms was measured using the ODs at 450 nm.

III. Results

A. Antigen Design

The sequences of both FP1 and FP2 were successfully designed and modelled using fold recognition approach. The predicted 3D structures were of high quality according to the QMEAN and Prosa Z-score and the results of Ramachandran plots (Fig. 1). The antigenicity scores of the FP1 and FP2 were both 0.77 which indicates they are strong antigens. Moreover, it has been shown that 31% and 32% of FP1 and FP2 amino acids are not surface accessible which indicates their high surface accessibility. The gene sequence of the FP1 and FP2 antigens were successfully reverse transcribed according to the *E. coli* codon preference.

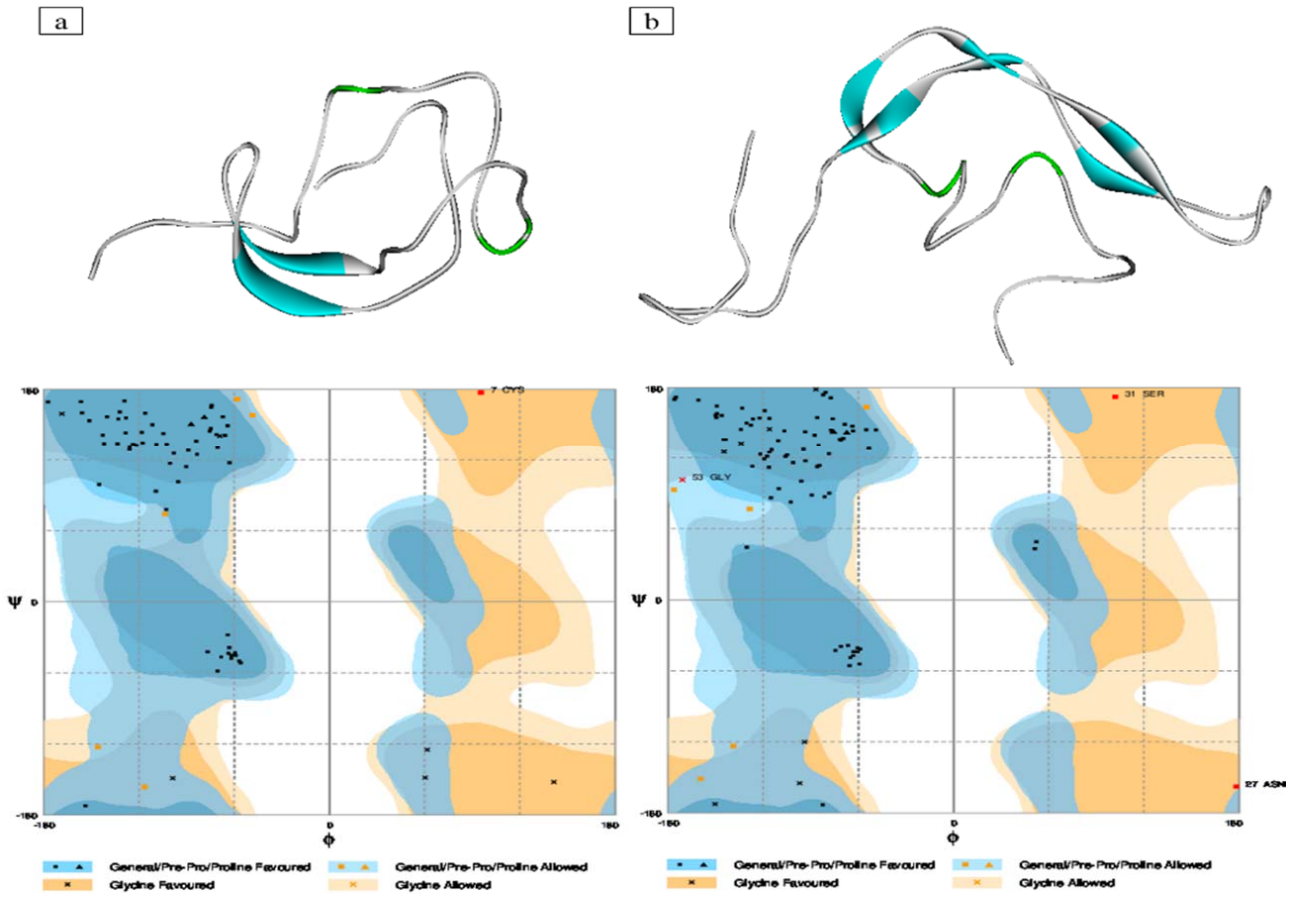

Fig. 1 The 3D structure of FP1 and FP2 and their quality assessment. (a) the FP1 3D structure (upper part) and its Ramachandran plot (lower part) and similarly (b) FP2 3D structure and its Ramachandran plot

B. Gene Cloning and Protein Expression

The synthesized genes were successfully sub-cloned into pET32a plasmid. The protein expression resulted in the expression of the FP2 antigen (25 kDa), while the FP1 protein could not be expressed. Therefore, the FP1 antigen was purchased from Pepmic Company (China), while FP2 antigen was overexpressed at the rate of 850 mg/ml (for 100 ml of induced *E. coli* culture). The FP2 antigen was purified by the NiNTA affinity chromatography column (Fig. 2).

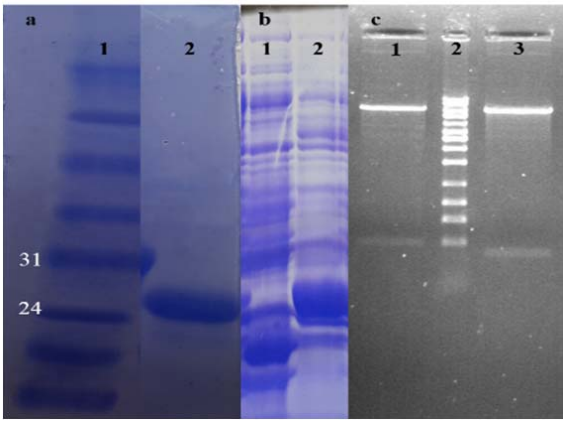

Fig. 2 Gene cloning and protein expression and purification results: (a) the FP2 purification results (lane 1 is the protein ladder lane 2 is the purified protein) which indicates over 90 % purification. (b) protein expression results for FP2 (lane 1 is before induction and lane 2 is after induction) which indicates successful protein expression. (c) gene cloning for the genes of FP1 and FP2 (lane 1 cloning confirmation by double endonuclease digestion on gene of FP2, lane 2 is the ladder and lane 3 is cloning confirmation by double endonuclease digestion on gene of FP1) which indicates their successful cloning

C. Rabbit Immunization Results

The immunization results indicated that both FP1 and FP2 antigens were capable of eliciting strong humoral responses. The antibody titration diagram for both pAB1and pAB2 is depicted in Fig. 3.

D. Antibody Purification and Biotinylation

The antibody purification step resulted in highly purified antibodies for both pAB1 and pAB2. The biotinylation of the purified antibodies resulted in covalently biotinylated pAB1 and pAB2.

E. Specificity and Sensitivity Assessment for Sandwich ELISAs

Our assessments for specificity of the design ELISA platforms indicted that the EP3 ELISA has the highest specificity to detect hTSH. The detailed results of cross reactivity are listed in Table I.

The sensitivity assessment for each ELISA platform has interestingly indicated that the EP3 ELISA platform have the highest sensitivity. This ELISA platform shows a linear increase of recorded ODs in response to increased THS concentration. Moreover, it's the only test without any hook effect (Table II).

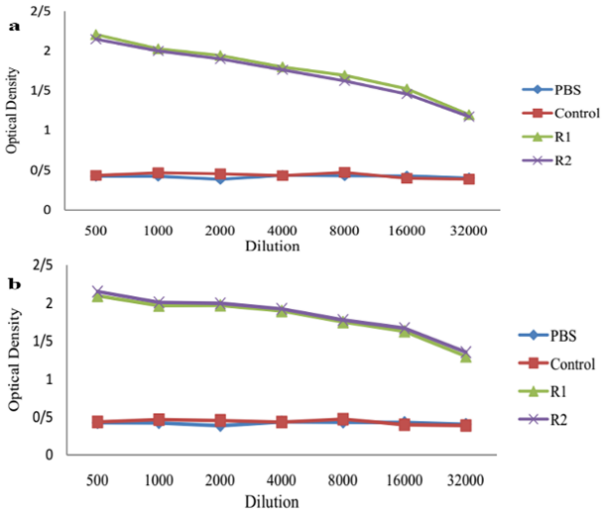

Fig. 3 Titration curve for both pAB1and pAB2. (a) the diagram for pAB1 and (b) the diagram for pAB2. Both antibodies could detect hTSH at serum delusions as high as 1/3200. Control stands for the control rabbit with no injections. PBS stands for the rabbit injected with PBS. R1 and R2 stands two rabbits of each test groups

TABLE I
THE SENSITIVITY RESULTS FOR SANDWICH ELISA. CR STANDS FOR THE CROSS REACTIVITY PERCENTAGE

	LH	LH	FSH	FSH	HCG	HCG	hTSH	PBS
EP1	0.351	0.301	0.388	0.367	0.349	0.326	1.389	0.419
CR%	~0	~0	~0	~0	~0	~0	100%	-
EP2	0.235	0.303	0.305	0.379	0.279	0.304	1.112	0.454
CR	~0	~0	~0	~0	~0	~0	100%	-
EP3	0.284	0.266	0.308	0.291	0.295	0.278	1.216	0.293
CR	~0	~0	~0	~0	~0	~0	100%	-
EP4	0.408	0.407	0.424	0.416	0.309	0.386	1.341	0.423
CR	~0	~0	~0	~0	~0	~0	100%	-

TABLE II
THE SENSITIVITY RESULTS FOR SANDWICH ELISA (OPTICAL ABSORBANCE AT 450 NM). THE EP3 SHOWS LINEAR INCREASE IN ODS WITH NO HOOK EFFECT

	0	3.125	6.25	12.5	25	50	PBS	serum
EP1	0.34	0.897	0.998	1.037	0.995	0.997	0.368	0.771
EP2	0.373	0.810	0.885	0.961	0.908	0.904	0.389	0.837
EP3	0.360	0.764	0.819	0.836	0.902	1.059	0.35	0.882
EP4	0.392	0.859	1.027	1.064	1.074	1.029	0.342	0.916

IV. DISCUSSION

hTSH is among the hormones frequently assessed to obtain data about the cases with hyperthyroidism and hypothyroidism [20]. The fluctuations in hTSH concentrations are most commonly assessed by ELISA tests. However, high similarities between glycoprotein hormones and the alternative sequences or posttranslational modifications like glycosylation patterns are the challenges ahead of getting specific antibodies against hTSH [21]. In the present study, we carefully have designed two antigens capable of eliciting specific antibodies for hTSH detection. Then, the combination of these antibodies

was used to develop ELISA platforms capable of specific and sensitive hTSH detection. Our results have indicated that both FP1 and FP2 could trigger proper immunizations. In the case of FP1, two repeats of 31 amino acids region from C terminal region of Beta chain have prompt elicitation of specific anti-hTSH antibodies. This seems only rational due to exclusion of any unrelated sequences within this antigen. In the case of FP2, inclusion of PII and P30 universal T cell epitopes from the C fragment of the tetanus toxin have made the FP2 to get a better 3D folding (data not shown). Moreover, the inclusion of these epitopes have been shown to produce the least amount of unspecific antibodies [22]. Although the production of minimum non-specific antibody seems to be inevitable, owing to natural low concentrations of glycoprotein hormones, this problem could be tolerated.

The polyclonal nature of attained antibodies helps the hTSH detection to be more sensitive. Low sensitivity is the problem associated with the ELISA detection kits based on monoclonal antibodies [4], [23]. Due to existence of various antibodies against different epitopes of hTSH, different natural variants of the antigen could be detected using our antibodies. Since the ELISA tests would most likely be employed for public screening, the sensitivity issue becomes even more important. Moreover, it have been shown that competition between two antibodies of a sandwich ELISA to bind to a common epitope could result in false negative or unreal lower concentrations [24], [25]. This complication could be circumvented using polyclonal antibodies. Our results revealed that the EP3 ELISA test based on pAB1 as capture antibody and pAB2 as detection antibody have the highest specificity and sensitivity. These results could be rooted in the high specificity of pAB1 and the good sensitivity of pAB2 antibody.

V. Conclusion

In conclusion, it should be noted that the approach of designing minimal fusion antigens could be used to overcome the problems associated with challenging antigens like hTSH. In silico methods could be harnessed to design these antigens. The raised antibodies following *in vivo* immunization with these antigens could be deemed as suitable candidates to develop specific and sensitive ELISA tests.

References

[1] S. Rabbiosi, M. C. Vigone, F. Cortinovis, I. Zamproni, L. Fugazzola, L. Persani, C. Corbetta, G. Chiumello, G. Weber, "Congenital hypothyroidism with eutopic thyroid gland: analysis of clinical and biochemical features at diagnosis and after re-evaluation," *The Journal of Clinical Endocrinology & Metabolism*, vol. 98, pp. 1395-402, 2013.

[2] R. A. McPherson, M. R. Pincus. Henry's Clinical Diagnosis and Management by Laboratory Methods E-Book: Elsevier Health Sciences; 2017.

[3] L. De Groot, G. Chrousos, K. Dungan, K. Feingold, A. Grossman, J. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, "Assay of Thyroid Hormones and Related Substances--Endotext," vol., pp., 2000.

[4] C. Ronin, M. Papandreou, S. Sergi, C. Labbe-Jullie, G. Medri, T. Hoffmann, H. Darbon, "Glycosylation-dependent epitope mapping of human TSH (hTSH) isoforms," *International Journal of Radiation Applications and Instrumentation Part B Nuclear Medicine and Biology*, vol. 17, pp. 651-6, 1990.

[5] J.-M. Bidart, F. Troalen, P. Ghillani, A. Puisieux, C. Bohuon, D. Bellet, "Immunochemical mapping of antigenic regions on the human thyrotropin beta-subunit by antipeptide antibodies," *Journal of Biological Chemistry*, vol. 266, pp. 19238-44, 1991.

[6] C. Cahoreau, D. Klett, Y. Combarnous, "Structure–function relationships of glycoprotein hormones and their subunits' ancestors," *Frontiers in endocrinology*, vol. 6, pp., 2015.

[7] C. A. Spencer, M. Takeuchi, M. Kazarosyan, "Current status and performance goals for serum thyrotropin (TSH) assays," *Clinical chemistry*, vol. 42, pp. 140-5, 1996.

[8] A. K. Abbas, A. H. Lichtman, S. Pillai. Cellular and Molecular Immunology E-Book: Elsevier Health Sciences; 2014.

[9] M. Tiwary, N. Agarwal, A. Dinda, S. C. Yadav, "Overexpression and purification of folded domain of prostate cancer related proteins MSMB and PSA," *Molecular biology reports*, vol. 43, pp. 349-58, 2016.

[10] H. Schellekens, N. Casadevall, "Immunogenicity of recombinant human proteins: causes and consequences," *Journal of neurology*, vol. 251, pp. ii4-ii9, 2004.

[11] A. Jahangiri, I. Rasooli, P. Owlia, A. A. I. Fooladi, J. Salimian, "In silico design of an immunogen against Acinetobacter baumannii based on a novel model for native structure of outer membrane protein A," *Microbial Pathogenesis*, vol., pp., 2017.

[12] E. Kazemi Moghaddam, P. Owlia, A. Jahangiri, I. Rasooli, M. R. Rahbar, M. Aghajani, "Conserved OprF as a Selective Immunogen Against Pseudomonas aeruginosa," *Iranian Journal of Pathology*, vol. 12, pp. 165-70, 2017.

[13] S. Khalili, A. Jahangiri, Z. S. Hashemi, B. Khalesi, M. Mardsoltani, J. Amani, "Structural pierce into molecular mechanism underlying Clostridium perfringens Epsilon toxin function," *Toxicon*, vol., pp., 2017.

[14] S. Khalili, M. Rasaee, T. Bamdad, "3D structure of DKK1 indicates its involvement in both canonical and non-canonical Wnt pathways," *Molecular Biology*, vol. 51, pp. 155-66, 2017.

[15] S. Khalili, A. Zakeri, Z. S. Hashemi, M. Masoumikarimi, M. R. R. Manesh, N. Shariatifar, M. J. Sani, "Structural analyses of the interactions between the thyme active ingredients and human serum albumin," *Turkish Journal of Biochemistry*, vol. 42, pp. 459-67, 2017.

[16] S. Khalili, H. Mohammadpour, M. S. BAROUGH, P. Kokhaei, "ILP-2 modeling and virtual screening of an FDA-approved library: a possible anticancer therapy," *Turkish journal of medical sciences*, vol. 46, pp. 1135-43, 2016.

[17] R. S. Chambers, S. A. Johnston, "High-level generation of polyclonal antibodies by genetic immunization," *Nature biotechnology*, vol. 21, pp. 1088-92, 2003.

[18] C. A. King, M. B. Spellerberg, D. Zhu, J. Rice, S. S. Sahota, A. R. Thompsett, T. J. Hamblin, J. Radl, F. K. Stevenson, "DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma," *Nat Med*, vol. 4, pp. 1281-6, 1998.

[19] M. Mard-Soltani, M. J. Rasaee, A. Sheikhi, M. Hedayati, "Eliciting an antibody response against a recombinant TSH containing fusion protein," *Journal of Immunoassay and Immunochemistry*, vol. 38, pp. 257-70, 2017.

[20] C. A. Spencer, M. Takeuchi, M. Kazarosyan, "Current status and performance goals for serum thyrotropin (TSH) assays," *Clinical chemistry*, vol. 42, pp. 140-5, 1996.

[21] N. Azzam, R. Bar-Shalom, Z. Kraiem, F. Fares, "Human thyrotropin (TSH) variants designed by site-directed mutagenesis block TSH activity *in vitro* and *in vivo*," *Endocrinology*, vol. 146, pp. 2845-50, 2005.

[22] R. S. Chambers, S. A. Johnston, "High-level generation of polyclonal antibodies by genetic immunization," *Nat Biotechnol*, vol. 21, pp. 1088-92, 2003.

[23] J. C. Drees, J. A. Stone, C. R. Reamer, V. E. Arboleda, K. Huang, J. Hrynkow, D. N. Greene, M. S. Petrie, C. Hoke, T. S. Lorey, "Falsely undetectable TSH in a cohort of South Asian euthyroid patients," *The Journal of Clinical Endocrinology & Metabolism*, vol. 99, pp. 1171-9, 2014.

[24] S. Maxeiner, A. Glassmann, H.-T. Kao, K. Schilling, "The molecular basis of the specificity and cross-reactivity of the NeuN epitope of the neuron-specific splicing regulator, Rbfox3," *Histochemistry and cell biology*, vol. 141, pp. 43-55, 2014.

[25] N. Sinha, S. Mohan, C. A. Lipschultz, S. J. Smith-Gill, "Differences in electrostatic properties at antibody–antigen binding sites: implications for specificity and cross-reactivity," *Biophysical journal*, vol. 83, pp. 2946-68, 2002.