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Original Research Article

Potential of B-Cell Epitopes Protein Ag85 Complex *Mycobacterium tuberculosis* as Serodiagnostic Antigen of Tuberculosis by In Silico Study

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Abstract

Background: The high case of tuberculosis which isn't followed by good detection becomes an urgency for the diagnostic developments. One of them with immunodiagnostic principle uses B-cell Ag85 complex epitope. The design of the diagnostic epitope was performed by mapping the B cell epitope used in silico studies.
Objective: The purpose of this research is to analyze antigenicity, physicochemical which affect immunogenicity, and homology of B-cell Ag85 complex epitope with the strain which circulates in Indonesia.
Methods: The samples used were taken from the NCBI protein bank with access numbers P9WQP3 for Ag85A, P9WQP1 for Ag85B, and P9WQN9 for Ag85C. The sequences were analyzed using IEDB (Bepipred) software as the epitope prediction, VaxiJen as antigenicity prediction, ProtParam as physicochemical properties prediction, and BLASTP NCBI as sequence alignment.
Results: Twenty seven epitopes were antigenic with 0.4297 to 2.6007 scores and the molecular weight was from 619.59 Da to 3145.36 Da. This research also obtained eleven stable and hydrophilic epitopes. The alignment of 11 candidate epitopes with the strain which circulates in Indonesia, had a similarity percentage of 85.71%-100% and 3 epitopes had a more significant score.
Conclusion: Three epitopes of Ag85 complex; Ag85A (212-235), Ag85B (209-237), and Ag85C (283-310), were universal antigens and can be developed into diagnostic antigens in Indonesia.

Keywords: *B-cell epitope; Ag85 complex; diagnostic antigen; in silico.*
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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a disease that mainly affects the lungs (pulmonary TB) and various organs (extrapulmonary TB). It is a world health problem and an infectious disease ranking among the top ten causes of death.[1] Indonesia is the 3rd country with the highest incidence of TB globally, contributing 8.4% of the cases. Furthermore, in 2020, only 351,936 (41.7%) incidences of TB were reported to the Ministry of Health, and 58.3% of other issues were undetected.[2] The use of *M. tuberculosis* antigen B cell epitope is one of the diagnostic developments performed with the immunodiagnostic principle.

The antigen 85 complex is the most commonly secreted protein from *M. tuberculosis* in liquid culture. It plays an essential role in the virulence of TB.[3] This immunodominant protein triggers the body's humoral immune response, which is characterized by the formation of protective antibodies and induce $TCD8^+$ Th1 $CD4^+$ for the production of cytokines such as IFN-γ and IL-2.[4] Based on antibody detection; commercial serodiagnosis has inconsistent sensitivity and specificity, namely 0-100% and 31-100%.[5]

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Table 1. Prediction and antigenicity test of B-cell linear epitope of Ag85 Complex

Name	Start	End	Peptide	Length	Antigenicity (T =0.4)	
					Score	Interpretation
Ag85A	35	49	VGGTATAGAFSRPGL	15	0.6074	**Antigen**
	54	63	LQVPSPSMGR	10	1.0688	**Antigen**
	70	76	QSGGANS	7	2.6007	**Antigen**
	86	99	RAQDDFSGWDINTP	14	0.958	**Antigen**
	101	104	FEWY	4	-	Non-Antigen
	106	106	Q	1	-	Non-Antigen
	115	123	VGGQSSFYS	9	0.5325	**Antigen**
	126	135	YQPACGKAGC	10	1.9389	**Antigen**
	137	137	T	1	-	Non-Antigen
	157	163	HVKPTGS	7	0.8572	**Antigen**
	196	203	LDPSQAMG	8	0.269	Non-Antigen
	212	235	GDAGGYKASDMWGPKEDPAWQRND	24	0.7449	**Antigen**
	256	267	NGKPSDLGGNNL	12	1.6823	**Antigen**
	286	308	AYNAGGGHNGVFDFPDSGTHSWE	23	0.8642	**Antigen**
	316	316	A	1	-	Non-Antigen
	319	320	PD	2	-	Non-Antigen
	322	322	Q	1	-	Non-Antigen
Ag85B	33	46	GGAATAGAFSRPGL	14	0.5111	**Antigen**
	51	60	LQVPSPSMGR	10	1.0688	**Antigen**
	67	74	QSGGNNSP	8	1.6042	**Antigen**
	83	96	RAQDDYNGWDINTP	14	1.2099	**Antigen**
	113	132	GGQSSFYSDWYSPACGKAGC	20	1.0201	**Antigen**
	134	134	T	1	-	Non-Antigen
	153	162	RAVKPTGSAA	10	0.4297	**Antigen**
	194	201	DPSQGMGP	8	0.7686	**Antigen**
	209	237	GDAGGYKAADMWGPSSDPAWERNDPTQQI	29	0.6896	**Antigen**
	253	264	NGTPNELGGANI	12	0.4363	**Antigen**
	283	289	AYNAAGG	7	1.5285	**Antigen**
	295	305	NFPPNGTHSWE	11	0.3702	Non-Antigen
	316	317	GD	2	-	Non-Antigen
Ag85C	15	16	TT	2	-	Non-Antigen
	37	52	TFGGPATAGAFSRPGL	16	- 0.0863	Non-Antigen
	59	59	V	1	-	Non-Antigen
	61	65	SASMG	5	-	Non-Antigen
	72	78	FQGGGPH	7	1.4682	**Antigen**
	87	105	RAQDDYNGWDINTPAFEEY	19	0.9835	**Antigen**
	107	107	Q	1	-	Non-Antigen
	117	139	GGQSSFYTDWYQPSQSNGQNYTY	23	0.5401	**Antigen**
	156	166	NKGVSPTGNAA	11	0.5434	**Antigen**
	172	172	S	1	-	Non-Antigen
	184	190	PQQFPYA	7	0.2671	Non-Antigen
	197	204	LNPSEGWW	8	- 0.7119	Non-Antigen
	213	239	NDSGGYNANSMWGPSSDPAWKRNDPMV	27	0.7312	**Antigen**
	257	269	NGTPSDLGGDNIP	13	1.1086	**Antigen**
	283	310	TFRDTYAADGGRNGVFNFPPNGTHSWPY	28	0.663	**Antigen**

Furthermore, the low sensitivity and specificity in using protein antigens in serological assays allow cross-reactions. The replacement of antigenic proteins with more specific epitopes recognized by the immune system can be used to develop more effective serological assays.[6] A study evaluated serodiagnosis ELISA to detect antibodies in *M. tuberculosis* using a combination of epitopes RD1 and RD2 antigens resulted in a sensitivity of 83.3% for acid fast bacilli (+), 62.5 % for acid fast bacilli (-), and a specificity of 100%.[7] In its application, Epitope Diagnostics, Inc., San Diego, USA has developed an epitope diagnostic kit product using the ELISA technique for the detection of *Novel coronavirus* antibodies with a sensitivity up to 94% for IgM and 100% for IgG detection.[8]

The initial concept of the diagnostic design was performed by mapping the B cell epitope within silico studies using computational and bioinformatics. Furthermore, antigenic and immunogenic B cell epitopes are used as diagnostic antigens.[9] There is currently little information about the B cell epitope of antigen 85 complexes that can be used to develop a tuberculosis diagnosis. Therefore, this study predicts the epitope of the Ag85 complex, which is a potential diagnostic antigen.

Table 2. Physicochemical properties of B-cell linear epitope of Ag85A

Name	Start	End	molecular weight (Da)	Instability Index		GRAVY score	
				Score	Interpretation	Score	Interpretation
Ag85A	35	49	1361.52	26.26	**stable**	0.42	hydrophobic
	54	63	1071.26	135.78	unstable	- 0.33	**hydrophilic**
	70	76	619.59	76.84	unstable	- 1.086	**hydrophilic**
	86	99	1621.68	20.41	**stable**	- 1.236	**hydrophilic**
	115	123	930.97	119.61	unstable	- 0.111	**hydrophilic**
	126	135	997.15	82.97	unstable	- 0.25	**hydrophilic**
	157	163	724.81	-18.44	**stable**	- 0.914	**hydrophilic**
	212	235	2651.81	28.06	**stable**	- 1.592	**hydrophilic**
	256	267	1185.26	2.54	**stable**	- 1.158	**hydrophilic**
	286	308	2422.47	35.92	**stable**	- 0.809	**hydrophilic**

Table 3. Physicochemical properties of B-cell linear epitope of Ag85B

Name	Start	End	molecular weight (Da)	Instability Index		GRAVY score	
				Score	Interpretation	Score	Interpretation
Ag85B	33	46	1232.36	33.49	**stable**	0.329	hydrophobic
	51	60	1071.26	135.78	unstable	- 0.33	**hydrophilic**
	67	74	759.73	123.41	unstable	- 1.812	**hydrophilic**
	83	96	1664.71	15.06	**stable**	-1.721	**hydrophilic**
	113	132	2071.22	112.9	unstable	- 0.47	**hydrophilic**
	153	162	957.1	-9.91	**stable**	- 0.23	**hydrophilic**
	194	201	787.84	56.9	unstable	- 1.238	**hydrophilic**
	209	237	3121.3	32.7	**stable**	- 1.193	**hydrophilic**
	253	264	1156.22	29.39	**stable**	- 0.617	**hydrophilic**
	283	289	622.64	26.2	**stable**	- 0.029	**hydrophilic**

Table 4. Physicochemical properties of B-cell linear epitope of Ag85C

Name	Start	End	molecular weight (Da)	Instability Index		GRAVY score	
				Score	Interpretation	Score	Interpretation
Ag85C	72	78	698.74	43.83	unstable	- 0.957	**hydrophilic**
	87	105	2304.37	41.02	unstable	- 1.463	**hydrophilic**
	117	139	2678.72	86.89	unstable	- 1.53	**hydrophilic**
	156	166	1015.09	47.41	unstable	- 0.636	**hydrophilic**
	213	239	2954.15	49.6	unstable	- 1.244	**hydrophilic**
	257	269	1256.29	47.03	unstable	- 0.892	**hydrophilic**
	283	310	3145.36	17.54	**stable**	- 0.939	**hydrophilic**

MATERIALS AND METHODS

Sequence Preparation

This research was going on November 2021-January 2022 online-virtually from the Faculty of Medicine, University of Jember, Jember, East Java. This study is an experimental research and the samples used were taken from the NCBI protein bank with access numbers P9WQP3 for Ag85A, P9WQP1 for Ag85B, and P9WQN9 for Ag85C. VaxiJen v2.0 was used to test the antigenicity of Ag85 complex sequence samples (Ag85A, Ag85B, Ag85C). VaxiJen v2.0 server (https://www.ddg-pharmfac.net/vaxijen) predicts protective antigens of bacteria, viruses, and tumors with an accuracy of 70-97%.[10] This works with an auto cross-covariance (ACC) transformation approach of protein sequences based on the primary physicochemical properties of amino acids. Protein sequences are entered in fasta form, and bacteria were selected as target organisms, while the threshold value was set at 0.4.

B-cell Epitope Prediction and Antigenicity Test

B-cell epitopes were predicted from protein sequence using the IEDB (http://tools.iedb.org/bcell/). The method used is linear BepiPred 1.0 with a threshold of 0.35. Furthermore, BepiPred by default is provided by the IEDB server for predicting B cell linear epitope locations of proteins with 75% specificity and 49% sensitivity at a threshold of 0.35. It predicts the location of the epitope using a combination approach of the HMM algorithm (Hidden Markov Model), Parker hydrophilicity, and Levitt secondary structure.[11] Then, the predicted epitopes were tested for antigenicity using the VaxiJen v2.0 server (https://www.ddg-pharmfac.net/vaxijen). The inputted sequence is a residual type with a threshold of 0.4.

Physicochemical Test

The physicochemical characteristics were determined by testing the linear epitopes of B cells classified as antigenic using ExPASY ProtParam (http://www.expasy.org/tools/protparam.html), which is

a reliable tool for computing various physicochemical properties that can be deduced from a protein sequence. Furthermore, ProtParam sums up the contributions of the different amino acids, not taking into account secondary or tertiary structure. A physicochemical test was used to determine the immunogenicity characteristics of the predicted epitope. Its sought were molecular weight, instability index, and GRAVY score.

Alignment of B-cell Epitope with Indonesian Strain

The linear epitopes of B cells Ag85 complex had a similarity percentage of 85.71%-100% with the strains in Indonesia. Also, the e-values obtained vary from the highest 85×10^{-3} to the smallest 3×10^{-26}. Table 5 shows details of alignment results with Indonesian strains.

Table 5. Alignment of epitope with *M. tuberculosis* Indonesian strain

Name	Start	End	Str. Beijing/NITR203		Str. CAS/NITR204		Str. Haarlem/NITR202		Str. EAI5/NITR206	
			e-value	Percent identity	e-value	Percent identity	e-value	Percent identity	e-value	Percent identity
	86	99	2×10^{-10}	100%	2×10^{-10}	100%	1×10^{-7}	85.71%	1×10^{-8}	92.86%
	157	163	85×10^{-3}	100%	85×10^{-3}	100%	85×10^{-3}	100%	85×10^{-3}	100%
Ag85A	212	235	3×10^{-21}	100%	3×10^{-21}	100%	3×10^{-21}	100%	3×10^{-21}	100%
	256	267	1×10^{-6}	100%	1×10^{-6}	100%	1×10^{-6}	100%	1×10^{-6}	100%
	286	308	3×10^{-19}	100%	3×10^{-19}	100%	3×10^{-19}	100%	3×10^{-19}	100%
	83	96	8×10^{-11}	100%	8×10^{-11}	100%	2×10^{-9}	92.86%	8×10^{-11}	100%
	153	162	4×10^{-4}	100%	4×10^{-4}	100%	4×10^{-4}	100%	4×10^{-4}	100%
Ag85B	209	237	3×10^{-26}	100%	3×10^{-26}	100%	3×10^{-26}	100%	3×10^{-26}	100%
	253	264	9×10^{-7}	100%	9×10^{-7}	100%	9×10^{-7}	100%	9×10^{-7}	100%
	283	289	1×10^{-2}	100%	1×10^{-2}	100%	1×10^{-2}	100%	1×10^{-2}	100%
Ag85C	283	310	2×10^{-25}	100%	2×10^{-25}	100%	2×10^{-25}	100%	2×10^{-25}	100%

Sequence Alignment

The candidate epitope were aligned from the strain of Ag85 complex in Indonesia (str. Beijing, str. Haarlem, str. CAS, str. EAI) using BLASTP NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Antigenicity of Ag85 Complex Sequence

The results showed that the total value exceeded the threshold of 0.4 with details of 0.5259, 0.5842, 0.4402 on Ag85A, A85B, and Ag85C, respectively.

Prediction and Antigenicity Test of Ag85 Complex B-cell Epitope

The prediction results using IEDB showed only 10 of 17 Ag85A epitopes, 10 of 13 Ag85B epitopes, and 7 of 15 Ag85C epitopes are antigenic and can respond to B cells. Table 1 show the detailed score of predicted B cell epitope antigenicity.

Physicochemical Test of Ag85 Complex B-cell Epitope

The antigenic epitopes were subjected to a physicochemical test with ExPASy ProtParam. The results showed that each linear epitope of B cells had varying molecular weights from 619.59 to 3145.36 Da. The stability index and GRAVY score obtained 13 stable and 14 unstable epitopes, as well as 25 hydrophilic and 2 hydrophobic epitopes. Also, there are 11 stable and hydrophilic B cell epitopes, namely Ag85A and Ag85B with 5 epitopes for each and Ag85C with an epitope. Tables 2-4 show the results.

DISCUSSION

Antigenicity of Ag85 Complex Sequence

The three antigenicity values showed that the Ag85 complex protein sequence was antigenic, implying that it could bind to an antibody. TB antibodies formed due to the interaction of costimulatory signals of B and Th1 cells previously activated by antigens.[12] Furthermore, the serum immunoglobulin titers in TB patients are elevated by 90% against mycobacterial antigens at the time of clinical presentation. This correlation between the antibody response and active TB disease has led to the development of antibodies as diagnostic markers.[13] The results of this study are also in line with the development of the Ag85 complex as a diagnostic antigen for antibody detection. Meanwhile, the previous study by Kumar et al. (2008) successfully evaluated the accuracy of Ag85 complex for antibody detection in ELISA serodiagnosis in children aged 0-18 years.[14] Another study by Mani et al. (2016), using a microchip-based TB ELISA (MTBE) technique that can detect antibodies in less than 15 minutes, showed that the sensitivity and specificity of Ag85A are 52% and 76%, respectively.[15] The result showed that Ag85B has the highest antigenicity score, and it is a potential biomarker for the diagnosis of TB in its complexity because this protein is the most secreted (40%) of *M. tuberculosis*.[16]

Prediction and Antigenicity Test of Ag85 Complex B-cell Epitope

Fortyfive epitope regions of Ag85A, Ag85B, and Ag85C were identified through the prediction of B cell epitopes from Ag85 complex samples. As a candidate for

a diagnostic antigen epitope, antigenicity is essential. The epitope prediction on the Ag85 complex was continued with an antigenicity test using Vaxijen v2.0. The results of the B cell epitope showed that the antigens Ag85A, Ag85B, and Ag85C contain 10, 10, and 7 antigenic epitopes, respectively. This study's linear epitope antigenicity value of Ag85 complex B cells has a wide range of score variations from 0.4297 to 2.6007. It shows that antigenicity is not the only factor in planning the antigen epitope as diagnosis, but there is another factor that affects, like immunogenicity.[9] Because epitopes are immunogenic, they can activate specific antibodies in the body. By following multi-epitope design studies for diagnosis, antigens exhibiting high antigenicity and immunogenicity were utilized.[17–20]

Physicochemical Test of Ag85 Complex B-cell Epitope

A total of 27 antigenic epitopes were subjected to physicochemical tests to determine immunogenicity characteristics, such as molecular weight, instability index, and GRAVY score. These results obtained various molecular weights from 619.59 Da to 3145.36 Da. Furthermore, the immunological approach states that molecules less than 10 kDa are weakly immunogenic. Molecules with a molecular weight of more than 100 kDa (macromolecules) are highly potent immunogens.[21] It was discovered that the highest epitope molecular weight was 3145.36 Da. Meanwhile, several studies showed that using multiple linker-associated epitopes produces molecular weights above 10 kDa and results in higher ELISA sensitivity.[17,18,22] The diagnostic design uses multiple epitopes connected by a flexible linker (Gly-Ser-Gly-Ser-Gly).[18] Different epitopes can be used as diagnostic markers to achieve high performance, as the use of multi-epitope peptides can express high density resulting in increased sensitivity and specificity.[17]

The order physicochemical properties obtained from this study were 13 stable and 14 unstable epitopes. Meanwhile, the stable epitopes will retain their structure when binding to antibodies or in response to physical and chemical environment changes.[23] Several studies concluded that hyper-stable proteins lose their capacity to induce antibodies due to inefficient processing and presentation.[24] Similarly, low stability makes proteins less immunogenic in antibody production due to the formation of tertiary structures leading to loss of B cell epitope.[25]

Most of the epitopes in the GRAVY score were interpreted as hydrophilic residues. Several studies stated that the epitopes used as diagnostic antigens are hydrophilic.[18,20] The predicted hydrophilicity parameters show the position of the residue across the antigen protein sequence. Protein antigens have a globular (spherical) tertiary structure. The residue in this nonpolar (hydrophobic) and polar (hydrophilic) structure tends to be on the inside and outer surface of the protein. Hydrophilic residues are discovered on the surface of the antigen, which is more easily exposed (surface-exposed domain).[24] According to Abbas et al. (2014) and Ahmad et al. (2019), the linear epitope is on the outer surface of the antigen; hence, it is easily recognized by part of the antibody structure (antigen-binding site).[12,27]

Alignment of B-cell Epitope with Indonesian Strain

Eleven stable and hydrophilic epitopes were aligned using BLASTP on NCBI with complex Ag85 sequences from strains circulating in Indonesia. Therefore, the potential for developing serodiagnosis in Indonesia can be determined based on the percentage of similarity between each epitope derived from this alignment. The strains circulating in Indonesia and the NCBI database are the Beijing NITR203, the Central Asian NITR204, the Haarlem NITR202, and the East African Indian strain/NITR206. They were isolated from South India because the complex Ag85 protein sequences from Indonesian isolates were not yet available in the database.[28] The results indicated that the similarity was relatively high, ranging from 85.71% to 100%. Furthermore, a similarity percentage higher than 70% has a possibility of 90% similarity in the biological process, molecular function, and cellular component.[29]

Three epitopes are more significant because they have a low e-value, namely $_{212}$GDAGGYKASDMWGPKEDPAWQRND$_{235}$ from the Ag85A epitope with a value of 3×10^{-21}, $_{209}$GDAGGYKAADMWGPSSDPAW-ERNDPTQQI$_{237}$ from the Ag85B with a value of 3×10^{-26}, and $_{283}$TFRDTYAADGGRNGVFNFPPNGTHSWPY$_{310}$ from the Ag85C with a value 2×10^{-25}. The e-value is an estimate that provides a statistical measure of the two sequences. It was discovered that the lower the e-value, the more significant the score and its alignment.[30] Additionally, the three epitopes have 100% similarity with all strains; hence, they were universal antigens and can be developed into serodiagnostic antigens in Indonesia. However, the results of research using the in-silico method are only predictions of amino acid sequence results that need to be revalidated with other research methods such as in vivo and in vitro with Indonesian isolates to prove the accuracy of the predictions.

The limitation of the result of this study using the in-silico method is only predicting the result of antigenic and immunogenic amino acid sequence. As an immunoassay development, it is necessary to stimulate the interaction between the ligand protein and the receptor protein to assess the binding score in 3D between the epitope and BCR immunoglobulin. Further studies need to be carried out in vivo with the construction, expression, purification of recombinant protein, followed by ELISA antigen-antibody immunoassay test and western blot analysis to prove the accuracy of these prediction.

CONCLUSION

There are three B cell epitopes of the Ag85 complex, namely Ag85A (212-235), Ag85B (209-237), and Ag85C (283-310), which are universal antigens and can be developed into diagnostic antigens in Indonesia. However, the results of research using the in silico method are only predictions of amino acid sequence results that need to be revalidated with other research methods such as in vivo and in vitro with Indonesian isolates to prove the accuracy of the predictions.

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