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The antigenicity potential of multi-epitope peptide vaccine candidate in *Cryptosporidium hominis* infection

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Abstract

Cryptosporidiosis caused by the protozoan parasite *Cryptosporidium hominis*. This parasite causes a significant diarrheal disease in humans. This study was designed to find a multi-epitope peptide vaccine candidate targeting the 60-kDa glycoprotein (60 gp) of *C. hominis*. Bioinformatics approaches were employed to evaluate the antigenicity of the 60 gp sequence, identify conserved antigenic regions, and predict MHC class I and II epitopes. Five antigenic regions were identified, and two were selected for epitope mapping based on high antigenicity scores. The predicted epitopes were further evaluated for their antigenicity and binding affinities to MHC molecules through molecular docking simulations. Two multi-epitope vaccine constructs were designed by combining selected MHC class I and II epitopes, exhibiting high antigenicity scores of 0.6906 and 0.7197, respectively. Molecular docking revealed favorable interactions between the epitopes and their respective MHC molecules. These findings provide a promising foundation for the development of an effective multi-epitope peptide vaccine against *C. hominis* and offer potential therapeutic settings against cryptosporidiosis. Experimental validation, including *in vivo* studies and clinical trials, is necessary to assess the immunogenicity and protective efficacy of the proposed vaccine candidates.

Keywords: Antigenicity, Multi-epitope, Peptide, Vaccine, *Cryptosporidium hominis*, Infection

Introduction

Cryptosporidium hominis, a protozoan parasite, represents a significant global health burden, particularly in developing countries. It is estimated to be the leading cause of diarrheal illness in children under five years old (Kotloff, KL et al. 2013). This diarrheal disease, known as cryptosporidiosis, can cause significant morbidity, with symptoms including watery diarrhea, abdominal cramps, and dehydration (Pinto, DJ and Vinayak, S 2021). In immunocompromised individuals and malnourished children, cryptosporidiosis can be life-threatening (Mead, JR 2014). The lack of a commercially available vaccine to prevent this diarrheal disease directed the researchers to find new approaches to control cryptosporidiosis (Rappuoli, R et al. 2016). Traditionally, vaccine development has been a time-intensive and resource-intensive process that relies heavily on trial-and-error methods. However, the field of vaccine technology has been revolutionized by the emergence of in-silico approaches (Soltan, MA et al. 2021). These computational methods leverage computer modeling and simulation techniques to accelerate vaccine design (Lu, G et al. 2021). *In silico* approaches offer several advantages over traditional methods (Mushebenge, AG-A et al. 2023). They are typically more cost-effective and time-efficient, allowing for the rapid screening of a vast number of potential vaccine candidates (Ifeonu, OO et al. 2016)Additionally, in-silico analyses can integrate and analyze massive datasets, enabling the identification of vaccine targets with a high degree of accuracy (Lu, G et al. 2021). One promising avenue for a *C. hominis* vaccine focuses on the 60 kDa glycoprotein, a major surface antigen of the parasite. This protein plays a critical role in the interaction between *C. hominis* and its host, making it essential for successful infection (Altschul, SF et al. 1990). By targeting this protein with a vaccine, it could be a potential approach to prevent *C. hominis* from establishing itself within the host and causing disease.

This study aimed to leverage the power of *insilico* methods to design a potential vaccine candidate against *C. hominis*. A multi-step computational approach was employed to encompass several key techniques. Firstly, sequence analysis of the 60 kDa glycoprotein was employed to identify conserved regions that are essential for the function of the protein and are likely to be immunogenic. Secondly, is utilizing the antigen prediction algorithms to pinpoint specific regions of the protein that are most likely to be recognized by the immune system. Thirdly, epitope mapping was employed to identify and refine the most immunogenic B-cell and T-cell epitopes within the 60 kDa glycoprotein. Finally, molecular docking simulations were conducted to assess how these potential vaccine candidates interact with the immune system's receptors. Through this comprehensive *in silico* approach, we aimed to identify a refined vaccine target that could effectively stimulate a protective immune response against cryptosporidiosis.

Materials and methods

Sequence retrieval and analysis

The amino acid sequence of the 60 kDa glycoprotein from C. hominis (accession number ACQ82748.1) was retrieved from the NCBI database. BLASTp analysis was performed against the non-redundant protein sequence database to identify similar sequences and variants. Multiple sequence alignment was carried out using Clustal Omega to identify conserved regions within the protein sequence in 2022 by Clustal Omega software (Sievers, F and Higgins, D 2021).

Transmembrane topology and antigenicity prediction

The TMHMM server was utilized to predict the transmembrane topology of the 60 kDa glycoprotein and identify potential extracellular regions (Krogh, A et al. 2001). The VaxiJen server was employed to assess the antigenicity of the conserved regions identified through multiple sequence alignments (Doytchinova, IA and Flower, DR 2007).

Epitope mapping and 3D structure prediction

Three antigenic sequences with high antigenicity scores were selected for further analysis. The Immune Epitope Database (IEDB) (Kaabinejadian, S et al. 2022, Reynisson, B et al. 2020) was used to predict potential binding epitopes for major histocompatibility complex (MHC I and II was taken from the Immune Epitope Database (IEDB). The 3D structures of the selected epitopes were modeled using the PEP-FOLD server (Krogh, A et al. 2001).

Molecular docking

Molecular docking simulations were performed to investigate the interactions between the predicted epitopes and MHC class I and class II molecules. The H-DOCK server was employed for rigid-body docking, considering the flexibility of the epitope peptides and the static nature of the MHC molecules (Shen, Y et al. 2014, Yan, Y et al. 2017).

Result

Table 1 shows predicted antigenicity calculated from the Vaxijen server with parasite parameter and cutoff 0.5 score. The table result suggests high predicted antigenicity with a promising effect for this protein type. The table shows the results of an antigenicity prediction analysis performed on a 60gp protein sequence using the Vaxijen server. The protein sequence is provided, along with the predicted antigenicity score calculated by the server. The Vaxijen server is a bioinformatics tool used to predict the potential for a given protein sequence to elicit an immune response, specifically by evaluating its ability to bind to major histocompatibility complex (MHC) molecules and stimulate T-cell responses. The prediction is based on the physicochemical properties of the protein sequence, such as amino acid composition, secondary structure, and intrinsic disorder.

In this case, the antigenicity score for the provided 60gp protein sequence is 0.9607, which is classified as a "Probable ANTIGEN" by the server. A score above 0.5 is generally considered indicative of a potential antigen, with higher scores suggesting a higher likelihood of antigenicity. Furthermore, the docking between the regions PDB ID: 6W9V for MHC-I and PDB ID: 3L6F for MHC-II, which presented in brown color, peptides was represented in a yellow ribbon in the 2nd structure. The docking score has shown a negative value, with the most negative indicating greater interaction favorability.

The BLAST (Basic Local Alignment Search Tool) analysis is a widely used bioinformatics tool for comparing biological sequences, such as proteins or nucleic acids, to identify regions of similarity or conservation across different organisms or sequences. The hit parameter of 500 refers to the number of sequences included in the analysis, which allows for a comprehensive assessment of conserved regions. The Vaxijen server, as mentioned earlier, predicts the potential antigenicity of protein sequences based on their physicochemical properties. The antigenicity scores range from 0 to 1, with scores above 0.5 generally considered indicative of a potential antigen as shown in Table 2. The table presents the results of a BLAST analysis performed with a hit parameter of 500, which identifies conserved regions within the protein sequence. For each conserved region identified, the corresponding sequence and its predicted antigenicity score, calculated using the Vaxijen server, are provided.

The IEDB is a comprehensive resource for the analysis of epitopes (antigenic regions) and their interactions with major histocompatibility complex (MHC) molecules, which are essential for the presentation of antigens to T cells

and the initiation of an immune response. In this table, the analysis focused on a particular region of the protein sequence, denoted as region (1). The predicted MHC Class I binding peptides are listed along with their corresponding alleles, start and end positions within the protein sequence, peptide length, and the peptide sequence itself. The MHC Class I alleles included in the analysis are HLA-A02:03, HLA-A02:06, HLA-A02:01, HLA-B40:01, HLA-B44:02, and HLA-B44:03. These alleles represent different variants of the human leukocyte antigen (HLA) genes, which are responsible for encoding MHC molecules as predicted in Table 3.

For each predicted peptide, a score is provided, which indicates the likelihood of the peptide binding to the respective MHC allele. Higher scores generally indicate a stronger binding affinity. Additionally, the table includes the antigenicity scores for the predicted peptides, calculated using the Vaxijen server. These scores predict the potential of the peptides to elicit an immune response, with scores above 0.5 considered indicative of probable antigens. The table 3 presents the results of an analysis for predicting potential MHC Class I binding peptides within a specific region of the protein sequence. The analysis was performed using the Immune Epitope Database (IEDB) tool, and the predicted peptides were further evaluated for their antigenicity using the Vaxijen server. Moreover, MHC Class II molecules are involved in the presentation of extracellular antigens to CD4+ T helper cells, which play a crucial role in orchestrating adaptive immune responses. In this table, the predicted MHC Class II binding peptides are listed along with their corresponding alleles, peptide sequence, and the binding score. The binding score indicates the likelihood of the peptide binding to the respective MHC Class II allele, with higher scores suggesting a stronger binding affinity.

The MHC Class II alleles included in the analysis are HLA-DRB104:05, HLA-DPA101:03/DPB102:01, HLA-DQA105:01/DQB103:01, HLA-DRB104:01, and HLA-DRB1*09:01. These alleles represent different variants of the human leukocyte antigen (HLA) genes, which encode MHC molecules. Additionally, the table includes the antigenicity scores for the predicted peptides, calculated using the Vaxijen server. These scores predict the potential of the peptides to elicit an immune response, with scores above 0.5 considered indicative of probable antigens which shown in Table 4. Table 4 presents the results of an analysis for predicting potential MHC Class II binding peptides within region (1) of the protein sequence. The analysis was performed using the Immune Epitope Database (IEDB) tool, and the predicted peptides were further evaluated for their antigenicity using the Vaxijen server

Notably, the antigenicity score for this peptide is 0.8660, which is well above the 0.5 threshold, suggesting that it is a probable antigen capable of eliciting an immune response. The combination of a relatively high binding score and

a high antigenicity score makes this predicted peptide a promising candidate for further investigation as a potential epitope for CD4+ T helper cell recognition and activation.

However, it is important to note that these predictions are based on computational models and may not fully reflect the complex mechanisms involved in antigen processing, presentation, and immune recognition. Experimental validation, such as binding assays, T-cell activation assays, and in vivo studies, are necessary to confirm the actual immunogenicity and potential applications of this predicted epitope. Furthermore, the specific context, such as the target population's HLA allele distribution and the intended application (e.g., vaccine design, immunotherapy, or diagnostics), should be considered when interpreting and utilizing these results.

Overall, the analysis provides a valuable starting point for identifying a potential immunogenic epitope within region (2) of the protein sequence that may be recognized by the MHC Class II allele HLA-DRB3*02:02. Further experimental validation and consideration of the specific context are essential for developing practical applications based on these findings. The table 5 identifies one potential MHC Class II binding peptide, FSTLSANSSSPTKDN, within region (2) of the protein sequence. This peptide has a binding score of 0.6479 for the HLA-DRB3*02:02 allele, indicating a moderate to strong likelihood of binding to this MHC Class II molecule.

The multi-epitope peptide vaccine architecture presented in the table aims to incorporate multiple epitopes from different sources into a single construct. By including both CTL epitopes and HTL epitopes, the vaccine construct aims to stimulate both CD8+ cytotoxic T cell responses and CD4+ T helper cell responses, respectively. The inclusion of adjuvant linker sequences (EAAK) and specific linkers (AAY and GPGPG) is intended to enhance the immunogenicity, stability, and proper presentation of the epitopes within the construct. Both constructed peptide sequences have antigenicity scores above 0.5, indicating their potential to elicit an immune response. Specifically, the first sequence has an antigenicity score of 0.6906, and the second sequence has an antigenicity score of 0.7197.

It is important to note that these antigenicity predictions are based on computational models and may not fully reflect the complex mechanisms involved in antigen processing, presentation, and immune recognition. Experimental validation, such as binding assays, T-cell activation assays, and in vivo studies, are necessary to confirm the actual immunogenicity and efficacy of these multi-epitopes peptide vaccine constructs. Furthermore, the specific context, such as the target population, the intended application (e.g., prophylactic or therapeutic vaccine), and the delivery system, should be considered when evaluating and optimizing these vaccine constructs.

Overall, the proposed multi-epitope peptide vaccine architecture represents a promising approach for eliciting broad and robust immune responses by incorporating multiple epitopes targeting both CD8+ and CD4+ T cell responses.

However, further experimental validation and optimization are essential to develop an effective and safe vaccine based on this design as illustrated in Table 6. The table 6 presents the results of an analysis for predicting potential MHC Class II binding peptides within region (2) of the protein sequence. The analysis was performed using the Immune Epitope Database (IEDB) tool, and the predicted peptides were further evaluated for their antigenicity using the Vaxijen server. A multi-epitope peptide vaccine construct was designed by combining the selected MHC class I and class II epitopes, linked together using appropriate linkers (Table 7). The antigenicity of the final construct was predicted, and two potential vaccine candidates were proposed, with antigenicity scores of 0.6906 and 0.7197, respectively, indicating their probable antigenic nature.

To further assess the potential interactions between the selected epitopes and their respective MHC molecules, molecular docking simulations were performed using HDOCK. The docking scores for the interactions between MHC-I (PDB ID: 6W9V) and MHC-II (PDB ID: 3L6F) with the selected epitopes are presented in Table 8. Negative docking scores indicate favorable interactions, with lower values suggesting stronger binding affinities, shown in Table 7. The table 7 presents the architecture of a proposed multi-epitope peptide vaccine and the predicted antigenicity of the constructed peptide sequences using the Vaxijen server.

Discussion

The protein sequence 60 gp, is one of the key components of immune system. It has been recognized as a significant aim for vaccine development and/or immunotherapeutic approaches (Oli, AN et al. 2020). Current data, as computed, showed a high antigenicity score that proposes the capability of 60gp inducing immune response, making it a potential target for vaccine development. However, data interpretation of *Vaxijen* predictions should be carefully revised, so it is ideal to make experimental proof to confirm the definite antigenicity and immunogenicity of the 60gp protein (Doytchinova, IA and Flower, DR 2007). The Vaxijen server was used to assess the antigenicity of the 60gp protein sequence, predicting it as a probable antigen with a score of 0.9607. Transmembrane topology examination using TMHMM discovered surface exposure via the presence of transmembrane, inside, and outside regions. Identifying the conserved antigenic regions within the 60gp sequence using BLAST identified five regions that were found to have high predicted antigenicity. Two regions, symbolized as regions 1 and 2. Both were selected for additional investigation based on the high antigenicity scores. As well, both regions in combination with MHC class I and class II epitopes were speculated using the IEDB server for these regions (Kalemati, M et al. 2023).

Data analysis of the conserved regions provides practicality for the antigenicity of these regions to be used in the mapping epitopes, design of vaccines, and recognizing the potential immunogenic properties of this protein. We found three sequences (**IVYAPIKDQTDPAPRYISGEVTSVSFEK-SESTVTIKVNG**, **FSTLSANSSSPTKDN**, and **HVQSRSRRSLAE**) have a score of antigenicity scores above 0.5. This suggests that these sequences may be potential antigenic regions provoking an immune response. These regions could be further investigated as potential targets for vaccine development or as diagnostic markers. Moreover, the protein sequence upon recognition through specific MHC Class I alleles means the likely immunogenic epitopes and their capability of producing an immune response. Furthermore, the MHC binding scores for these peptides are relatively high, demonstrating a robust prospect of binding to the respective MHC Class II alleles (Rajapakse, M et al. 2007).

Notably, the 60gp protein classification according to the high antigenicity score, further investigations required to measure, validate, and characterize its potential applications as an immunogenic target. Paricularly, YISGEVTSV, a promising sequence due to the high antigenicity score and robust binding affinity to multiple MHC I. However, it is important to note that these predictions are based on computational models and may not entirely imitate the underlying and complex mechanisms involved in antigen processing, presentation, and immune recognition. Experimental validation, such as binding assays, T-cell activation assays, and in vivo studies, is necessary to confirm the actual immunogenicity and potential applications of these predicted epitopes (Peters, B et al. 2020, Hudson, D et al. 2023) .

Conclusion and future perspectives

The study identified antigenic regions in C. hominis' 60gp protein and designed a multi-epitope peptide vaccine using predicted MHC class I and II epitopes. The vaccines showed high antigenicity scores and favorable interactions with MHC molecules, paving the way for further experimental validation and the development of an effective vaccine against cryptosporidiosis.

Computational approaches provide valuable insights, nevertheless further experimental validation is necessary to confirm the immunogenicity and protective efficacy of the proposed vaccine candidates. Including *in vivo* studies, and clinical trials. This will be crucial to assess the safety, immunogenicity, and protective potential of the multi-epitope vaccine constructs against Cryptosporidium hominis infection.

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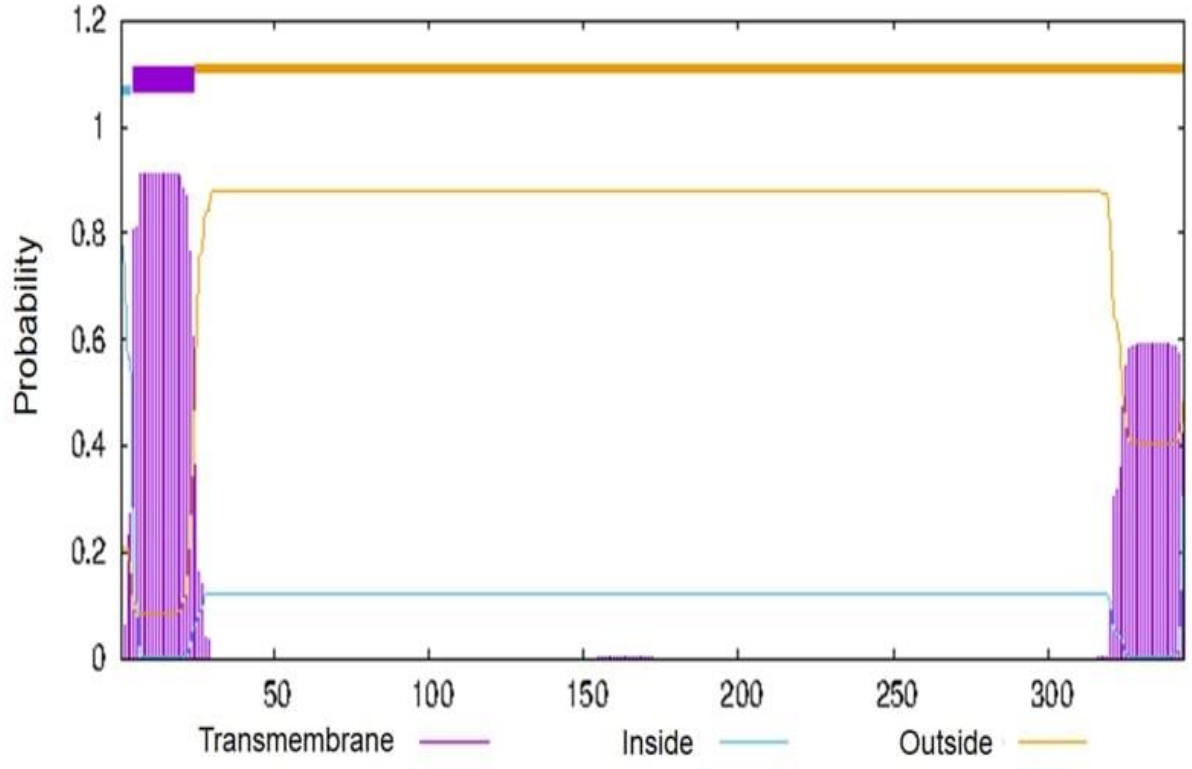

Figure 1. TMHMM probability for WEBSEQUENCE (transmembrane regions) 60gp. Figure illustrates the transmembrane in purple, the inner in blue, and the surface regions in yellow for 60 gp *C. hominis.* The data showed strong estimated antigenicity with a promising consequence for the three locations chosen for HTL and CTL screening.

Table 1. Showed 60gp protein sequence antigenicity calculated from Vaxijen server.

Sequence	Vaxijen antigenicity
MRLSLIIVLLSVIVSAVFSAPAVPLRGTLKDVSVEGSSSSSSSSSSSTVAPAPK KERTVEGGTEGKNGESSPGSEEQDGGKEDGGKEDGGKEDGGKEDGGKEN GEGDTVDGVQTGSGSQVTPSESAGTATESTATTTPKEECGTSFVMWFEKGT PVATLKCGDYTIVYAPIKDQTDPAPRYISGEVTSVSFEKSESTVTIKVNGKEFS TLSANSSSPTKDNGESSDSHVQSRSRRSLAEENGETVATVDLFAFTLDGGRR IEVAVPKDENADKRSEYSLVADDKPFYTGANSGITNGVYKLDENGNLVDKD NEVLLKDAGSSAFGFKYIVPSVFAIFAALFVL	0.9607 (Probable ANTIGEN).

Table 2. conserved regions extracted from BLAST with 500 hit parameters with antigenicity for all of them.

Sequence	Antigenicity
P--KEECGTSFVMWFEKGTP	0.0499 (Probable NON-ANTIGEN).
IVYAPIKDQTDPAPRYISGEVTSVSFEK-SESTVTIKVNG	0.7457 (Probable ANTIGEN).
FSTLSANSSSPTKDN	0.8660 (Probable ANTIGEN).
HVQSRSRRSLAE	0.8239 (Probable ANTIGEN).
ETVATVDLFAFTLDGG	0.2537 (Probable NON-ANTIGEN).

Table 3. MHC I peptides predicted with IEDB and Antigenicity predicted using Vaxijen for region (1).

Allele	#	Start	End	Length	Peptide	Score	Antigenicity
HLA-A*02:03 HLA-A*02:06 HLA-A*02:01	1	16	24	9	YISGEVTSV	0.964345	0.9026 (Probable ANTIGEN).
HLA-B*40:01 HLA-B*44:02 HLA-B*44:03	2	29	37	9	SESTVTIKV	0.842162	0.7366 (Probable ANTIGEN) .

Table 4. MHC II peptides and predicted antigenicity for region 1.

Allele	#	Peptide Sequence	Score	Antigenicity
HLA-DRB1*04:05	1	IVYAPIKDQTDPAPR	0.9590	0.6560 (Probable ANTIGEN).
HLA-DRB1*04:05	1	VYAPIKDQTDPAPRY	0.7647	0.5706 (Probable ANTIGEN).
HLA-DPA1*01:03/DPB1*02:01	1	SGEVTSVSFEKSEST	0.7315	0.6933 (Probable ANTIGEN).
HLA-DQA1*05:01/DQB1*03:01	1	APRYISGEVTSVSFE	0.7006	0.5876 (Probable ANTIGEN).
HLA-DRB1*04:01	1	PRYISGEVTSVSFEK	0.6914	0.5875 (Probable ANTIGEN).
HLA-DPA1*01:03/DPB1*02:01	1	ISGEVTSVSFEKSES	0.6855	0.6489 (Probable ANTIGEN).
HLA-DRB1*09:01	1	APRYISGEVTSVSFE	0.6500	0.5876 (Probable ANTIGEN).
HLA-DQA1*05:01/DQB1*03:01	1	PAPRYISGEVTSVSF	0.5963	0.5732 (Probable ANTIGEN).

Table 5. MHC I peptides predicted with IEDB and antigenicity predicted using Vaxijen for region (2).

Allele	#	Peptide	Score	Antigenicity
HLA-A*02:03	1	YISGEVTSV	0.964345	0.9026 (Probable ANTIGEN).
HLA-A*02:06	1	YISGEVTSV	0.941515	
HLA-A*02:01	1	YISGEVTSV	0.921678	
HLA-B*40:01	1	SESTVTIKV	0.842162	0.7366 (Probable ANTIGEN).
HLA-B*44:03	1	SESTVTIKV	0.770872	
HLA-B*44:02	1	SESTVTIKV	0.666586	

Table 6. MHC II peptides predicted with IEDB and Antigenicity predicted using Vaxijen for region (2).

Allele	#	Start	End	Length	Core Sequence	Peptide Sequence	Score	Antigenicity
HLA-DRB3*02:02	1	1	15	15	LSANSSSPT	FSTLSANSSSPTKDN	0.6479	0.8660 (Probable ANTIGEN).

Table 7. Multiepitope peptide vaccine architecture and antigenicity prediction.

EAAK	CTL(AAY)	HTL(GPGPG)	EAAK
Sequence			**antigenicity**

EAAKYISGEVTSVAAYSESTVTIKVAAYIVYAPIKDQTDPAPRGPGPGSGEVTSVS FEKSESTGPGPGAPRYISGEVTSVSFEGPGPGPRYISGEVTSVSFEKGPGPGISGEVTS VSFEKSESGPGPGEAAK	0.6906 (Probable ANTIGEN).
EAAKYISGEVTSVAAYSESTVTIKVAAYFSTLSANSSSPTKDNGPGPGEAAK	0.7197 (Probable ANTIGEN).