

Design of a predicted MHC restricted short peptide immunodiagnostic and vaccine candidate for Fowl adenovirus C in chicken infection

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Abstract:

Fowl adenoviruses (FAdVs) are the etiologic agents of multiple pathologies in chicken. There are five different species of FAdVs grouped as FAdV-A, FAdV-B, FAdV-C, FAdV-D, and FAdV-E. It is of interest to develop immunodiagnostics and vaccine candidate for Peruvian FAdV-C in chicken infection using MHC restricted short peptide candidates.

We sequenced the complete genome of one FAdV strain isolated from a chicken of a local farm. A total of 44 protein coding genes were identified in each genome. We sequenced twelve Cobb chicken MHC alleles from animals of different farms in the central coast of Peru, and subsequently determined three optimal human MHC-I and four optimal human MHC-II substitute alleles for MHC-peptide prediction. The potential MHC restricted short peptide epitope-like candidates were predicted using human specific (with determined suitable chicken substitutes) NetMHC MHC-peptide prediction model with web server features from all the FAdV genomes available. FAdV specific peptides with calculated binding values to known substituted chicken MHC-I and MHC-II were further filtered for diagnostics and potential vaccine epitopes. Promiscuity to the 3/4 optimal human MHC-I/II alleles and conservation among the available FAdV genomes was considered in this analysis. The localization on the surface of the protein was considered for class II predicted peptides. Thus, a set of class I and class II specific peptides from FAdV were reported in this study. Hence, a multiepitopic protein was built with these peptides, and subsequently tested to confirm the production of specific antibodies in chicken.

Background:

FAdVs are widely distributed, and some species are associated with important poultry diseases, representing current threats of serious economic losses for the aviculture industry. Some of the diseases include the Inclusion body hepatitis (IBH) related with FAdV-C and D infections, hepatitis-hydropericardium syndrome (HHS) related with FAdV-C infections [1,2], gizzard erosion (GE) related with FAdV-A infections [3-6] among others. The family Adenoviridae is divided into five genera: Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus and Ichtadenovirus (Harrach 2011). Currently, Fowl adenovirus (FAdV) is classified into five different species (FAdV-A to

FAdV-E) on based upon the DNA restriction patterns generated by endonucleases BamHI and HindIII [7]. Recently, at least 12 genotypes within the five species were revealed by sequence analysis of the hexon loop 1 (L1) gene region [8].

Proteins are the essential immune-target structures, which in the MHC class I/II (MHC-I/II) pathway are processed to 8-11/15-18 mer peptides. In this way, peptides that bind to MHC molecules are presented and potentially recognized by cytotoxic T cells (CD8/CD4), which can lead to an immune response [9]. The most selective step in this antigen presentation is the peptide binding to MHC [10]. Each MHC

molecule has a potentially unique binding affinity motif [11] and the characterization of this motif for each MHC molecule prevalent in a given population is a central aspect of rational T cell epitope discovery. Due to the immense MHC polymorphism [12,13], an exhaustive characterization of all MHC molecules is a high cost-intensive effort, and as of today in spite of significant advances in high-throughput immune assays only a little more than 100 MHC molecules, including 25 non-human molecules have been experimentally characterized at a detail allowing to describe their binding specificity (IEDB date 2012).

To face this problem, several in silico prediction methods have been developed in the last decades [14-18]. Of these methods, NetMHCpan is the current in state-of the art method for predicting binding affinity of peptides to any MHC-I/II molecule with a known protein sequence [19]. In this study we sequence the complete genome of a FAdV-C from Peruvian wild strains, and perform an immunoinformatics analysis to predict immunogenic MHC-I and MHC-II T-cell linear epitopes for immunodiagnostics and potential vaccine.

Methodology:

FAdV isolation and DNA preparation

Field samples from a local chicken farm let us isolate a FAdV strain. Inclusion body hepatitis (IBH) was confirmed by clinical symptoms and by real-time PCR [20]. Liver from each individual was blended with phosphate buffered saline using individual sterile blenders. Then, for virus isolation, IBH PCR positive homogenized livers were ultracentrifuged by CsCl gradient as follows, cellular lysis was reached given tissues three freeze-thaw cycles (-80°C to 37°C). Cellular debris was eliminated through centrifugation of samples to 5,500 g for 30 minutes at 4°C. Five milliliters of supernatant were added into CsCl gradient ($\delta = 1.25$ g/mL and $\delta = 1.4$ g/mL) and ultracentrifuged in a SW28 rotor (Beckman Coulter, Indianapolis, USA) at 35000 RPM for 1 hour at 15°C in a Optima L-100K (Beckman Coulter). Capsids and complete viral particles were clearly identified. An aliquot of complete virus was used for DNA isolation as mentioned before and quantitation by real-time PCR, [20]. For DNA extraction, an aliquot of 200 μ l was used to DNA isolation by DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following manufacturer instructions.

FAdV genome sequencing, assembly and annotation

For the genome sequencing we obtained 2 mg of the Phi29-amplified viral DNA, and used for the construction of the library, which was sequenced using the 454-Roche GS-FLX Titanium System (454 Life Sciences, Roche). The reads obtained from the sequencing were assembled de-novo using GS De Novo Assembler 2.7. Additionally, we assembled the reads using as reference the FAdV-C serotype 4 strain ON1 (Accession Code: GU188428) in the software GS Reference Mapper. Genome annotation and manual revision were performed using the software CLC Main Workbench 6.8.3. All the possible ORFs were predicted. We used as criteria to validate an ORF, the fact of encoding peptides longer than 30 aa, thus generating putative proteins. The amino acid sequences of all the putative proteins were compared to the available protein sequences in GenBank using the Basic Local Alignment Search Tool (BLAST). The presence of homologous genes in other adenoviruses was used as evidence to support our potential genes.

Sequencing of the Cobb chicken MHC-I and MHC-II coding genes

We collected twelve breeding commercial Cobb chickens. All animals were sampled from farms located in Chinchá, an important city of chicken breeding located in the mid-southern coast of Peru. Spleen samples were taken from each selected chicken described above. The tissue was preserved in RNA-Later (Ambion) and stored at -80°C until further processing. Spleen RNA was purified by homogenizing 20 mg of excised tissue in 600 μ l of RLT buffer (Qiagen). The sample was mechanically disrupted with the TissueLyser system (Qiagen), then proceeding (the RNA extraction) according to the manufacturer's protocol (RNeasy mini kit; Qiagen) and resuspended in 50 μ l of DEPC-treated water. Excess DNA was eliminated by Turbo DNase (Ambion) treatment.

cDNA synthesis was performed according to the manufacturer's instructions (Transcriptor First Strand cDNA Synthesis Kit, Roche); second, to amplify the entire ORF, cDNA was used as template in a conventional PCR, carried out by a High Fidelity polymerase and PCR Enhancer (Invitrogen). Primers used for alpha subunit of MHC class I were: (Forward) 5'-ATGGGCTCGTGCGGGG-3' and (Reverse) 5'-CAGATGGAGGGGTTGCTC-3'; and for the beta subunit (β -microglobulin) were: (Forward) 5'-AATGGGGAAGGCGGCG-3' and (Reverse) 5'-GGATCCCACTGTAGACCT-3'. Primers used for alpha subunit of MHC class II were: (Forward) 5'-ATGGCGGTGCTGAGCG-3' and (Reverse) 5'-CAGAGCAGCCCCGGTT-3'; and for the beta subunit: (Forward) 5'-AATGGGAGCGGGCGCGT-3' and (Reverse) 5'-TTCAGCATCCCTGGAGCGGC-3'. PCR products were cloned into pGEM-T Easy plasmid (Promega). DNA was sequenced using 454 GS Junior sequencer (Roche). Each amplicon of MHC subunits were sequenced using a different multiplexer identifier (MID). Unlike MHC-I α and MHC-II subunits, the MHC-I β amplicon is small (350 bp length), so this allowed us to discriminate between different subunits; therefore there was no need to clone it because the entire amplicon could be sequenced in one read.

The sequencing experiment produced .sff files containing the information of the reads and the quality by each position. These reads were processed in the software GS Amplicon Variant Analyzer 2.7 to obtain contigs, using as reference the reported sequences of MHC-I alpha subunit (HQ435266.1, EF554701.1, HQ435266.1), MHC-I beta subunit (AB162661.1, EF554713.1, AB162661.1), MHC-II alpha subunit (EF554701.1) and MHC-II beta subunit (EF554713.1). The variations in the sequences were revised in detail in the software GS Amplicon Variant Analyzer 2.7 and CLC Main Workbench 6.8.3, and the most frequent haplotype for each subunit were determined.

Selection of human MHC-I/II alleles that best substitute its chicken homologues

We used the NetMHCcons and NetMHCIIpan immunoinformatics software to predict potential epitopes with high affinity to MHC class I and II, respectively. The MHC alleles available in these tools belong mainly to humans, macaques, mice, and pigs. Because there is no data of alleles of chicken MHCs, we selected "the best human substitute alleles" for the chicken MHC. To find these substitute alleles, all the sequences of the human MHC-I and II alleles available in NetMHCpan and NetMHCIIpan were compared with the most frequent haplotypes of the chicken MHC alleles. After multiple alignments, we selected the human MHC alleles that showed

an identity greater than 70%. The selected human MHC alleles and the most frequent haplotypes of the chicken MHC alleles were compared at their peptides “predictive level”, using as criteria, the similarity of the predicted affinities with a random

set of 100,000 peptides. This was performed using the software MHCcluster [21].

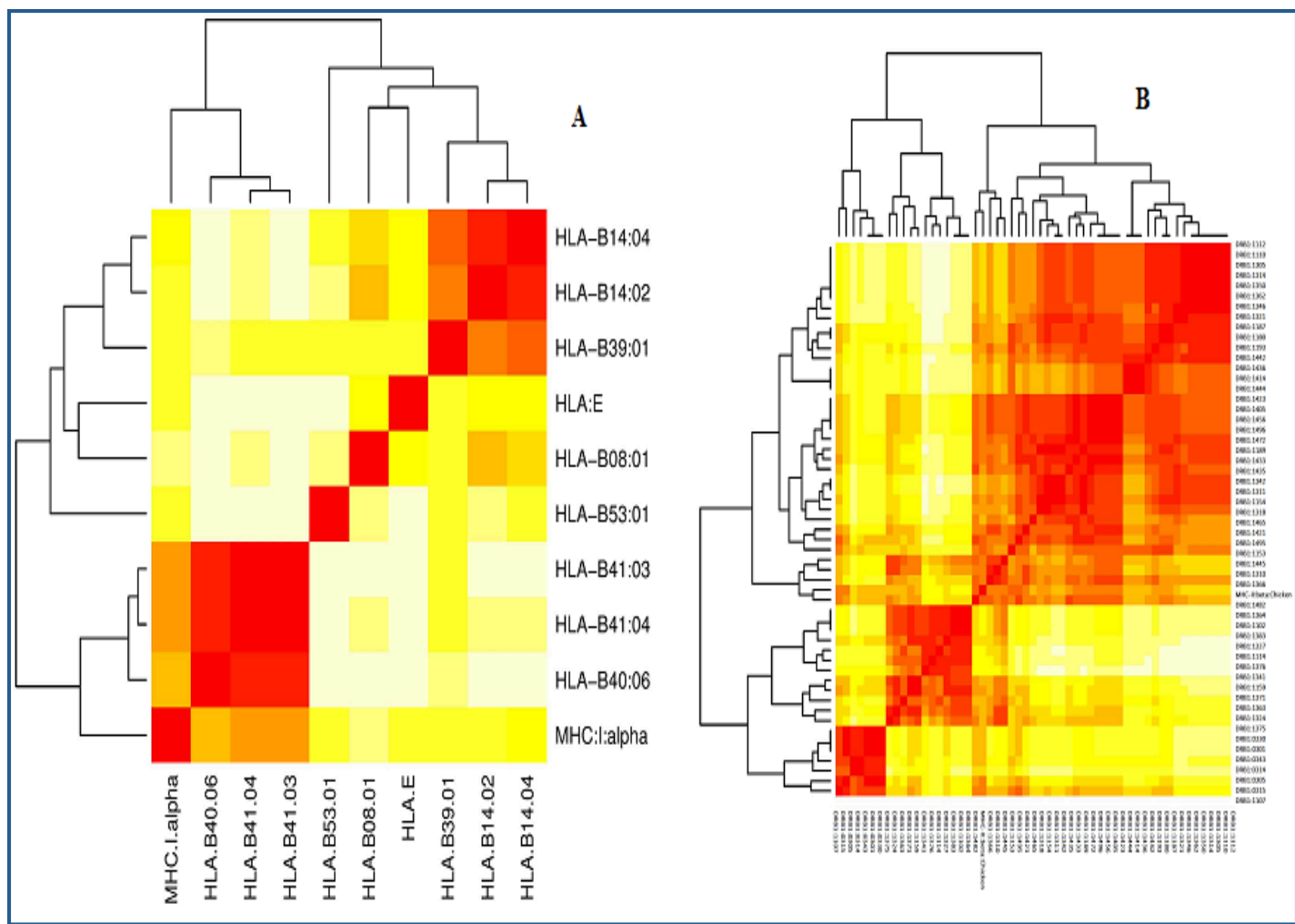


Figure 1: Heatmaps of the MHCcluster. Figure A and B show the heatmaps for the MHC class I and II, respectively. The regions in red represent the warm regions where the alleles of MHC with similar capacity of anchor epitopes with high affinity, the regions of the color yellow represent cold spots where the similarity of affinity epitopes are more distant from each other. The intensity of color is related to the behavior of the predictive ability of epitopes between different MHCs.

Prediction of immunogenic MHC-I and MHC-II FAdV peptides Prediction of peptide affinity to MHC-I and MHC-II

We explored the FAdV genomes looking for potential immunogenic peptides. The FAdV proteins were analyzed with NetMHCcons v1.1 (for MHC-I) [18] and NetMHCIIpan v2.2 (for MHC-II) [22], for the prediction of MHC-peptide binding affinity. For MHC-I peptides, NetMHCcons cuts the proteins in all the 9 amino acids length possible peptides. These peptides and “the best human substitutes” MHC-I alleles were used to predict the MHC-peptide binding affinity. Similarly, the NetMHCIIpan software cuts the proteins in all the 15 amino acids length possible peptides and uses “the best human substitutes” MHC-II alleles to predict the binding affinity. The predictions were made for all the human-substitute alleles. These predicted binding affinities were estimated as IC50 concentration (in nM). Additionally, these software provide a pre-defined cut-off for the categorization of the peptides according to the predicted affinity: “SB” or Strong Binder (for $IC_{50} \leq 2$ nM) and “WB” or Weak Binder (for 2 nM $< IC_{50} \leq 50$

nM). Higher values of IC50 are considered as evidence of lack of binding.

Filtering of promiscuity, conservation, host proteome, and surface accessibility

Promiscuity: Promiscuous peptides are defined as those recognized by multiple MHC alleles. We determined the peptides with high affinity to the several MHC-I optimal human alleles selected. Only the peptides predicted as strong binders for all the MHC-I alleles were selected. The same procedure was conducted for MHC-II alleles; **Conservation:** The peptides obtained after the promiscuity filter were aligned with all the proteins of FAdV available in GenBank. Only the strictly conserved epitopes were selected; **Host proteome:** The peptides filtered at this point were aligned with the chicken proteome, to avoid selecting epitopes present in the host. **Surface accessibility:** The localization of the selected peptides in their corresponding native proteins was determined to verify if the peptide is exposed to the surface and thus could be

recognized by an antibody. This step was performed only for MHC-II peptides.

Design of the FAdV multi-epitopic protein and its encoding gene

The selected MHC-I and MHC-II peptides were concatenated linearly in two synthetic designed protein constructs, called "multi-epitopic proteins", one for the MHC-I peptides and the other for the MHC-II peptides. These proteins were designed avoiding the appearance of strong binder peptides in the junctions between two consecutive peptides. To produce the recombinant multi-epitopic proteins, ad-hoc designed genes encoding them were synthesized, including and optimization of the codon usage for E. coli K12, where these genes were expressed. The synthetic genes were cloned in pET28a-c plasmid, using the NcoI and XhoI restriction sites, including a six-His-Tag at the 3' end for affinity chromatography purification. These genes were synthesized by the company Genemed Synthesis Inc. (<http://www.genemedsyn.com/>).

Expression and purification of the FAdV MHC-I and MHC-II multi-epitopic proteins

The plasmids with the synthetic genes were inserted in E. coli competent cells (Novablue) by electroporation. The correctly transformed colonies were selected in petri dishes with Luria-Bertani agar culture supplemented with 30 µg/mL of kanamycin. To produce each MHC-I and MHC-II multi-epitopic protein, the transformed E. coli cells were induced by the addition of isopropyl-1- β -D-galactopyranoside (IPTG) 1mM. The expressed protein was isolated from the bacteria by thermal shock, sonication and centrifugation. The thermal shock consisted in incubation at -70°C during 10 min, then 37°C for 10 min, and this procedure was repeated three times. The sonication was performed using 15,000 g at 4°C during 20 min. The multi-epitopic proteins will be obtained in the no-soluble phase of the inclusion bodies. This phase was solubilized adding urea 8 M. The solubilized fraction was purified by affinity chromatography using His-trap columns. To remove any E. coli protein of low-med affinity to the His-trap columns, solutions with discrete incremental concentrations of imidazole were eluted in the column. The solutions were used in a progressively increasing imidazole concentration, until the multi-epitopic protein was eluted. The presence of the multi-epitopic protein was verified in a SDS-PAGE electrophoresis revealed with Coomassie blue. The resulting protein was aliquoted in siliconated tubes of low protein-retention in volumes of 500 µl and stored at -70°C.

Immunization of SPF chickens with the FAdV MHC-II multi-epitopic protein and antibody detection

Forty-eight, 3-week-old SPF chickens were selected and separated into four groups. Twelve were immunized subcutaneously once with 25 µg of multi-epitopic protein with QuilA adjuvant at day 1, while 24 chickens were immunized with the same volume of adjuvant, and the 12 remaining chickens remained with no immunization. The pre-immune sera were collected on the first day. Post-immune sera was collected at the second, third and fourth week for detection of antibodies

Serum antibodies to multi-epitopic proteins were detected using a standard ELISA assay. Briefly, after washing the fixed plates with the multi-epitopic proteins, 50 ml of the test serum diluted 1:10 in phosphate buffer pH 7.2 was added. After incubation at 37°C for 1 h, 100 µL horseradish peroxidase conjugate, diluted

1:1000, was added to each well and plates were further incubated at 37° C for 1 h. After two washing steps, 100 mL of TMB substrate was added and incubated at room temperature for 10 min. The reaction was stopped by adding 100 ml of H2SO4 2M. Absorbance was measured at 490 nm with a micro ELISA reader (Bio-Rad).

Results:

Genome sequence

The full genome of FAdV-C was found to be 45,673 bp with a G+C content of 54.63%, and very similar to the other FAdV strains, comprising a total of 44 coding genes.

Human MHC-I and MHC-II best chicken-substitute-alleles

We found nine human MHC-I alleles highly similar to the chicken MHC-I. These sequences were used in the MHCcluster, where by means of the heatmap were determined three human alleles as best substitutes. These alleles were HLA*B 40:06, HLA*B 41:04 and HLA*B 41:03 (**Figure 1A**). In the same way, 56 MHC-II human sequences similar to the alpha chain of the chicken MHC II were selected and analyzed by the heatmap of the MHCcluster. DRB1 alleles 1482, DRB1:1366, DRB1:1310 and DRB1:1445 were found as the best human substitute alleles of the chicken MHC class II (**Figure 1B**).

FAdV MHC-I and MHC-II peptides predicted as immunogenic in chicken

After all the analyses and filters described before, a list of 29 MHC-I peptides and 9 MCH-II peptides from FAdV were selected as potentially immunogenic in chicken (**Table I**)

Immunogenicity of FAdV MHC-II multi-epitopic protein in chickens

Antibody stimulation experiments were conducted using the multi-epitopic MHC-II protein. Chickens were immunized and anti- MHC-II-multi-epitopic-protein antibodies were measured. Compared to the control group, a significant increase in the anti MHC-II-multi-epitopic protein antibodies was observed in the immunized group four weeks after the first immunization. After the booster, the anti-MHC-II-multi-epitopic protein antibodies level increased notably and remained at a high level throughout the duration of the experiment. This confirms that the immune system of the chicken was activated by the immunization with the MHC-II multi-epitopic protein (**Table 2**).

Discussion:

This study shows for the first time an immunoinformatics analysis of the complete FAdV genome to determine potential MHC-I and MHC-II immunogenic peptides for the chicken immune system. The genome sequence of the Peruvian FAdV strain confirmed its global conservation while clustering within other strains. Linear peptides with predicted high affinity to the Cobb chicken MHC-I and MHC-II complexes were found to also meet criteria of specie specificity, MHC allele promiscuity, and conservation through the FAdV genome sequences analyzed here. This is an important evidence for these peptides to be potentially immunogenic [23]. In the case of MHC-II linear T-cell predicted peptides, these were filtered for surface accessibility. MHC-II linear epitopes that are exposed to the surface are potential candidates to elicit a neutralizing antibody response, which is important during viral infections, in order to control the circulating viruses [24,25].

Our methodology has many similarities with other studies that perform the prediction of MHC-epitope affinity. Rai et al. prioritize the proteins that are secreted or exposed on the cell surface, because these are more accessible to the immune system [26]. Molero-Abraham et al. evaluates the variability of the sequences of the epitopes to select the most conserved [24]. And Moise et al. applied the filter of the host proteome, to avoid the tolerance that may generate autoimmunity. And also selects the epitopes with high affinity to different MHC alleles (promiscuity) [27].

The prediction of conformational B-cell epitopes is still under development, and the results are not as accurate as the corresponding to linear T-cell MHC-I and MHC-II epitopes [28]. However, it is important to remark that linear T-cell MHC-II peptides that are located on the surface of a protein are likely to correspond to the main linear component of the B-cell conformational epitopes [29]. Although the recognition of peptides with high affinity to the MHC-I or MHC-II complex is necessary to define an epitope, this is not a sufficient condition. In order to achieve a complete immune stimulation, it is necessary that once the peptides are presented by the MHC-I / MHC-II complex in the surface of the antigen presenting cells, they need to be recognized by the T-cell receptor of CD8/CD4 T-lymphocytes [30,31]. Although this last requirement could be determined experimentally, there are no approaches available yet to predict this in-silico. Current immunoinformatics efforts are devoted to create predicting tools of T-cell receptor recognition. In conclusion, we predicted potentially immunogenic T linear MHC-I and MHC-II epitopes from FAdV for the chicken immune system. We confirmed that the MHC-II multiepitopic protein was able to elicit specific antibodies. The finding of a group of peptides with high affinity to the Cobb chicken MHC-I and MHC-II complexes, that meet the conditions of conservation, promiscuity, surface accessibility and specificity is an important step towards the development of novel antigens that will help the immunodiagnostics and vaccine development against this disease in chicken.

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Supplementary material:

Table I: List of the potential MHC-I and MHC-II FAdV immunogenic peptides for the chicken immune system, predicted by the immunoinformatics approach described in this study

Nº	Peptide Sequences	Bind Level	MHC	Nº	Peptide Sequences	Bind Level	MHC
1	TELSYQYML	SB	I	1	SEAAKAAAA	WB	I
2	KESLSRFVV	SB	I	2	IEMTYEEAT	WB	I
3	IEYHLTLAI	SB	I	3	SESPDAFPA	WB	I
4	TEYLGAVAV	SB	I	4	QESLFKYLM	WB	I
5	SELELMAEL	SB	I	5	MEAPLLPPI	WB	I
6	MEWCMDTAL	SB	I	6	QEFVFPYPRF	WB	I
7	REHLASVPI	SB	I	7	TELGWIVKL	WB	I
8	FEFQHEDSI	SB	I	8	SETAPGQNV	WB	I
9	RELNLCLPA	SB	I	9	AEREASDTA	WB	I
10	LEFINGRAL	SB	I	10	GENSLTSGL	WB	I
11	RENWFYQLL	SB	I	11	TEISLKMVL	WB	I
12	AEISPITPL	SB	I	12	SELEYGGPS	WB	I
13	SELAFHLQL	SB	I	13	LELMLRNAT	WB	I
14	HEREYVLSV	SB	I	14	AELADSDVL	WB	I
				15	KEYCEVLYL	WB	I

Table 2: Antibody titers estimated as optical density (OD) elicited in the control and vaccinated groups.

		MHC-II multi epitopic protein		P-value
		Control		
OD Mean (OD SD)	2nd Week	0.252 (0.124)	0.324 (0.222)	0.381
	3rd Week	0.237 (0.118)	0.404 (0.271)	0.050
	4th Week	0.258 (0.141)	1.728 (1.569)	0.024

A greater difference in the optical density (proportional to antibody titer) is observed at the third and fourth week in comparison with the control group.