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RESEARCH

Epitope characterization, docking and molecular dynamic simulation studies on two main immunogenic Canarypox virus proteins

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ABSTRACT

The Canarypox virus (CNPV) infects captive and wild canaries and cause high mortality and substantial economic losses especially in Middle East countries. Currently, unapproved and illegally imports of embryo propagated, freeze-dried, and live CNPV vaccines are being used by individuals for personal uses in Iran and the region against canarypox disease. The aim of this work was to prepare the stage for the design of a peptide vaccine against canarypox disease. Two immunogenic CNPV proteins were chosen based on homology to antigens from Poxine®, HP1-440 and FP9 strains in fowlpox virus. MHC II specific epitopes of candidate proteins were characterized using various bioinformatics tools. The predicted epitopes were modeled and docked to HLA-DRB1 0101, 0301, 0401, 0405 and 1501 receptors. The stability of docked complexes was evaluated through molecular dynamic simulations. Also, an experimental epitope in vaccinia virus for MHC I receptors was chosen and its canary homolog was docked to two BF receptors in chicken. Due to the critical role of MHC class II in confronting with poxvirus, the IFNAIILWITYAL, LRQLYDVIIPPR, YYNRITSIHM and YRHDDIIAT epitopes were selected among 13 predicted epitopes for MHC class II receptors after docking and MD evaluations. Moreover, due to its long-lasting CD8+ T cell memory responses, the homolog of an experimental epitope from Vaccinia virus (VP35#1) in Canary was evaluated and proposed as potential epitope SLSAYIVSK. These candidate epitopes of high binding affinities may be considered to be included as most effective epitopes for designing epitope-based vaccines against CNPV infection.

Keywords: Canarypox virus, epitope prediction, dynamic simulation, docking, MHC I, MHC II

RESUMEN

Estudio de caracterización epitópica, acoplamiento molecular y simulación dinámica molecular de dos proteínas inmunogénicas principales del virus de la viruela del canario. El virus de la viruela del canario (CNPV) infecta a estas aves, con alta mortalidad y pérdidas económicas significativas especialmente en los países del Medio Oriente. Existen reportes de importaciones no autorizadas e ilegales de vacunas contra el CNPV propagadas en embriones, liofilizadas y vivas para su uso contra la enfermedad en Irán y en la región. El propósito de este trabajo fue preparar las condiciones para el diseño de una vacuna peptídica contra la enfermedad causada por el CNPV. Se seleccionaron dos proteínas inmunogénicas del CNPV homólogas a los antígenos de Poxine®, HP1-440 y las cepas de virus fowlpox. Se identificó a los péptidos específicos por el MHC II mediante varias herramientas bioinformáticas, los que se modelaron y acoplaron a los receptores HLA-DRB1 0101, 0301, 0401, 0405 y 1501. La estabilidad de los complejos acoplados se evaluó mediante simulaciones de dinámica molecular. También se seleccionó un epitopo experimental del virus Vaccinia para los receptores del MHC I, y su epitopo homólogo en CNPV se acopló a dos receptores BF de pollos. De los 13 epitopos predichos para los receptores del MHC II e identificados mediante acoplamiento y dinámica molecular se escogieron cuatro: IFNAIILWITYAL, LRQLYDVIIPPR, YYNRITSIHM y YRHDDIIAT. El epitopo homólogo al epitopo experimental VP35#1 del virus Vaccinia del canario se seleccionó, dada su respuesta de células T CD8+ de memoria de larga duración, y se propuso el epitopo SLSAYIVSK. Los epitopos candidatos de alta afinidad de unión pudieran incluirse como los más efectivos para diseñar vacunas peptídicas contra la infección por el CNPV.

Palabras clave: Virus de la viruela del canario, predicción de epitopo, simulación molecular, acoplamiento molecular, MHC I, MHC II

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Introduction

Avipoxvirus is a member of the family Poxviridae that infects in particular non-avian species [1, 2]. Avian poxvirus infection is observed in more than 232 species of wild birds all over the world [2-4]. Among them, canarypox virus (CNPV) is the etiologic agent of canarypox, and the causative agent of viral disease of wild and captive birds that can cause significant losses. While live CNPV vaccines have proven to be an alternative against canarypox disease, there are currently several limitations for its application: i) unapproved and illegally imports in some countries; ii) need of cold chain to maintain vaccine potency and of skilled health care workers which adds extra cost; iii) the risk of infection by the live vaccine in canary when getting into contact with other animal areas aside the injection site. Hence, investigation of the best prevention method and strategy for CNPV infection is required [5].

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In this scenario, peptide vaccines have been recently explored in several studies as a plausible alternative to live attenuated vaccines, due to their easy production, high chemical stability and lack of infectious potential [6]. In fact, peptide vaccines are intended to present appropriate B- and T-cell epitopes to stimulate the adaptive (specific) immune system and subsequently induce immune protective and long-lasting immune responses [7].

Regulation of cellular communication in the immune response is a critical function of the avian major histocompatibility complex (MHC) [8, 9]. The chicken MHC (B-complex) gene cluster is located on a micro chromosome (chromosome 16) and contains MHC class I (B–F) and class II (B-LB) genes that are similar to those of the mammalian species in the encoded protein structure [10]. Furthermore, MHC class II has been shown more determinant than other molecules involved in specific immunity in immunization of vertebrates against poxviruses [11-13]. However, there are no data on the most immunologically relevant MHC II epitopes in CNPV proteins for using them to design CNPV peptide vaccines in canary.

In this regard, Boulanger *et al*. [14] showed that production of monoclonal antibodies (MAbs) in immunized chicken by fowlpox virus (FWPV) strains allowed the identification of three immunodominant FWPV proteins: a 39-kDa core protein (encoded by FPV168 ORF) a 30-and 35-kDa protein doublet, and a 63-kDa protein (encoded by FWPV 191ORF). Their results indicated that two of them, the 39-kDa and 63-kDa proteins, were also recognized by anti-CNPV polyclonal serum which obtained by immunized chicken through CNPV [14-16]. Comparison of FLPV168 and FWPV191 ORFs with genome of canarypox virus [17] showed that these two coding sequence are homologous to CNP241 and CNPV265 ORFs, respectively [17].

Advantageously, the progress of bioinformatics techniques and applications opened a new field in immunology called immunoinformatics [18], which is considered as a subject area of bioinformatics [19]. In this regards, B and T-cell epitopes could be predicted using computational approaches for different purposes such as: antibody production, immune diagnostics and epitope-based vaccine design [20, 21].

Therefore, in this work, the MHC class II epitopes associated with CNPV241 and CNPV265 ORFs were established using in silico approaches. The epitopes were modeled and a docking study was carried out on these epitopes with human MHC receptors to study their binding affinities.

Materials and methods

Determination of CNPV immunogenic proteins

Two immunogenic canary proteins were used, that had been previously determined by producing monoclonal antibodies in several animals [14]. These two proteins are encoded by CNPV241 and CNPV265 ORFs, homologs to FWPV168 and FWPV191 in FWPV and also A4L and A26/27 in Vaccinia virus [17]. CNPV241 and CNPV265 ORFs code for 39-kDa and 63-kDa proteins, respectively. The sequence of these two proteins were retrieved from the NCBI database with NP_955264.1 and NP_955288.1 accession numbers, respectively.

MHC class II epitope prediction

T-cell epitopes against MHC class II receptors were predicted by uploading the respective sequences of the two CNPV proteins into three epitope prediction servers and analyzing it through three different algorithms. First, protein sequences were uploaded to the SYFFPEITHI server [19], which benefits from a Motif Matrices (MM) algorithm. Afterwards, PROPRED server [22] with a QM (Virtual QM) algorithm was applied for these two proteins and finally, IEDB server [23] with ANN-regression and SMM-QM algorithms were used. Due to the lack of special epitope prediction servers for avian alleles and also high homology between HLA-DR1 and HLA-DR4 to B-LB alleles, epitope prediction were carried out against HLA-DRB1 (0101, 0301, 0401, 0701, 0802, 0405, 1501) receptors [15].

The final epitopes which were predicted by all three servers against MHC class II receptors were validated using VaxiJen 2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), an alignment-independent prediction of protective antigens.

Epitope modeling and dynamic simulation

For docking analysis, 3D structures of predicted epitopes were needed. In this regard, the amino acid sequences of epitopes were modeled by PEP-FOLD as an online webserver, a de novo approach for predicting peptide structure [24].

The predicted structure of epitopes was used as input for 2 ns MD simulations, this short time applied due to the short length of peptide sequences. All MD calculations were done in water cubic boxes using GROMACS 5.0.1 [25], GROMOS 54a7 [26] protein force field and well-tested SPC/E model for water molecules. Proteins were solvated in explicit solvent box with 1.0 nm distance from each box wall with periodic boundary conditions. Charges of each simulation box were neutralized using Na^+ and Cl^- ions. The Particle Mesh Ewald (PME) summation method was used for calculating the total electrostatic energy in each periodic box. The other non-bonded interactions were calculated by L-J model with a cutoff distance of 10Å. A steepest-descent algorithm was used to minimize the energy of each system and to relax the solvent molecules. The LINCS algorithm [25] was applied to fix the chemical bonds between the atoms of the protein and SETTLE algorithm in the case of solvent molecules. To maintain a constant temperature (312 ºK, 39 ºC) and pressure of each system during simulations pressure and temperature both were applied using the Berendsen coupling algorithm [25]. A weak-coupling algorithm was used for the temperature and pressure regulation with a coupling time of 1.0 ps.

Docking

The Gasteiger charge and polar hydrogen were added to the peptides and receptors using the Chimera 1.11.2 [27]. The epitope-receptor pairs were docked by PYRX-Autodock Vina [28] and MOE software [29].

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The high rank predicted epitopes for MHC class II receptors were docked to human HLA-DRB1 receptors (0101, 0301, 0401, 0405 and 1501) with similar grid size and grid center parameters (Table 1). Moreover, to choose a proper MHC class I epitope, first an experimental epitope from H3L protein of vaccinia virus was defined as candidate, namely VP35#1, which was previously considered as a dominant epitope for evoking CD+8 T cells [16, 12]. Secondly, the homolog of this epitope in Canary poxvirus (SLSAYIVSK) was retrieved (NP_955209.1) and modeled by PEP-FOLD webserver. Afterwards, the modeled epitope was docked to the groove binding site of BF2*2101 and BF2*0401 as two main chicken MHC class I receptors (MMDB ID: 61647/PDB ID: 3BEW and MMDB ID: 105232/PDB ID 4G42, respectively). The epitopes with lower ΔG (higher binding affinity) and more stable RMSD in docking and molecular dynamic studies were identified. Residue involvement of epitope-receptor complex was determined after evaluation of stability through MD simulations.

Validation for docking analysis

Considering that Pyrx is mainly a protein-ligand docking software, apart from validation of binding energies through MOE software, we have confirmed our docking study through experimental records. In the first step, nine complexes of MHC receptors consisting of three chicken MHC class I (4CW1, 3BEW and 4G42), three human MHC class I (3UTQ, 1A1N and 1ZHL) and three human MHC class II (1AQD, 2SEB and 5V4M) were retrieved from the RCSB database. The ligands were separated from the complexes and saved as distinct PDB files. Subsequently, receptors and ligands were docked by PYRX-Autodock Vina after appropriate preparation for docking. Each docking was repeated three times and the average of binding affinity (–ΔG) was presented as a quantitative result for docking validation. Relevant docked and crystallography complexes were aligned through Pymol software to visualize the capability of Pyrx software to perform MHC receptor-epitope docking properly, and also to prove the ability of this software to mimic natural pose of epitopes in the binding groove of MHC receptors.

The position of epitopes in the binding groove of MHC receptors were further confirmed by calculating the RMSD between the same epitopes, prior and after docking studies, through CE and Alignment algorithms. For more certainty, this alignment and RMSD calculation was done again by Chimera 1.11.2 under the Smith-waterman and needleman-Wunsch algorithms and results were compared.

Evaluation of stability for complexes by dynamics simulations

The stability of the docked complexes was further investigated by molecular dynamic (MD) simulations using the GROMACS 5.0.1 [30]. After a stepwise energy minimization and equilibration protocol the system (with SPC/E water) was submitted to a 5 ns simulation at 312 K and at 1 bar pressure (see Epitope Modeling and dynamic simulation for the detailed protocol). Accordingly, root-mean-square deviation (RMSD) and Radius of gyration (Rg) were plotted versus time during the 5 ns MD simulation. All graphical representations were constructed by PyMOL [31]. After ending simulation, the output data were analyzed according to root-mean-square (RMSD) and Gyration radius. Finally, the residue involvement of epitope-receptor complexes was determined by PyMOL software.

Table 1. Grid size and grid center for the MHC I and MHC II human HLA receptors in the docking process of predicted canarypox virus epitopes

Coordinates	Peptide/MHC I		Peptide/MHC II HLA-DRB1 allele			
Grid size	SLSAYIVSK BF2*0401	SLSAYIVSK BF2*2101	YYNRITSIHM 0405	YRHDDIIAT 0401	LRQLYDVIIPPR 0101	IFNAIILWITYAL 0101
x	39.788	29.920	30.788	50.1210	27.9720	27.9720
y	33.333	41.150	43.333	54.1200	29.5760	29.5760
z	34.184	29.750	30.184	77.1830	41.1750	41.1750
Grid center						
x	–28.036	11.175	12.036	3.5744	12.3750	12.3750
y	0.270	16.044	85.237	111.5000	25.0540	25.0540
z	–28.796	58.347	45.296	29.7020	37.1070	37.1070

Results

Prediction and modeling for MHC Class II and I epitopes

During the study, no clinical symptoms were shown The highest ranks of predicted MHC class II epitopes were selected and listed in the Table 2. For MHC class I epitopes, two epitopes from canary poxvirus and Vaccinia virus were selected, modeled and dynamically simulated (Table 3).

Docking

The results of molecular docking for the best selected MCH class II and MHC class I epitopes are shown in Table 3. The position of the modeled and VP35#1 epitope in the HLA*A-0201 antigen- binding groove was depicted in the figure 1.

There was selected the highest ranks of candidate epitopes which had maximum binding affinity in three runs of docking. The list of the residues which had the major role in the binding affinity for MCH class II and

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Table 2. Candidate MHC class I and II canarypox virus epitopes

Receptor	Epitope	Receptor allele	Vaxijen rank	Position	Protein ID
MHC I	SLSAYIIRV	HLA-A 0201.	Experimental	45-53	NP_955288.1
	SLSAYIIRV	HLA-A 0201.	Modeled	153-166	NP_955288.1
	SLSAYIVSK	BF2*2101	Modeled	157-166	NP_955288.1
	SLSAYIVSK	BF2*0401	Modeled	156-167	NP_955288.1
MHC II	HRYYNRITSIHMRFR	HLA-DRB1-0101	1.5952	155-169	NP_955288.1
	LRQLYDVIIPPR	HLA-DRB1-0101	0.6232	202-214	NP_955264.1
	IFNAIILWITYAL	HLA-DRB1-0101	0.5406	179-191	NP_955264.1
	MRFRCKYMF	HLA-DRB1-0301	1.5641	166-174	NP_955288.1
	YRHDDIIAT	HLA-DRB1-0301	1.4542	252-260	NP_955288.1
	LRQSMVNLA	HLA-DRB1-0301	0.7844	453-460	NP_955288.1
	YRHDDIIAT	HLA-DRB1-0401	1.4542	252-260	NP_955288.1
	YRHDDIIATET	HLA-DRB1-0701	1.2159	252-262	NP_955288.1
	RYYNRITSIHM	HLA-DRB1-0701	1.0078	156-166	NP_955288.1
	IFNAIILWITYALKE	HLA-DRB1-0701	0.6794	179-193	NP_955264.1
	INRYYACCI	HLA-DRB1-1501	1.3549	45-53	NP_955288.1
	IRHRYYNRITSIHM	HLA-DRB1-1501	1.0709	153-166	NP_955288.1
	YYNRITSIHM	HLA-DRB1-0405	1.0321	157-166	NP_955288.1
	RYYNRITSIHMR	HLA-DRB1-0802	1.3795	156-167	NP_955288.1

MHC class I are shown in tables 4 and 5, respectively. The candidate epitopes based on the binding affinity results were all in the correct position in the antigen-binding groove (Figures 2 and 3).

Validation for docking analysis

The results of docking validation indicated that our procedure for docking was appropriate and reliable (Table 6). The range of obtained binding affinity) –ΔG (for all MHC complexes were reasonable [32]. Considering that the box of our docking was large enough to let the epitope to interact with any site of receptor, all peptides were positioned in the docking groove which is the specific site for the presentation of epitope to the cellular immunity during the docking studies. In previous research, all the three epitope prediction servers that we used in this study were confirmed by our experimental data [33].

Moreover, the alignment visualization of docked complexes and relevant crystallography complexes which were done by Pymol software (supplementary materials), proved that Pyrx software can dock the epitopes exactly at the same position of crystallography complexes. On the other hand, the RMSD between the same epitopes, prior and after docking studies (Table 6), revealed that epitopes changed their conformation during docking studies but are still in the appropriate position (supplementary data).

Molecular dynamic analysis

The alignment of IFNAIILWITYAL-HLA-DRB1-0101 complex (as an example for all six complexes) before and after molecular dynamic simulation (RMSD: 2.5) revealed that the 3-D structure of this complex has not changed much during simulation (Figure 4A). Molecular dynamic analysis for all six complexes revealed the variation range for backbone's RMSD and the radius of gyration during 5 ns simulation were 0.1 (Figure 4B) and less than 0.1 (Figure 4C), respectively. These results proved the stability of all complexes during simulation.

Discussion

In the present study, a 39-kDa and 63-kDa proteins were selected which were considered as common FWPV antigens in several investigations [14, 34, 35]. Both of these antigens strongly evoke chicken's immune system. In this regard, Boulanger *et al*. also observed that the injection of these antigens induced high polyclonal antibody titers in canary blood [14]. Considering the fact that these two proteins are conserved among avian poxviruses [14], the homologous peptides in CNPV were selected and used in *in silico* analysis aim for peptide vaccine design.

Four epitopes were selected for MHC class II receptors after docking and MD evaluations. Choosing the MHC class II as the main receptors for our *in silico* analysis was based on the previous studies that indicated the role of MHC class II pathway in presenting Vaccinia virus epitopes to CD4+ T cells is critical in compare to humoral immunity and MHC class I pathway [11, 12, 36-38]. They showed B cell-deficient mice unable to generate antibodies and β2-microglobulin-deficient mice not expressing MHC class I molecules for a CD8+ T cell responses were both protectively vaccinated by modified vaccinia virus Ankara vaccine (MVA). Additionally, double-knockout mice for MHC II and I as well knockout mice for only MHC II could not be protected by MVA against a lethal strain of Vaccinia virus [12]. Moreover, in another study showing vaccination in HLA transgenic mice with a smallpox vaccine (VennVax), the T cell immune responses considerably stimulated [37]. No antibody response pre-challenge was observed,

Table 3. Details of docking studies for the best predicted canarypox virus epitopes for two different docking software

Parameters	MHC I peptide binding affinity (kJ/mol)/receptor/software			
	SLSAYIVSK BF2*2101		SLSAYIVSK BF2*0401	
	Pyrx	MOE	Pyrx	MOE
Run 1	–8.50	–18.236	–7.00	–16.465
Run 2	–8.60	–17.961	–7.50	–16.847
Run 3	–8.80	–17.532	–7.50	–17.492
Mean	–8.66	–17.906	–7.33	–16.934
Variance	0.023	0.122	0.083	0.260

	MHC II peptide binding affinity (kJ/mol)/receptor/software							
	YYNRITSIHM HLA-DRB1-0405		YRHDDIIAT HLA-DRB1-0401		LRQLYDVIIPPR HLA-DRB1-0101		IFNAIILWITYAL HLA-DRB1-0101	
	Pyrx	MOE	Pyrx	MOE	Pyrx	MOE	Pyrx	MOE
Run 1	–8.30	–18.236	–7.80	–16.966	–8.00	–19.125	–8.40	–19.612
Run 2	–7.70	–17.961	–7.80	–17.235	–8.00	–17.981	–9.30	–18.632
Run 3	–8.00	–17.532	–8.00	–17.782	–8.80	–18.365	–8.50	–18.163
Mean	–8.00	–17.906	–8.00	–17.327	–8.26	–18.490	–8.73	–18.802
Variance	0.09	0.12	0.01	0.17	0.21	0.33	0.24	0.54

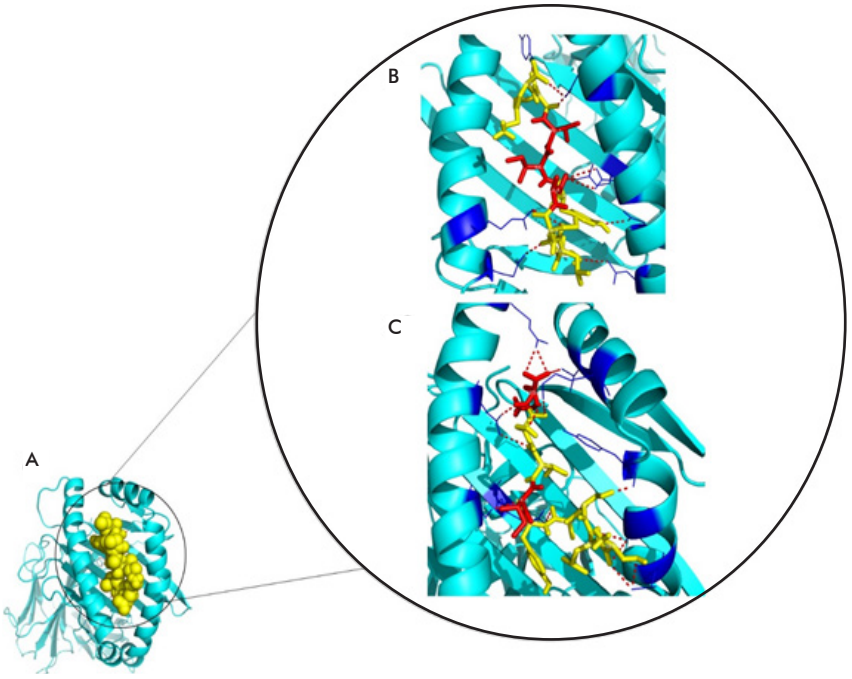

Figure 1. Structures of the docked modeled and VP35#1 epitope in the HLA*A-0201 antigen-binding groove of MHC class I complex. A) MHC I docked complex. B) Experimental epitope (VP35#1) SLSAYIIRV _ HLA*A-0201 complex. C) Modeled SLSAYIIRV _ HLA*A-0201 complex. Dark blue: the residues of alleles that interact with the epitope. Yellow: The residues of epitopes that interact with the receptor. Red (dotted lines): Hydrogen bonds between alleles and epitopes.

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Table 4. List of residues of Canarypox virus epitopes and MHC II receptor alleles playing a major role on its binding affinity

Receptor HLA-DRB1-0101	Peptide IFNAIILWITYAL	Receptor HLA-DRB1-0101	Peptide LRQLYDVIIPPR	Receptor HLA-DRB1-0405	Peptide YYNRITSIHM	Receptor HLA-DRB1-0401	Peptide YRHDDIIAT
ASN (258)	ILE (1)	GLN (246)	ILE (1)	SER (53)	TYR (1)	SER (53)	TYR (1)
ASN (258)*	ILE (1)*	ASN (60)	ARG (2)	ASN (82)	TYR (2)	ASN (82)	ARG (2)
HIS (257)	ILE (1)	ASN (60)*	ARG (2)*	ASN (82)*	TYR (2)*	ASN (82)*	ARG (2)*
SER (51)	PHE (2)	GLY (56)	ARG (2)	GLN (9)	ARG (4)	THR (77)	ARG (2)
SER (51)	ASN (3)	N (258)	LEU (4)	ASN (62)	ARG (4)	GLY (58)	HIS (3)
ASN (258)	ASN (3)	HIS (256)	LEU (4)	GLN (9)*	ARG (4)*	GLN (9)	ASP (4)
ASN (258)*	ASN (3)*	ASN (258)	TYR (5)	LYS (71)	THR (6)	GLN (9)*	ASP (4)*
GLN (7)	ILE (5)	ASN (258)*	TYR (5)*	TYR (30)	THR (6)	LYS (71)	ASP (4)
GLN (7)*	ILE (5)*	ASN (258)*	TYR (5)*	ASN (62)	THR (6)	LYS (71)	ASP (5)
ASN (60)	ILE (5)	ASN (67)	ASP (6)	ASN (62)*	THR (6)*	GLN (70)	ILE (7)
GLN (7)	ILE (5)	GLN (7)	ASP (6)	GLN (64)	HIS (9)	GLN (70)	THR (9)
ARG (247)	ILE (6)	GLU (9)	ASP (6)	TYR (60)	MET (10)		
ARG (247)*	ILE (6)*	GLN (7)*	ASP (6)*	GLN (64)	MET (10)		
ASN (67)	TRP (8)	GLN (7)*	ASP (6)*				
TRP (237)	ILE (9)	GLN (246)	ILE (8)				
ASN (67)	THR (10)	ASN (60)	ILE (8)				
ASP (233)	TYR (11)	GLN (246)	ILE (9)				
ILE (70)	ALA (12)	ARG (247)	ILE (9)				
ARG (74)	ALA (12)	ASN (67)	PRO (11)				
ARG (74)*	ALA (12)*	TRP (237)	ARG (12)				
		ASP (233)	ARG (12)				
		ASN (67)	ARG (12)				

* Repeated receptor and epitope residues pairs indicate their identification with different interactions with several other residues.

neither against whole vaccinia antigens nor vaccine epitope peptides. Remarkably, 100 % of vaccinated mice survived lethal vaccinia challenge, demonstrating that protective immunity to vaccinia does not require B cell priming [36].

Although MHC II pathway has demonstrated to play a critical role in establishing prevention against Poxvirus infections, Drexler *et al*. reported that the MHC I is also relevant in poxviruses prevention due to long-lasting memory cytotoxic T cell (CTLs) responses in comparison to helper T cells [12]. Therefore, in other section of our investigation we used the Canary homologous protein of epitope VP35#1 which was an experimental epitope able to induce high levels of CD8+ T cells in human blood sera [12, 16].

The sequence of canary MHC I alleles is barely available while there is no data for MHC II alleles due to its significant variability. Canary MHC II alleles have been searched for extensively, with unsuccessful results. The same problem has been found with MHC II for FWPV and in sheep for MHC I alleles [39]. There seems that this experimental problem would not be satisfied unless further report on the canary MHC allele frequencies will be available. Considering this, epitopes were predicted for human alleles, despite the lack of information on relevant canary MHCII alleles and their frequency. Notably, canary was chosen based on its relevant geographic distribution in the Middle East, being further proposed these epitopes as probable candidates. Due to the absence of the 3-D structure of Canary MHC class II receptor, we used HLA-DR1 and HLA-DR4 alleles in epitope prediction and their X-ray structures for docking and molecular dynamic analysis. The selection of HLA-DR1 alleles in our study was based on previous investigations which similarly used HLA-DR1 instead of chicken B-L alleles due to the lack of 3-D structure for MHC II chicken alleles and also structural similarities between B-L and HLA-DR1 alleles [15, 40]. In this regard, we used HLA-DR1 and HLA-DR4 alleles due to their high similarity to Canary (approximately, 70 % identity (NCBI data base: Canary: XP_018781121.1; Human: ARB08440.1, P01903.1).

Table 5. List of residues of Canarypox virus epitopes and MHC I receptor alleles playing a major role on its binding affinity

Receptor BF2*0401	Peptide SLSAYIVSK	Receptor BF2*2101	Peptide SLSAYIVSK
GLU (169)	SER (1)	GLN (155)	SER (1)
ASN (76)	SER (1)	GLN (155)*	SER (1)*
ARG (152)	LEU (2)	GLY (152)	SER (1)
ARG (152)	SER (3)	GLY (152)	LEU (2)
ASN (69)	TYR (5)	TRY (149)	SER (3)
ASN (69)*	TYR (5)*	ARG (9)	ALA (4)
ARG (9)	TYR (5)	SER (69)	TYR (5)
GLN (62)	SER (8)	ASN (76)	VAL (7)
GLN (7)	ILE (5)	LYS (143)	SER (8)
TYR (168)	SER (8)	LYS (143)*	SER (8)*
GLN (62)	LYS (9)	ASN (76)	LYS (9)
		THR (140)	LYS (9)
		ARG (83)	LYS (9)
		ARG (83)*	LYS (9)*

* Repeated receptor and epitope residues pairs indicate their identification with different interactions with several other residues.

In order to validate the Pyrx software for protein-peptide docking, the RMSD between docked and relevant crystallography complexes were not calculated, because the macromolecule, which is MHC receptors, would remain constant during docking studies and the only flexible component is epitope. According to the size of epitopes in comparison with receptors, these changes would be considered insignificant. So, we just visualized the considerable similarity between the pose of epitopes in these structures (the same

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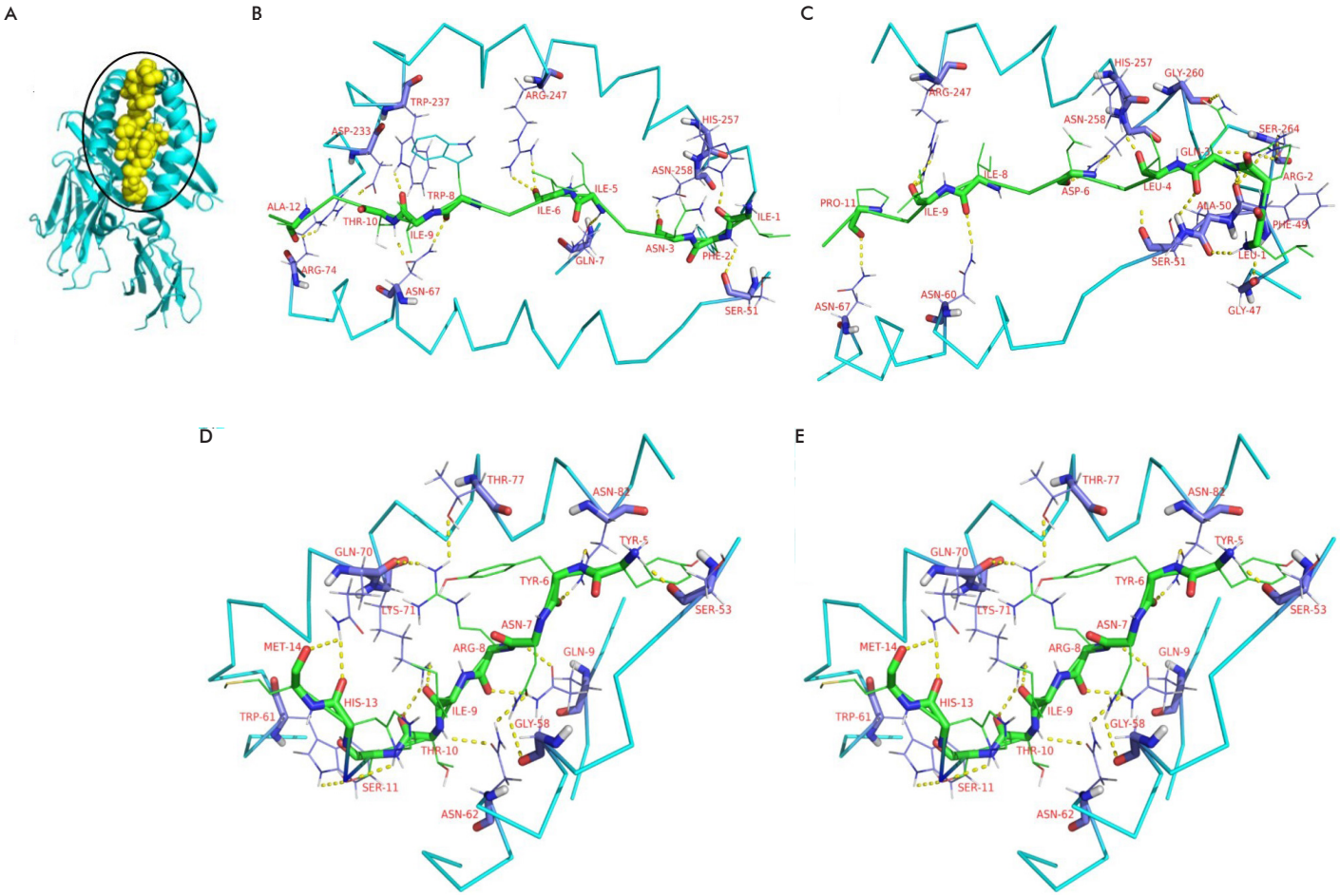

Figure 2. Structures of the docked MHC class II-Canarypox virus epitope complexes. A) MHC II docked complex. B) Epitope IFNAIILWITYAL-HLA-DRB1-0101 MHC II receptor complex. C) Epitope LRQLYDVIIPPR-HLADRB1-0101 MHC II receptor complex. D) Epitope YYNRITSIHM-HLA-DRB1-0401. E) Epitope YRHDDIIAT-HLA-DRB1-0405 MHC II receptor complex. Cyan color: residues of the MHC II receptor allele. Green: epitope residues. Stick shapes: residues from epitopes and receptors that have interaction with each other. Yellow (dotted lines): hydrogen bonds between alleles and peptides.

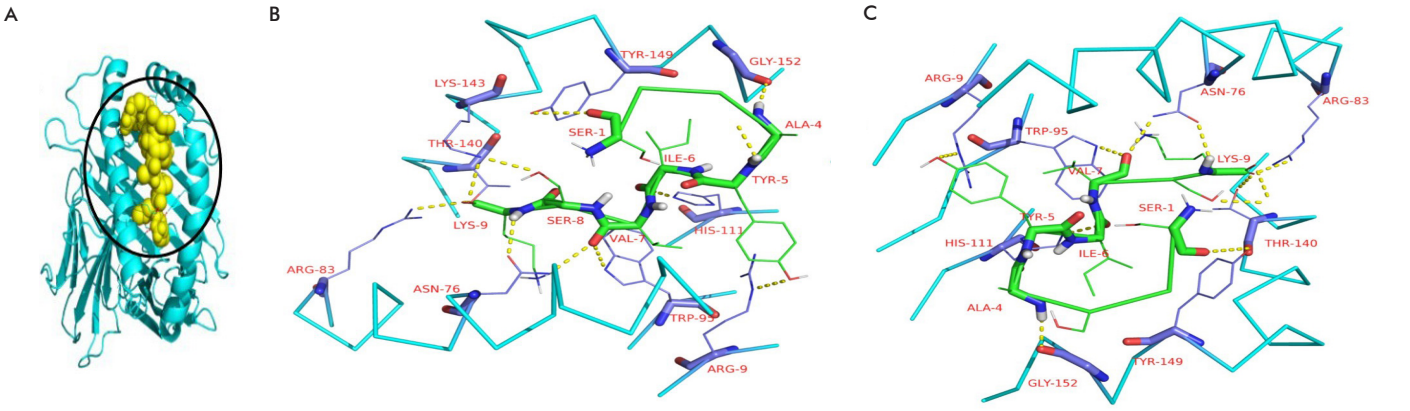

Figure 3. Structures of the docked MHC class I-Canarypox virus epitope complexes. A) MHC I docked complex. B) Epitope SLSAYIVSK and chicken MHC I allele BF2*0401. C) Epitope SLSAYIVSK and chicken MHC I allele BF2*2101. Cyan color: the residues of alleles. Green: the residues of epitopes. Stick shapes: residues from epitopes and receptors that had interaction with each other. Yellow (dotted lines): Hydrogen bonds between alleles and peptides.

crystallography and docked complexes) by alignment procedure. In addition, we have calculated the RMSD between the same epitopes, prior to and after docking. The significant changes of RMSD were due to the linear structure of epitopes. But interestingly, in spite of changing the structure, the pose of epitopes in the binding groove of MHC receptors were the same prior and after docking. This fact revealed that structural

changes in a linear epitope are inevitable while the essential parameter is the correct placing of the epitope in the binding groove of the MHC receptor as to be presented to CD4+ and CD8+ T cells.

Hence, four of 14 candidate epitopes were selected from epitope prediction analysis, which had the best ranking score through molecular docking and dynamic simulation studies. One of the main features for proper epitope is correct placing of its side chains of 'anchor' residues. Pocket 1, 4 and 9 residues are the most important [41-46] for side chain-binding pocket in HLA-DR1. In this regard, for the IFNAIILWITYAL epitope, ILE-1 and PHE-2, ILE-6, THR-10 and ILE9 were located in pocket 1, 4 and 9, respectively. In addition, in the LRQLYDVIIPPR epitope, ARG-2, ILE-8, ILE-9 and PRO-11 were located in pocket residues 1, 3, 4 and 6, respectively. In the YRHDDIIAT, related to HLA-DR4, TYR-1, ASP-5 and ASP-4 were collated in pocket 1, 6 and 7. In the HLA-DR4, a previous study indicated that locating GLY in position β86 markedly affected T cell recognition [47-49]. By placing GLY in β86 position of our receptor, TYR-1 could be located in pocket 1 due to its large side chain and GLY small side chain. This situation in the complex could be lead to better T cell recognition. In the YYNRITSIHM epitope, related to HLA-DR4, TRP-5, TYR-6, ILE-9, SER-11 and THR-10 were located in pocket 1, 2, 4, 7 and 6. Dessen *et al.* [49] reported that the side chain of position 2 Arg in epitope ligated to HLA-DR4 extends across the binding cleft, so that it can be better recognized by a T cell receptor [49]. This result was consistent to our positioning analysis for the epitope YRHDDIIAT - HLADRB1-0405 complex (Figure 4 D). Overall, these results suggested all four candidate epitopes for MHC class II not only had strong binding affinity but also located in correct positions in determined pockets.

In chicken MHC class I BF2*2101, anchor residues are found usually at peptide position 2 interacting with pocket B, sometimes at position 5 or 6 interacting with pocket C or E, and usually at the C-terminal residue binding in pocket F [50]. In this regard, in SLSAYIVSK epitope ILE-6 was positioned in pocket C and LYS-9 and also VAL-7 was located in pocket F. Moreover, in this epitope TYR-5, SER-8 and LYS-9 were placed in pocket B of BF2*0401. Generally, the results of positioning analysis for epitope residues in chicken MHC class I revealed correct locating of epitope in determined pockets [50].

Several studies investigated the dynamics of peptide binding in the MHC binding groove by means of MD simulations [13, 51-53]. Generally, these studies suggested that a low-affinity peptide or the absence of a peptide widens the groove. In our work, results from RMSD and radius of gyration in MD simulation analysis showed that all complexes had minimum changes during simulation in comparison to starting structures (docking complexes). Therefore, these evidences suggested that all complexes were stable due to high-affinity epitopes locating in binding groove.

In conclusion, due to the risks for the application of CNPV attenuated vaccines, considerable efforts have been currently performed in designing and production of a recombinant vaccine. For that purpose, we propose for the first time five potent and reliable CNPV epitopes identified by computational analysis, which could be further validated in experimental immunization studies. In this regard, further selection of immunodominant antigens and reliable epitope characterization are critical steps for developing effective recombinant vaccines. There is ongoing research to try to achieve these goals.

Table 6. List of residues of Canarypox virus epitopes and MHC I receptor alleles playing a major role on its binding affinity

MHC class (species)	PDB entry	Receptor	Ligand	RMSD (nm)	Average binding affinity (-ΔG)
I (chicken)	4CW1	BF2*1401	SWFRKPMTR	0.771	–9.9 ± 0.2
	3BEW	BF2*2101	REVDEQLLSV	0.406	–7.4 ± 0.1
	4G42	BF2*0401	IDWFDGKD	0.243	–8.7 ± 0.1
I (human)	3UTQ	HLA A 0201	ALWGPDPAAA	0.138	–10.2 ± 0.2
	1A1N	HLA B 3501	VPLRPMTY	0.269	–10.3 ± 0.3
	1ZHL	HLA B 3508	LPEPLPQGQLTAY	1.896	–9.6 ± 0.1
II (human)	1AQD	HLA DRB1 0101	GSDWRFLRGYHQYA	1.897	–9.5 ± 0.2
	2SEB	HLA DRB1 0401	AYMRADAAAGGA	1.406	–8.5 ± 0.3
	5V4M	HLA DRB1 1501	GWISLWKGFSF	0.372	–8.6 ± 0.4

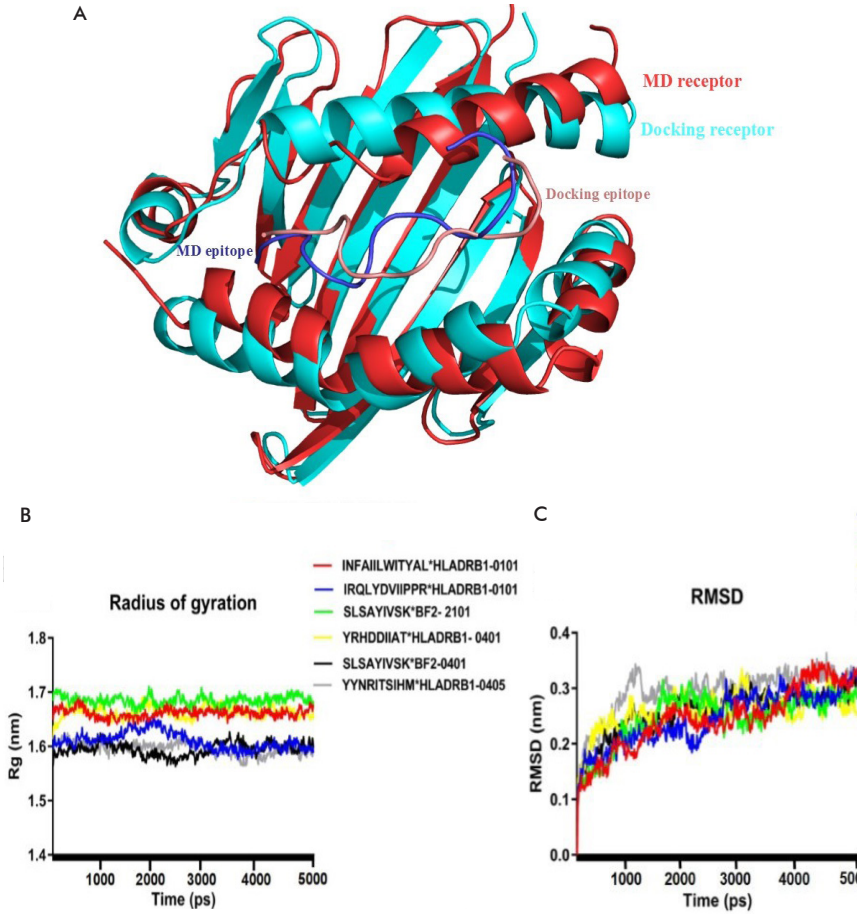

Figure 4. Complex stability analysis using molecular dynamic simulation for all docking complexes of canarypox virus (CNPV) candidate epitopes and the human MHC I receptor. A) Alignment of docking complex of the CNPV epitope IFNAIILWITYAL and the HLA-DRB1-0101 receptor before and after MD simulation in water during 5 ns. B) Radius of gyration. C) RMSD analysis.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary information

Epitope characterization, docking and molecular dynamic simulation studies on two main immunogenic Canarypox virus proteins

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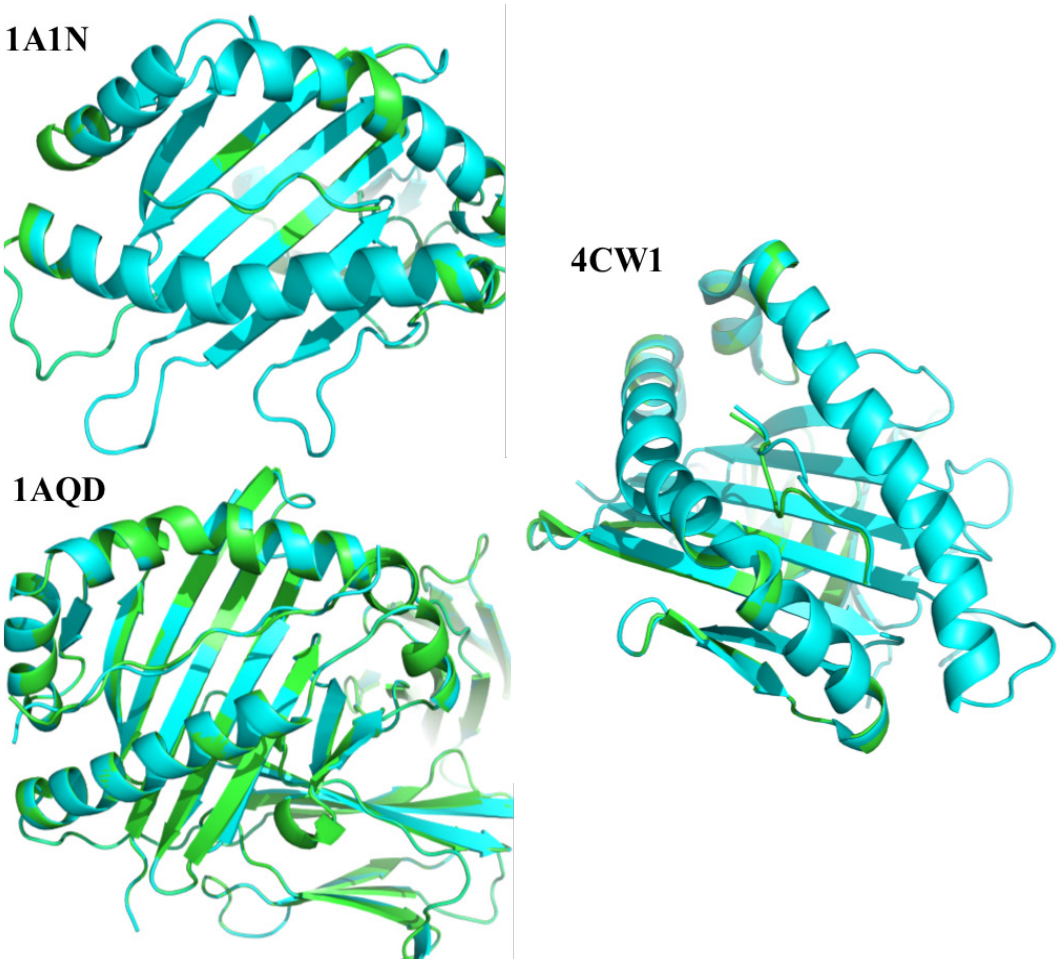

Supplementary material. Alignment of three pairs of epitope-receptor complexes. Docked and crystallography complexes are shown in green and blue respectively. 1A1N: MHC Class I (human). 1AQD: MHC Class II (human). 4CW1: MHC Class I (chicken).