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Integrative Structural & Immunoinfortmatics-Assisted Docking Analysis of VP1-TLR3 Interactions in Chicken Anemia Virus

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Abstract

Chicken anemia virus is a significant immunosuppressive pathogen of poultry that causes anemia, lymphoid atrophy, and increased susceptibility to secondary infections, leading to significant economic losses. The current study aimed to investigate the genetic diversity and structural characteristics of indigenous CAV isolates. VP1 gene sequences of indigenous CAV isolates OP11 - OP19 were amplified, sequenced, and comparatively analyzed with reference and vaccine strains using bioinformatics tools. Multiple sequence alignment and phylogenetic analysis were performed using MEGA 11. Alignment revealed amino acid substitutions at various positions of VP-1 Protein, indicating genetic variability among circulating isolates. Phylogenetic analysis showed that OP11 - OP17 clustered with international strains such as Egy-4-CAV and Arg728, whereas OP18 and OP19 were more closely related to the vaccine strain Del-Ross. Selected isolates OP12 and OP14 were further analyzed for structural and immunological studies. Three-dimensional structures predicted using AlphaFold2 showed conserved folding patterns, while epitope prediction identified surface-exposed antigenic regions. Docking Analysis of VP-1 protein with Toll-like receptor 3 indicated variations in binding patterns. Overall, the study highlights the genetic diversity and structural characteristics of circulating CAV isolates.

Keywords

Chicken Anemia Virus, 3D Protein Structure, Antigenic Epitope Mapping, Molecular Docking, TLR3

1. Introduction

Chicken infectious anemia (CIA) is an economically significant immunosuppressive disease of poultry, primarily affecting young chickens. The condition is marked by aplastic anemia, significant immunosuppression, growth retardation, and atrophy of lymphoid tissues, leading to increased susceptibility to secondary infections and poor vaccine responses. Consequently, CIA is responsible for significant economic losses to the global poultry industry. Chicken anemia virus (CAV), belongs to the genus *Gyrovirus* within the family *Anelloviridae*.

Chicken anemia virus is a small, non-enveloped virus with icosahedral capsid of about 23 - 25 nm in diameter. It has a negative-sense, covalently closed, circular DNA genome [1]. The compact genome of CAV contains three majors partially overlapping open reading frames (ORFs), designated as VP1, VP2, and VP3, with nucleotide lengths of approximately 1350, 651, and 366, respectively. These ORFs encode three viral proteins with distinct molecular weights and biological functions: VP1 (51.6 kDa), VP2 (24 kDa), and VP3 (13.6 kDa).

VP1 is the sole structural and major capsid protein of CAV which is essential for viral assembly, encapsidation of the single-stranded DNA genome, and interaction with host cells. Molecular characterization of the VP1 gene has shown the presence of a hypervariable region, particularly between amino acid positions 139 - 151, along with additional upstream amino acid substitutions. These variations contribute to genetic diversity, viral evolution, and differences in pathogenicity among CAV strains [2] [3]. Notably, amino acid substitution at position 394 of the VP1 protein has been identified as a significant factor of virulence. The presence of glutamine (Q) at position 394 is linked with highly pathogenic strains, whereas histidine (H) at the same position is indicative of moderately pathogenic strains.

VP2 functions primarily as a scaffold protein and helps in the proper folding of VP1 during viral particle assembly. In addition, VP2 exhibits protein phosphatase activity, which is considered to be important for viral replication. Co-expression of VP1 and VP2 has been shown to stimulate the production of neutralizing antibodies in the host, highlighting their importance in protective immunity and vaccine design. VP3, commonly referred to as apoptin, is a non-structural protein that triggers apoptosis in infected and transformed cells. Due to its selective apoptotic activity in cancerous cells, VP3 has also attracted attention as a potential anticancer therapeutic agent [4].

Based on genetic variability and phylogenetic analysis, CAV strains are divided into three major genotypes: I, II, and III. These genotypes exhibit varying geo-

graphic distributions and genetic divergence, which may influence viral pathogenicity, transmission dynamics, and vaccine efficacy [4] [5].

In recent years, bioinformatics approaches have become essential tools for understanding viral evolution and host pathogen interactions. In the present context, bioinformatics analyses; including phylogenetic reconstruction, three-dimensional protein structure prediction, physicochemical property analysis, antigenic epitope mapping, and molecular docking provide valuable insights into the molecular characteristics of VP1 protein from indigenous CAV isolates. Comparative analysis with the commercial vaccine strain Nobilis allows identification of genetic, structural, and antigenic differences that may influence immunogenicity and vaccine performance [6]. The aim of the present study is to investigate the genetic diversity, evolutionary relationships, and structural characteristics of indigenous isolates of Chicken anemia virus through comparative bioinformatics analysis of the VP1 gene.

2. Materials and Methods

2.1. Sequence Alignment and Phylogenetic Analysis

PCR amplicons of the VP1 gene from indigenous chicken anemia virus (CAV) isolates (OP11 - OP19) [7] were commercially sequenced by Apical Scientific Sdn. Bhd. (Malaysia). The obtained nucleotide sequences were analyzed using BLAST against sequences available in the GenBank database of the National Center for Biotechnology Information to confirm sequence identity and similarity with previously reported CAV strains.

Multiple sequence alignment of the VP1 amino acid sequences was performed using Clustal Omega [8] implemented in MEGA 11 [9]. The sequences were aligned with selected reference strains (Del-Ross/OP4, AO/77) and vaccine strains (Del-Ross, Cux-1, and Nobilis P4) retrieved from the GenBank database.

Phylogenetic analysis was conducted using the Maximum Likelihood method in MEGA 11. The reliability of the tree topology was assessed using 100 bootstrap replicates. The resulting phylogenetic tree was visualized and statistically evaluated within the MEGA 11 software environment.

2.2. Physicochemical Properties Analysis

ExPASy ProtParam calculated physicochemical properties of the predicted protein sequences, including molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, extinction coefficient, and grand average of hydropathicity (GRAVY), based on their amino acid sequences [10] [11]. The analysis was performed to determine the physicochemical characteristics of the predicted proteins.

2.3. Protein Selection and Structural Refinement

Protein sequences were retrieved in FASTA format from the UniProt database. AlphaFold2 was used to predict the three-dimensional structures of the proteins

using the Google Colab AlphaFold2 notebook [12]. The prediction utilized the amino acid sequence together with multiple sequence alignment (MSA) data. The predicted structures were further refined using the AMBER force field through energy minimization to optimize molecular geometry and reduce steric clashes. The final structures were visualized using PyMOL.

2.4. B-Cell Epitope Prediction

Conformational B-cell epitopes of the OP12 and OP14 constructs were predicted using the ElliPro server [13]. ElliPro, which has a high reliability with an AUC score of 0.732, identifies antibody-accessible epitopes from protein 3D structures. Using default parameters, five conformational B-cell epitopes were predicted from the refined 3D models, with scores ranging from 0.5 to 0.8.

2.5. Selection and Retrieval of Receptor

The Protein Data Bank provided the three dimensional structure of TLR3, *i.e.*, the receptor protein, based on literature and structural information [14] [15]. The 3D chemical structure was visualized and prepared for analysis using PyMOL [16].

2.6. Docking Analysis

Protein–protein docking was performed between the VP1 and Toll-like receptor 3. TLR3 has a three-dimensional structure that was obtained at the RCSB Protein Data Bank [14]. Docking was carried out using ClusPro to predict possible binding orientations and interaction conformations [17]. The docked complexes were subsequently visualized and analyzed using PyMOL to examine the interaction interfaces [16].

3. Results

3.1. Alignment

Multiple sequence alignment of the VP1 protein revealed several amino acid substitutions among indigenous CAV isolates compared with reference and vaccine strains. At amino acid position 57, indigenous isolates OP11 and OP12, along with reference strains Del-Ross/OP4 and AO/77 and vaccine strains Del-Ross, Cux-1, and Nobilis P4, exhibited valine (V) instead of isoleucine (I) as shown in **Figure 1**.

Substitutions were also identified at positions 79 and 121, where methionine (M) and lysine (K), respectively, were present in several strains including OP11, OP12, Del-Ross/OP4, Del-Ross, Cux-1, and Nobilis P4. At position 126, a substitution from glutamine (Q) to glutamate (E) was observed in the indigenous isolates OP11 and OP12 and the reference strains, whereas the vaccine strain Cux-1 showed aspartate (D) at this site, as depicted in **Figure 1**.

Amino acid position 107 was conserved as isoleucine (I) in OP11, OP12, and the reference strains, while variation was observed in isolates OP13 - OP19. Furthermore, analysis of positions 74 and 139 showed that indigenous isolates and

reference strains contained glycine (G) and valine (V), respectively, whereas the vaccine strain Nobilis P4 exhibited substitutions to aspartate (D) at position 74 and methionine (M) at position 139. At amino acid position 131, a substitution from cysteine (C) to methionine (M) was detected in OP11, OP12, and Del-Ross/OP4, while other strains retained cysteine (C) at this position, as illustrated in Figure 1.

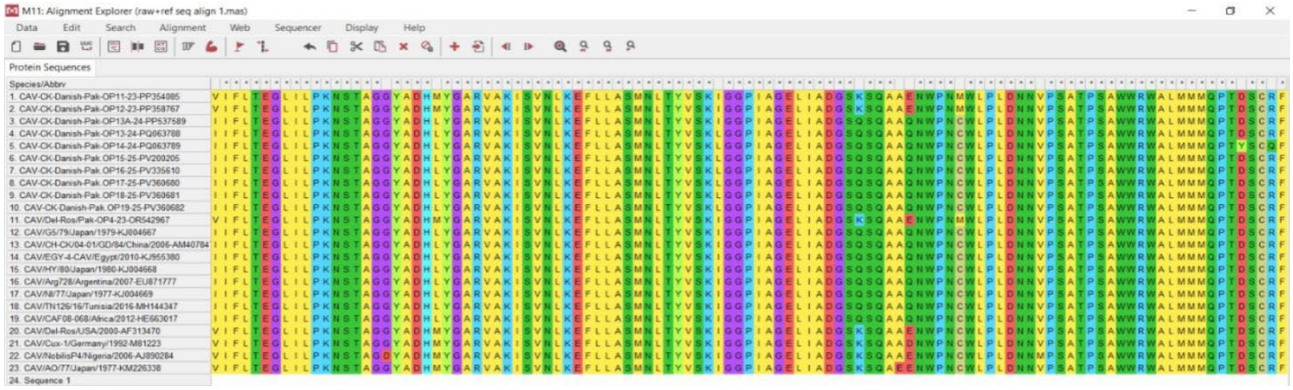

Figure 1. Alignment of amino acids of the hypervariable region of the VP1 protein of the chicken anemia virus (CAV). Amino acid sequences were aligned and represented using single-letter codes. Substitutions at positions **57**, **74**, **79**, **121**, **131**, **and 139** are highlighted by colored backgrounds.

3.2. Phylogenetic Analysis

Phylogenetic analysis of VP1 nucleotide sequences revealed that several indigenous CAV isolates clustered closely with previously reported strains. The isolates PQ063788, PQ063789, PV335610, PV200205, PV360680, PV360682, and PV360681 grouped with international strains Egy-4-CAV, Arg728, and NI/77 (KJ955380, EU871777, and KJ004669) within the same clade, showing zero branch length and indicating high sequence similarity as described in Figure 2.

Another isolate, PP537589, clustered with strain CK/04-01/GD/84 (AM407827) with a branch length of 0.00074. The remaining isolates, PP358767 and PP354085, grouped with the national strain Del-Ross (OR542967) within the same clade. Additionally, the vaccine strains Nobilis (AJ890284.1) and Cuxhaven-1 (M81223) formed a separate clade and showed phylogenetic relatedness with isolates PP358767 and PP354085 with a bootstrap value of 49, as shown in Figure 2.

3.3. Physicochemical Properties Evaluation

The physicochemical properties of the VP1 proteins from OP12, OP14, and the vaccine strain Nobilis were analyzed using ExPASy ProtParam. The extinction coefficients were calculated as 5806 for OP12, 11460 for OP14, and 107955 for Nobilis, indicating differences in protein absorbance properties.

Protein half-life analysis predicted that OP12 had a half-life of 7.2 h in mammalian cells (*in vitro*), 20 h in yeast (*in vivo*), and 10 h in *E. coli* (*in vivo*), while OP14 showed shorter half-lives of 1.1 h, 3 min, and 10 h, respectively. Nobilis exhibited half-lives of 30 h, 20 h, and 10 h in the same systems.

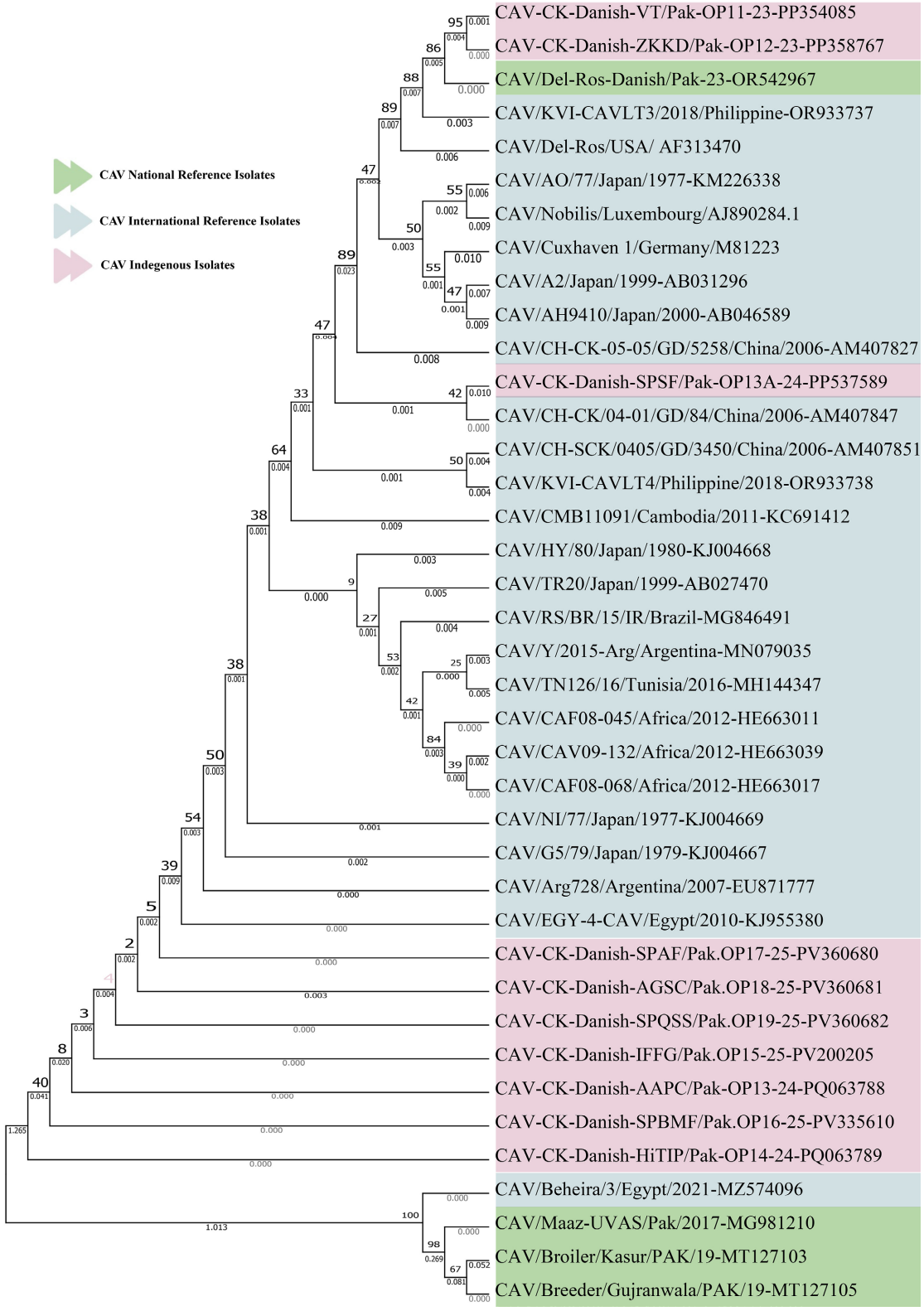

Figure 2. Phylogenetic tree of indigenous and reference CAV VP1 nucleotide sequences constructed using the Maximum Likelihood method in MEGA 11 with 100 bootstrap replicates. Clustal Omega was used to perform sequence alignment. The tree illustrates the genetic relationships among indigenous isolates and reference strains, with color-coded clades representing different phylogenetic groups.

The instability index values for OP12 (58.09), OP14 (52.80), and Nobilis (64.55) were all above 40, indicating that the proteins are predicted to be unstable. The aliphatic index values were 109.07 for OP12, 66.65 for OP14, and 55.96 for Nobilis, suggesting variation in thermostability among the proteins. Additionally, the GRAVY scores for OP12 (−0.547), OP14 (−0.565), and Nobilis (−0.701) were negative, indicating that all proteins are hydrophilic in nature as explained in Table 1.

Table 1. Physicochemical properties of VP1 proteins from CAV isolates.

Property	OP12	OP14	Nobilis
Extinction Coefficient	5806	11,460	107,955
Half-life (mammalian, *in vitro*)	7.2 h	1.1 h	30 h
Half-life (yeast, *in vivo*)	20 h	3 min	20 h
Half-life (*E. coli*, *in vivo*)	10 h	10 h	10 h
Instability Index	58.09 (Unstable)	52.80 (Unstable)	64.55 (Unstable)
Aliphatic Index	109.07	66.65	55.96
GRAVY	−0.547	−0.565	−0.701

3.4. Structure Prediction

The three-dimensional structures of VP1 proteins from indigenous isolates OP12 and OP14 and the vaccine strain Nobilis were predicted using AlphaFold2 through the ColabFold notebook [18]. The predicted models showed folded three-dimensional conformations of the VP1 proteins for all three strains, as depicted in Figure 3.

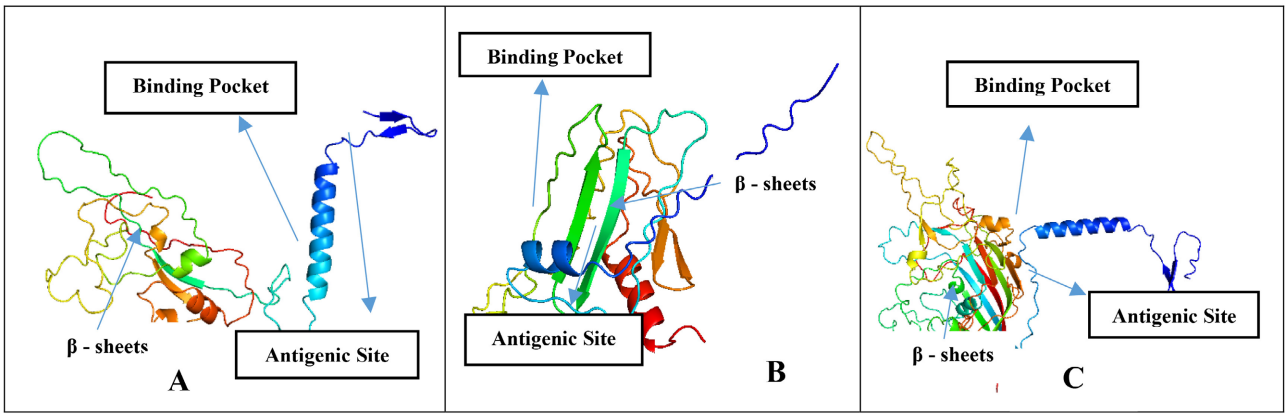

Figure 3. Predicted three-dimensional (3D) structures of chicken anemia virus (CAV) VP1 proteins generated using AlphaFold2. The models include (A) indigenous isolate OP12 (Del-Ross–like strain), (B) indigenous isolate OP14 (field strain), and (C) the Nobilis P4 vaccine strain. The predicted structures represent the folded conformations of the VP1 proteins for the respective strains.

3.5. Conformational B-Cell Epitope Prediction

The modeled VP1 protein structures were predicted as conformational B-cell epitopes with ElliPro. Several surface-exposed epitopes were identified in the pre-

dicted structures of OP12, OP14, and the Nobilis strain. The top-ranked epitope in OP12 consisted of a 29-residue segment spanning positions 1 - 29 with a protrusion score of 0.84. In OP14, the highest-scoring epitope corresponded to an 18-residue segment located at positions 85 - 102 with a score of 0.806. In the Nobilis strain, the major epitope comprised a 45-residue segment spanning positions 1 - 45 with a score of 0.885. These epitopes were mapped onto the three-dimensional protein structures and were found on the surface of the VP1 protein models as represented in Figure 4.

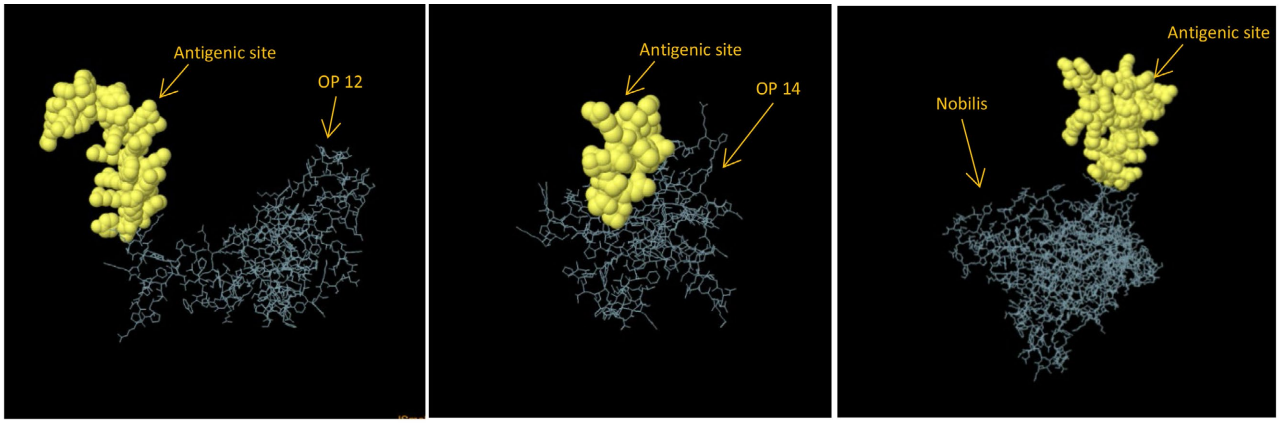

Figure 4. Predicted conformational B-cell epitopes in the three-dimensional VP1 protein structures. Yellow regions represent predicted conformational B-cell epitopes, while grey regions represent the remaining residues. (A) Indigenous isolate OP12 (Del-Ross-like strain), (B) Indigenous isolate OP14 (field strain), and (C) Nobilis vaccine strain.

3.6. Molecular Docking

Protein-protein docking analysis was performed between the VP1 proteins of OP-12, OP-14, and the Nobilis P4 strain with the receptor Toll-like receptor 3 using ClusPro. Three docking complexes were generated for OP-12, OP-14, and Nobilis P4.

The OP-14 complex formed the largest docking cluster containing 99 members with a weighted score of −846.6. In comparison, the OP-12 and Nobilis P4 complexes produced clusters containing 46 and 69 members, respectively. The lowest-energy structures for OP-12 and Nobilis P4 showed weighted scores of −1135.2 and −1130.8, respectively, as shown in Figure 5.

4. Discussion

Chicken anemia virus (CAV) is a pathogen that suppresses immunity resulting in high mortality and poor growth and secondary infections in infected flocks, leading to substantial economic losses in the poultry industry. Continuous monitoring of circulating CAV strains is therefore essential to understand viral evolution and genetic diversity [1].

In the present study, amino acid alignment of the VP1 protein revealed several substitutions among indigenous isolates when compared with reference and vaccine strains. Mutations were observed at amino acid positions 57, 74, 79, 121, 131,

and 139. The VP1 protein contains a hypervariable region that plays a crucial role in viral replication and pathogenicity. Previous studies have reported that mutations within this region can influence virulence and antigenic properties of CAV strains as represented in **Figure 1** [2] [6]. The presence of these substitutions in indigenous isolates therefore suggests ongoing genetic variation among circulating CAV strains.

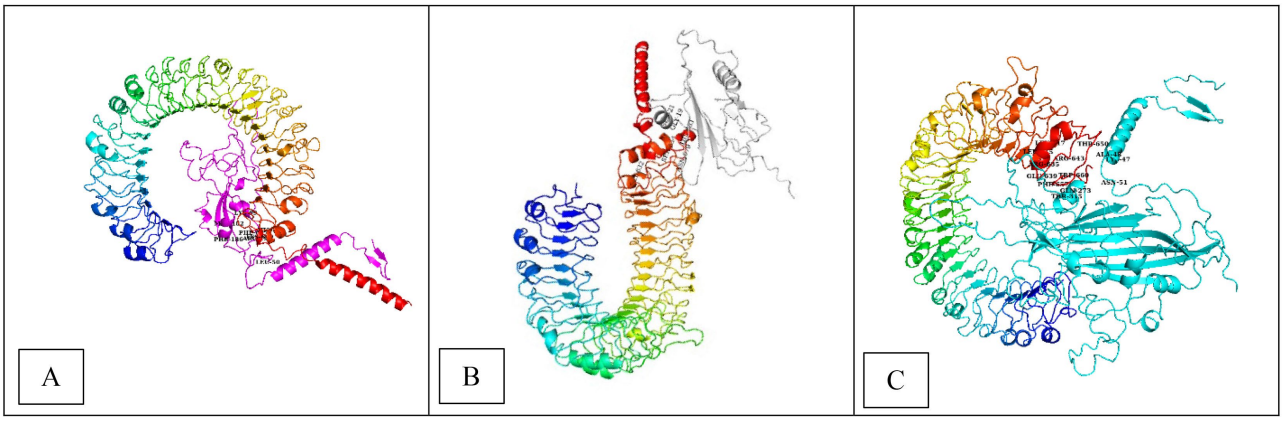

Figure 5. Protein–protein docking complexes between the VP1 proteins of chicken anemia virus (CAV) and Toll-like receptor 3 generated using ClusPro. The figure illustrates the predicted interaction complexes of (A) indigenous isolate OP-12 (Del-Ross-like strain) with TLR3, (B) indigenous isolate OP-14 (field strain) with TLR3, and (C) the Nobilis P4 vaccine strain with TLR3, showing the docked conformations of the VP1 proteins with the receptor.

Phylogenetic analysis further demonstrated that several indigenous isolates clustered closely with international strains such as Egy-4-CAV and Arg728, while others showed similarity with the Del-Ross vaccine strain. Similar clustering patterns have been reported in previous molecular characterization studies of CAV circulating in poultry populations as shown in **Figure 2** [1] [3]. These findings indicate that indigenous isolates share evolutionary relationships with previously reported strains and may have originated from common ancestral lineages.

Analysis of physicochemical properties revealed differences in stability and structural characteristics among OP12, OP14, and the Nobilis strain. All three proteins exhibited instability index values above the stability threshold, indicating predicted structural instability. Such behavior has also been reported for several viral proteins that require structural flexibility for effective host interaction and viral assembly [11]. Furthermore, negative GRAVY values indicated that all proteins are hydrophilic, which may facilitate their interaction with host cellular components.

Three-dimensional structural modeling of VP1 proteins revealed similar overall folding patterns among indigenous isolates and the vaccine strain. The use of deep learning-based structure prediction tools such as AlphaFold2 has significantly improved the accuracy of protein structure prediction and is increasingly used in structural bioinformatics studies [10] [12]. The predicted structural similarity among the analyzed proteins suggests structural conservation within the

VP1 capsid protein.

Conformational B-cell epitope prediction identified several surface-exposed antigenic regions in the VP1 protein structures. These epitopes were distributed across different regions of the protein and were located on surface-accessible areas. Surface-exposed residues are important for antibody recognition and immune response generation. Structure-based epitope prediction tools such as ElliPro have previously been used for identifying potential antigenic determinants in viral proteins [1].

The molecular docking was carried out to examine the interaction of VP1 proteins with Toll-like receptor 3 (TLR3), which is a key factor in innate antiviral immunity [14]. The docking results showed that OP12 and the Nobilis strain exhibited more favorable binding energy scores compared with OP14. Protein–protein docking tools such as ClusPro are widely used to predict molecular interactions between proteins and to analyze binding conformations [17]. These results suggest that structural variations in VP1 may influence its interaction with host immune receptors.

Overall, the findings of this study highlight the genetic variability and structural characteristics of indigenous CAV isolates. The observed mutations, phylogenetic relationships, and predicted structural features provide valuable insights into the molecular diversity of circulating CAV strains and may contribute to improved understanding of virus evolution and host immune interactions.

5. Conclusion

Comparative bioinformatics analysis of indigenous isolates of Chicken anemia virus revealed genetic diversity and evolutionary relationships with reference and vaccine strains. Phylogenetic analysis showed that OP11 - OP17 clustered closely with international strains such as Egy-4-CAV and Arg728, whereas OP18 and OP19 showed closer similarity to the vaccine strain Del-Ross. Further structural and immunoinformatics analyses focused on OP12 and OP14, which were compared with the Nobilis vaccine strain. Structural prediction indicated conserved three-dimensional conformations of VP1 proteins, while epitope prediction identified surface-exposed antigenic regions. Docking analysis of VP1 with Toll-like receptor 3 suggested differences in binding patterns among the proteins, indicating that sequence variations may influence host virus interactions. Overall, these findings highlight the genetic relationships and structural characteristics of indigenous CAV isolates.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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