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SEROLOGICAL AND MOLECULAR RELATIONSHIPS OF TWO EGYPTIAN CYANOPHAGE ISOLATES

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Abstract- This research aimed to investigate the serological relationship between two Egyptian cyanophage isolates (virulent and temperate) infecting *Anabaena* sp. A simple purification procedure, based on ultracentrifugation of the clarified lysates was applied, and diagnostic antisera were prepared by immunizing New-Zealand (NZ) rabbits using the purified preparations. Ouchterlony and microprecipitin tests were performed to check the serological relationship as well as discontinuous non-denaturing gel analysis. Molecular analysis of the amino acid sequences of coat protein genes was applied using DNA star lasergene 10 suite.

The purified preparations revealed A260/A280 ratios of 1.4 and 1.1, concentration was 1.005 and 0.58 mg/ml, and precipitation dilution end point of 1/32 and 1/16 for virulent and temperate isolate respectively. The purified suspensions (7x 10^4 and 5x 10^4 pfu/ml, estimated by plaque assay, for virulent and temperate isolate respectively) were used to obtain the specific antisera. The purified preparations are found immunogenic, producing specific polyclonal antibodies.

Data indicated a serological relationship between the two isolates which was discussed on the basis of discontinuous non-denaturing gel electrophoresis analysis and bioinformatic analysis of coat protein genes i.e antigenic indexes, secondary,3D and space fill structures.

Keywords- Cyanophage, Purification, Immunogenicity, Serology, Molecular analysis

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Introduction

Cyanophages are the most important factor influencing the activity of cyanobacteria which have the capability to fix atmospheric nitrogen especially in rice field ecosystem [1,2]. They also have several therapeutic effects ranging from reduction of cholesterol and cancer to enhancing the immune system [3]. Huang, et al [4], suggested that cyanopodoviruses are widely distributed in the ocean but their community composition varies with local environments.

Marei [5], isolated and identified two Anabaena cyanophages as virulent and temperate isolates. The isolates contain double stranded linear DNA with isometric heads and short tails belong to family podoviridae. Virulent phage has three structural proteins with molecular weights of 111, 106 and 102 KDa, whereas the temperate phage has two structural proteins with molecular weights of 111 and 106 KDa.

Sequence of coat protein gene of the virulent phage (published in Genbank under Accession no. Jn014839) revealed a moderate degree of similarity (49%) to temperate phage and cyanophage AN-15 [5].

Translation of partial nucleotide sequences of cp-gene for two Egyptian isolates (virulent and temperate) were found to be 65 and 67 amino acids starting with leucine and proline and ending to valine and leucine respectively. Andrea, et al [6], found that the N-terminal sequence of the putative major capsid protein of cyanophage strains, N-1 and A-1 (L) contained of seven amino acid residues (alanine - leucine - threonine - leucine - threonine - threonine - proline).

Singh, et al [7], purified Syn5 cyanophage isolated from Sargasso sea on *Synechoccocus* strain WHS109. Phage with short tailed and the tail fibers is highly antigenic stimulate antibody production when NZ white rabbits were immunized. Othman, et al [8], used two rabbits for each phage of *Erwinia carotovora* to prepare the phage specific antiserum. They used double diffusion and rocket tests to determine the titer of anti-phage sera and the antigen dilution end point. Nien, et al [9], recorded that cyanophages were purified by sedimentation at high speed centrifugation followed by equilibrium ultracentrifugation in CsCl. David, et al [10] studied the serological characterization of blue green algae virus (BGAV), using neutralization test for studying the homology of bacteriophages T1, T2 and T3 with antiserum for (BGAV). No relationship was found between antigens T1, T2 and T3 and BGAV.

This research aimed to investigate the immunogenicity and serolog-

ical relationship between two cyanophage isolates (virulent and temperate) infecting *Anabeana* sp. in Egypt. Therefore, rabbits were immunized with purified preparation of each isolate and the produced antisera were elvaluated. The serological relationship was checked using outerchlony, microprecipitin tests, discontinuous non -denaturing gel electrophoresis analysis and molecular analysis of amino acid sequences of coat protein genes using DNA star lasergene 10 suit.

Material and Methods

Purification of two Cyanophage Isolates Specific for *Anabaena* sp.

High titer of crude phage lysates were prepared using liquid culture propagation method as described by Franche [11]. The crude phage lysates of virulent and temperate isolates were ultracentrifuged at 30000 rpm / 1.15 hrs. / 4C (Beckman L7-35 Ultracentrifuge). The pellets were gently re-suspended in 0.2 M phosphate buffer at pH 7.2.

Evaluation of Purified Cyanophage Preparations

The purified preparations were evaluated according to David, et al [10]. UV absorbance was measured at 200 to 300 nm (Ascending with rate of 4 nm) using UV Spectrophotometer (ct-2200). Concentrations of the purified phages were calculated using the following equation:

$$\text{Phage conc.}\,(\text{mg}/\text{ml}) = \frac{\text{Absorbance reading at 260nm} \times \text{Dilution Factor}}{\text{Extinction coefficient}\,(2)}$$

Preparation of Antisera Specific for Anabaena Phage Isolates

Prior to rabbit immunization (white New Zealand species), preimmune sera (normal sera) were collected as a control. The purified phage suspensions; 7x104 and 5x104 pfu /ml estimated by plaque assay test; for virulent and temperate cyanophages respectively were used to obtain the virus specific antiserum for each isolate. Four hundred micro liters of each purified isolate was emulsified with equal amount of Freund's complete adjuvant and used for subcutaneous and intramuscular injections. Each rabbit received five injections (Four subcutaneous and one intramuscular). The rabbits were bled seven days after the last injections.

Serological Relationship Between the Two Isolates

The relationship was clarified using double diffusion test in plates according to Ouchterlony [12]. Determination of precipitation end point of antigens was performed by Ouchterlony and microprecipitin test (in plates) according to Matthews [13].

Discontinuous Non-denaturing Gel Electrophoresis of the Specific Antisera for the Two Isolates

It was performed by native gel electrophoresis (Bio-Rad GelDoc 2000 and analyzed with the quantity one software package supplied from the manufacturer) method according to Hames & Rickwood [14].

Molecular Studies of cp-genes of Two Cyanophage Isolates

Bioinformatic studies were applied to demonstrate the relationship between coat proteins of virulent and temperate isolates of cyanophages infecting *Anabeana* sp. using of DNA STAR Lasergene Core Suite 10.

Results

Evaluation of Purified Preparations of Cyanophage Isolates

The results show that the purification procedure applied for cyanophage isolates is satisfactory where virus particles suspended in 0.2 M phosphate buffer pH 7.2 had absorption ratio A260/A280 of 1.4 and 1.1for virulent and temperate isolates respectively. While the purified phage concentration of the virulent and temperate isolates was found to be 1.005 and 0.58 mg/ml, respectively. The characteristics of the UV spectra showed that the purified preparations of both cyanophage isolates are quite pure and concentrated.

Serological Relationship of *Anabaena* sp. Cyanophage Isolates

The titers of the purified cyanophages isolates using the produced polyclonal antibodies for each isolate in microprecipitin and Ouchterlony tests were found to be 1/32 and 1/16 for the virulent and temperate isolate, respectively. [Fig-1](A&B) shows that the serological reactions are specific, in which antiserum prepared for virulent phage 1 (AS1) reacts with virulent phage antigen 1 (Ag1), and that produced for temperate phage 2 (AS2) reacts with phage temperate antigen 2 (Ag2).

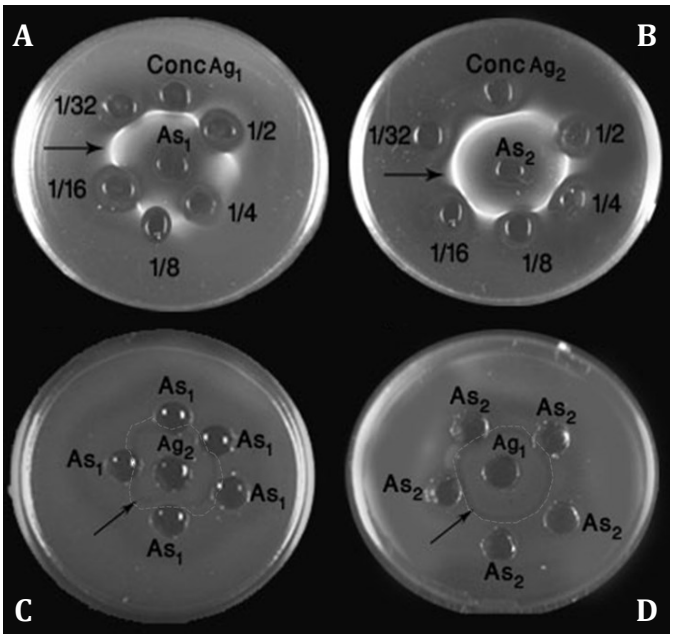

Fig. 1- Homologous serological reactions of the cyanophage isolates (indicated by arrows) in Ouchterlony test. Central wells contain antiserum of phage 1 **(A)** and phage 2 **(B)**. Peripheral wells contain concentrated and two fold dilutions of antigens (Ag_1 and Ag_2). Phage 1 (Ag_1) = virulent phage. Phage 2 (Ag_2) = temperate phage.

Serological relationship of cyanophage isolates (indicated by arrows) as tested by Ouchterlony test. Central wells contain Ag_2 **(C)** and Ag_1 **(D)**. Peripheral wells contain antisera to phage 1 **(C)** and phage 2 **(D)** antigens (Ag_1 and Ag_2). Antigen 1 (Ag_1) = virulent phage. Antigen 2 (Ag_2) = temperate phage. (Dashed lines are drawn to guide the readers' eyes to the reaction region).

There is a serological relationship between the two cyanophage isolates since antigen of virulent isolate react with the antiserum of temperate phage and vice versa [Fig-1](C&D). The isolated phages are serologically related to each other. Polyclonal specific antibodies are successfully produced for the two Egyptian isolates of cyanophages. Each virulent and temperate phage antigens produced

specific antiserum when NZ rabbits were immunized using subcutaneous and intramuscular injections.

Discontinuous Non-denaturing Gel Electrophoresis Analysis of the Polyclonal Antisera

Results shown in [Fig-2] and [Table-1] reveal the presence of 14 bands for the antiserum belonging to each isolate; mainly in beta-globulin, post-transferen, transferen alpha-globulin, albumin and pre -albumin. It is clear that the normal serum does not contain three bands that belongs to post-transferen (with RF: 0.2,0.31 &0.38) and bands with RF 0.41, 0.43,0.46 and 0.49 have lower intensity in comparison with the two antisera. Alpha and beta globulins are transport proteins, but gamma globulins have an important role in natural and acquired immunity. Transferen is a glycoprotein that binds to iron strongly but reversibly. Although iron bound to transferen is less than 0.1% (4mg) of the total iron, it is the most important iron peal with the highest rate of turnover (25 mg/24h).

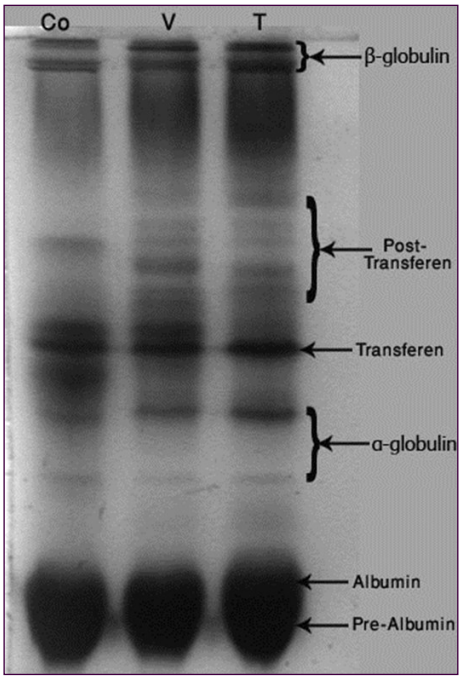

Fig. 2- Plasma proteins electrophoresis pattern of normal serum (Co), antiserum (v) virulent and antiserum (T) for temperate isolate

Table 1- Native gel analysis for protein banding pattern of sera collected from rabbits non immunized and immunized with virulent and temperate cyanophage isolates

RF	Normal serum	Virulent phage (Antiserum 1)	Temperate phage (Antiserum2)
0.02	+	+	+
0.05	+	+	+
0.07	+	+	+
0.2	-	+	+
0.31	-	++	++
0.38	-	++	++
0.41	+	++	++
0.43	+	++	++
0.46	+	++	++
0.49	+	++	++
0.5	+	+	+
0.57	+	+	+
0.6	+	+	+
0.68	+	+	+

Molecular Analysis of Amino Acid Sequences of cp-genes of the Two Isolates

Bioinformatics studies was applied using DNA STAR Lasergene Core Suite 10 to illustrate the relationship between coat proteins of the two cyanophage isolates under investigation.

Studying the RNA Folding: Data herein indicate that RNA folding of cp-genes for both cyanophage isolates are stable; with hairpin, internal, stability, exterior and multi branch loops, however the virulent cp-DNA shows higher stability that's shown in [Fig-3](a & b) than that of temperate isolate [Fig-4](a & b). The present data reveal a serological relationship between e two cyanophage isolates.

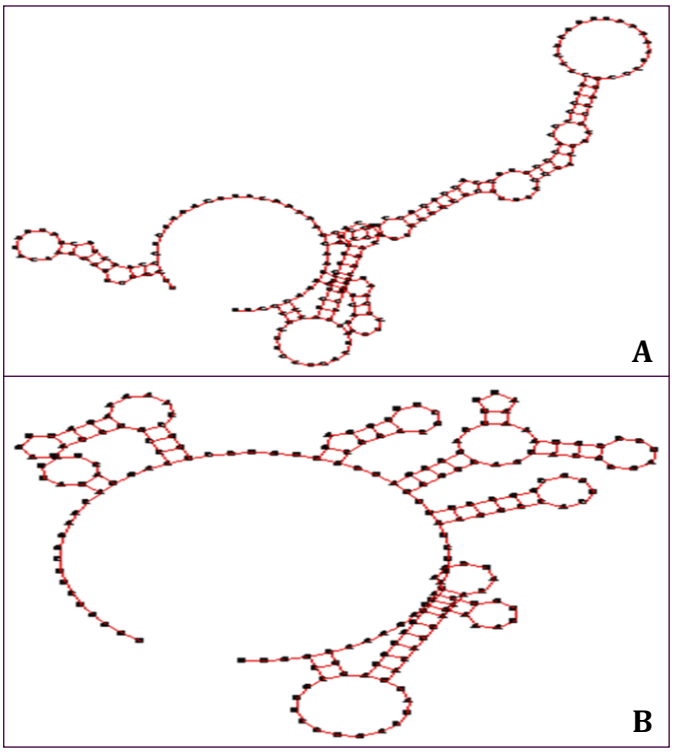

Fig. 3(A & B)- RNA folding of virulent cyanophage

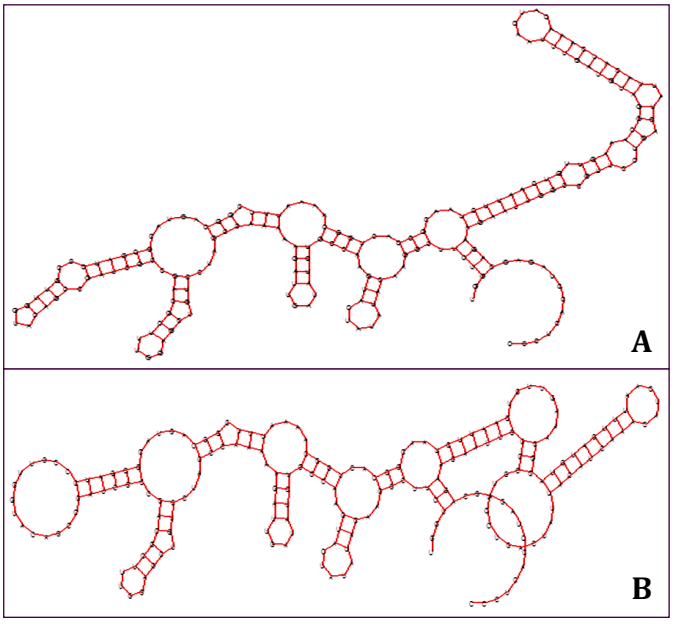

Fig. 4(A & B)- RNA folding of temperate cyanophage

Protein Folding: Data herein indicate that the amount of tryptophan by weight is 10.92 and 6.59 for virulent and temperate isolates, respectively. Antigenic indexes are revealed in [Fig-5](a & b) using protein program it is apparent that antigenic or immunogenic determinant of the virulent isolate consists of five segments No 1 consist of Gly, Lus, Asp, Glu, No 2 consist of: Ala, Thr, No 3 consist of: Ser, Thr, Arg, Lys, Asn, Phe, No 4 consist of : Trp and No 5 consist of: Leu, Lle. The Temperate isolate has three antigenic determinant, No 1 consist of: Pro, Ser, Pro, Arg, Ala, Glu, No 2 consists of: Ala, Thr, Gly, Lys, No 3 consist of : Pro, Val, Gly, Thr, Ala, Asp, Arg, Arg, Arg. The positive contribution of hydrophilic amino acids in a surface is acknowledged here for CP of cyanophage as aspartic, glutamic, histidine, lysine, asparagine, glutamine, serine and theronine, which all of them are in higher concentrations in the virulent isolate (expect theronine) than the temperate isolate. The antigenic index present the protein surface contours (potential antigenic determinant) directly from its primary amino acid sequences.

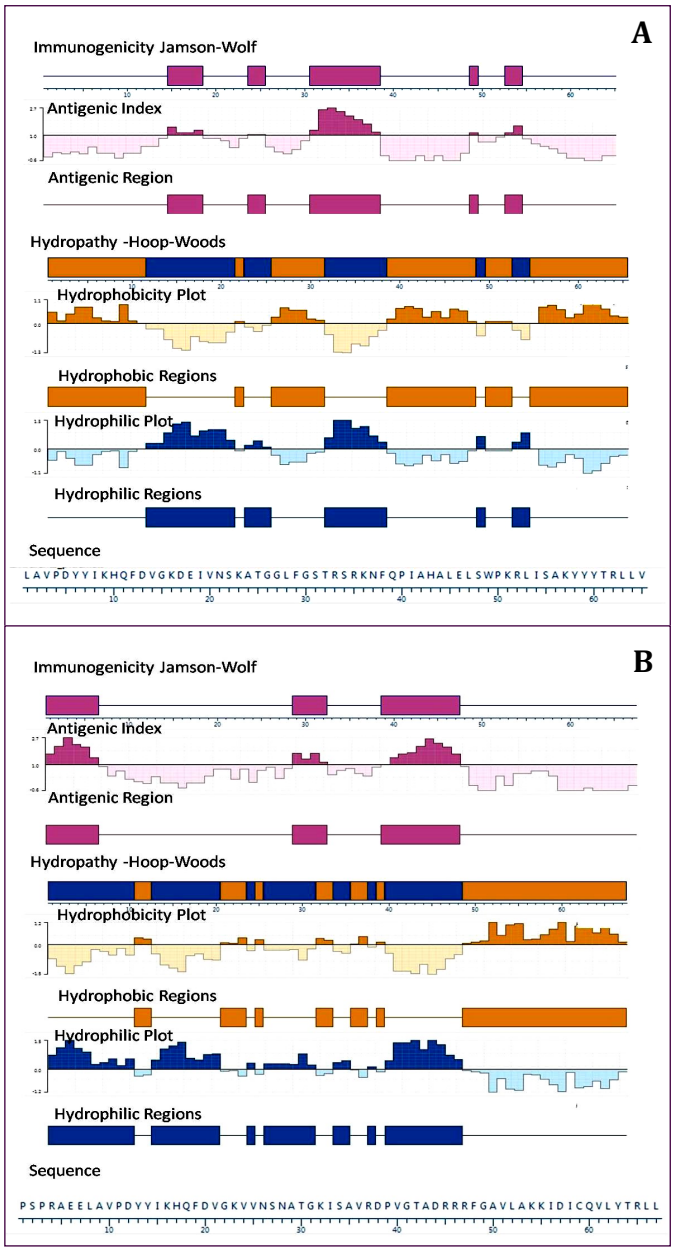

Fig. 5- Secondary structure of **(A)** virulent cyanophage coat protein; **(B)** temperate cyanophage coat protein.

The space-filling model is known as calotte model which is a type of three dimensional molecular, model where the atoms are presented by spheres whose radii are proportional to the radii of the atom and whose center to center distances proportional to the distances between atomic nuclei. The helical wheel is a type of plot in a rotating manner where the angle of rotation between consecutive amino acids is 100. It is used to illustrate the properties of alpha or amphipatic helices which consist of hydrophopic-non polar residues on one side of the helical cylinder and hydrophilic and polar residues on the other side. which are shown in [Fig-6](a & b). The three dimensional structures of coat proteins are shown in [Fig-7](a & b) using protein 3D program.

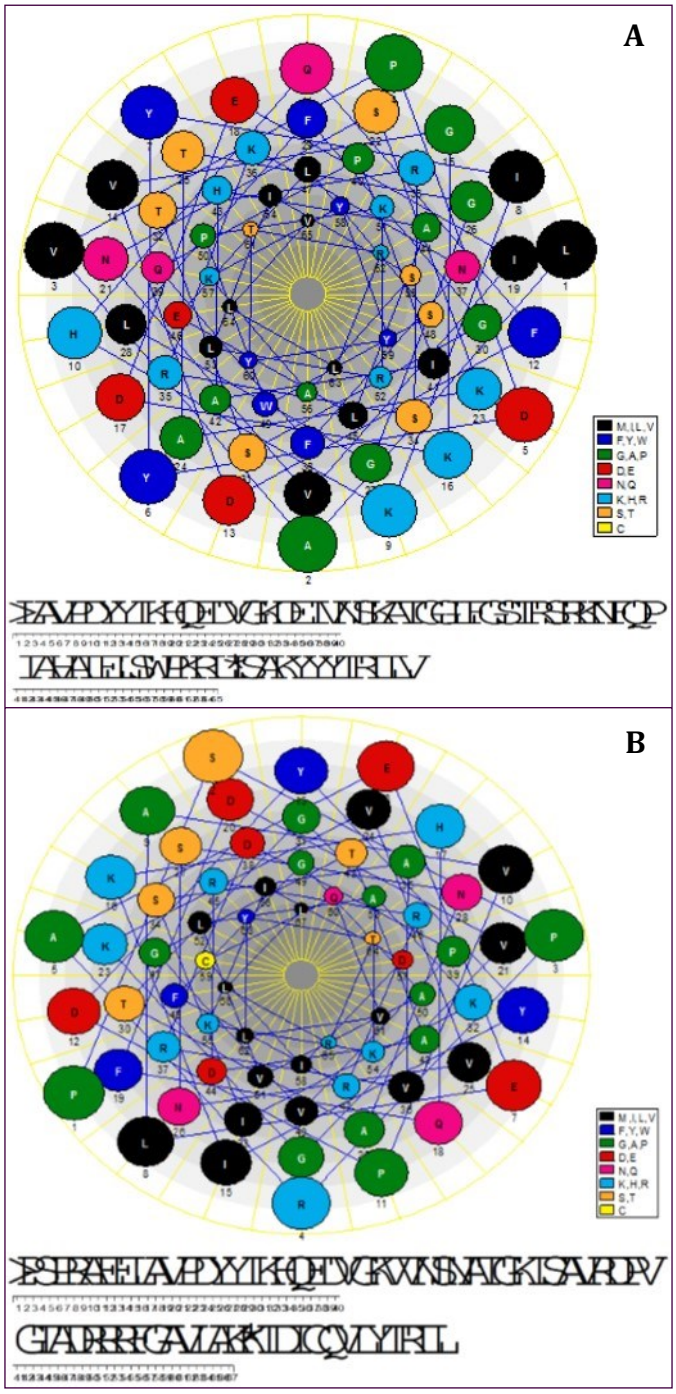

Fig. 6- Helical wheel and space fill model of **(A)** virulent cyanophage coat protein; **(B)** temperate cyanophage coat protein.

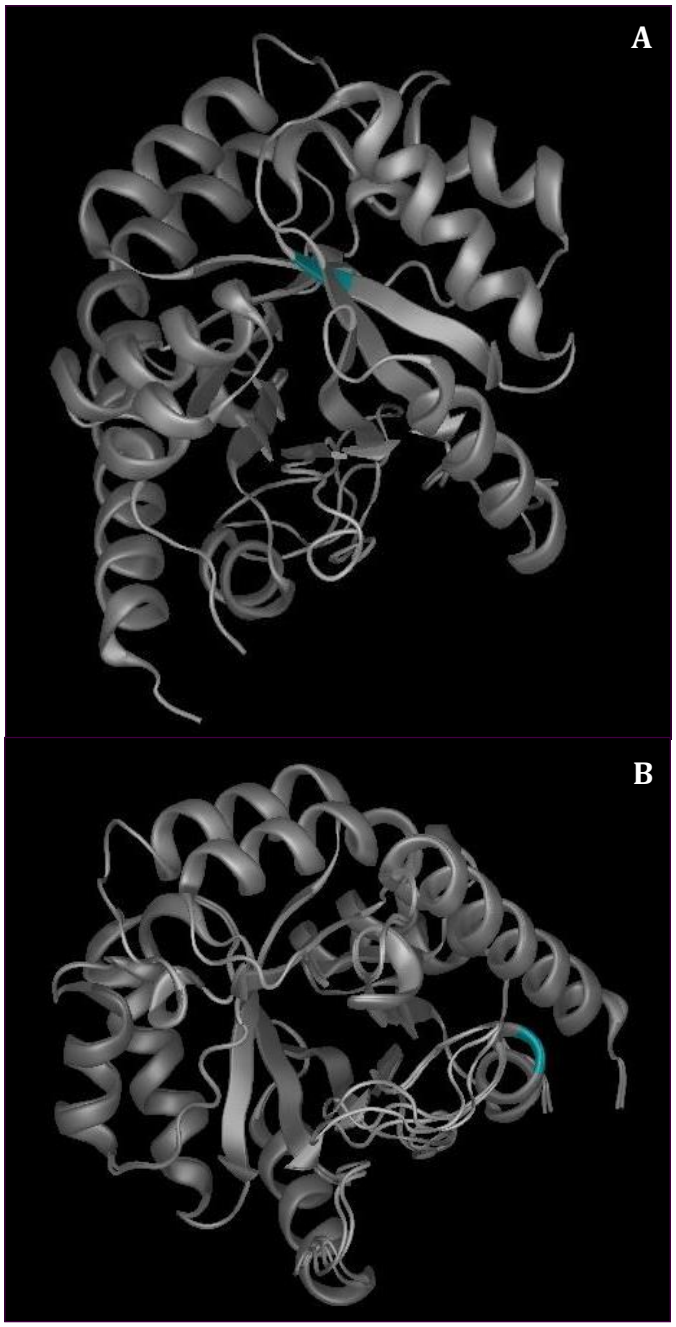

Fig. 7- 3D structure of **(A)** virulent cyanophage coat protein; **(B)** temperate cyanophage coat protein.

Discussion

The titers of the purified cyanophages isolates using the produced polyclonal antibodies for each isolate in microprecipitin and Ouchterlony tests were found to be 1/32 and 1/16 for the virulent and temperate isolate, respectively. The results of the current study are in agreement with those of Bachrach & Friedmann [15]. They compared various methods for phage concentration and purification and recorded that differential centrifugation is efficient when the sediment phages were gently dispersed before repeating the centrifugation cycle. The results are also aligned with the conclusion of Pope, et al [16], who recorded the production of significant quantities of highly concentrated cyanophage virions (10 virions / ml), when purified and concentrated from large volume of crude lysate with ultracentrifugation.

Polyclonal specific antibodies are successfully produced for the two Egyptian isolates of cyanophages. Each temperate and virulent phage antigens produced specific antiserum when NZ rabbits were immunized using subcutaneous and intramuscular injections. The dilution end point for the antigen is 1/32 and 1/16 for virulent and temperate isolates respectivelly. The reactions of ouchterlony and microprecipitin tests revealed specific serological reactions for each isolate. In addition the reactions in ouchterlony reveal that the two isolates have identical antigens. Ouchterlony and microprecipitin tests are the most widely used techniques for virus assay, detection, diagnosis, determination of dilution endpoint and relationship between antigens [8]. Using SDS-PAGE, Marei [5], revealed that Egyptian isolates of cyanophages (virulent & temperate) have two monomorphic structural protein bands with MW 111 and 106 KDa with 80% similarity In addition, upon studying the phylogenetic tree of the two Egyptian *Anabaena* cyanophage isolates based on the amino acid sequences of the cp-gene, Marei [5], found a moderate degree of similarity (49%) between the virulent and the temperate isolates.

Pope, et al [16], reported that proteins of cyanophage syn5 encoded by genes 43 and 44 may not have been detected as they are predicted to be internal virion proteins and therefore may not have well exposed to the immune system of the rabbit during antibody production. It was concluded that the protein encoded by gene 45 would travel out of the virion and could have been exposed to the immune system of the rabbit and have antibodies created against it. It was also clarified that the intensity of most of the bands of western blot varied in through proportion to the intensity of the protein bands seen on the SDS-gel. Phage tail fibers are known to be highly antigenic and may stimulate antibody production at higher rates than other phage proteins.

Alpha and beta globulins are transport proteins, but gamma globulins have an important role in natural and acquired immunity. Beta-globulin and post transferen are found with innate immune system and some antibodies are found in beta-globulin zone [17].

Data herein indicate that RNA folding of cp-genes for both cyanophage isolates are stable; with hairpin, internal stability, exterior and multi branch loops, however the virulent cp-DNA shows higher stability. Zhong, et al [18], found that P-ssp7 cyanophage is T7-like phage that infects the *Cyanobacterium prochlorococcus* MED4. P-ssp7 is classified as T7- like based on genome content and organization. However, because its genome assembled as circularly molecule, it was thought to be circularly permeated and to lock the direct terminal repeats found in other T7- like phage. P-ssp7 genome map is linear and contains a 206 bp repeat both ends. RNA folding (secondary structure) which is the number of wobble base pairs is higher in virulent cyanophage isolate. These base pairs or stem - loop structures are fundamental in RNA secondary structure, proper translation and stabilized base stacking. Marei [5], revealed that the DNA of cp-gene of virulent isolate is more stable than that of the temperate since it is rich in GC content.

The secondary structure of biological RNA can often be uniquely decomposed into stem and loops which can be further classified into tetra loops pseudoknots and stem-loops. A pseudoknots is a nucleic acid secondary structure containing at least two stem-loops structures in which half of one stem is interacted between the two halves of another stem. It is recognized first in turnip yellow mosaic virus [19]. Pseudoknots fold into knot-shaped three dimensional conformations but are not true topological knots.

The present data reveal a serological relationship between the two cyanophage isolates. This indicates that the structural proteins belonging to heads and tails have a degree of similarity, since the purified preparations. Electron micrographs of negative staining revealed that two Egyptian cyanophage isolates have heads and short tails differing in size, with similar protein bands in SDS-PAGE with MW 111&106 KDa, the virulent isolate has an additional protein band with MW 102 KDa [5]. In addition, a moderate degree of similarity (49%) was found between the proteins of the two isolates based on amino acid sequences of the cp-genes. The major head proteins of LPP-1 are 39.000 and 13.000 MW, the major tail protein has a molecular weight of 80.000. Reports have shown that immunoglobulin (Ig) domains occur quite frequently on the surface of tailed ds DNA bacteriophages [8].

The herein indicate that the amount weight of tryptophan for virulent and temperate phages is 10.92 and 6.59 for virulent and temperate isolates, respectively, it is shown that although the molecular weights of coat proteins is 7.472 and 7.428 for virulent and temperate cyanophage isolate respectively their molar extinction coefficients are 12950 and 4595 (data not shown). The extinction coefficient of a protein at 280 nm depends exclusively on the number of aromatic residues particulary tryptophan. Gill [20] On the basis of the amino acid sequences of coat proteins which was performed by Marei [5] for the two cyanophage Egyptian isolates (virulent Accession no. Jn014839) and temperate. Antigenic index was designed to integrate flexibility components such as averaged backbone B factors and predicted B-turns with surface exposure parameters such as hydropathy and solvent accessibility values [21]. Highly mobile segments seem to correlate with known antigenic determinant [22]. The helical wheel is a type of plot in a rotating manner where the angle of rotation between consecutive amino acids is 100. It is used to illustrate the properties of alpha or amphipatic helices which consist of hydrophopic-non polar residues on one side of the helical cylinder and hydrophilic and polar residues on the other side. The hydrophobic effect which formed the side-chains, gives tendency to sequester themselves in the interior of the protein, away from contact with water, providing an important component of the driving force for protein folding to be in the secondary structure [21,23].

Space-filling model is known as calotte model which is a type of three dimensional molecular, model where the atoms are presented by spheres whose radii are proportional to the radii of the atom and whose center to center distances proportional to the distances between atomic nuclei. Zhong, et al [18], reported that phylogenetic analysis of isolated cyanophages revealed that marine cyanophages were highly diverse yet more closely related to each other than enteric coliphage T4. Sullivan, et al [24], presented the first genome of cyanobacterial siphoviruses-P-ss2 which was isolated from Atlantic slope water using *Prochlorococcus* host (MII933). The P-ss2 genome is larger than and considerably divergent from previous sequenced siphoviruses. While only 6 structural proteins were identified, 35 proteins were detected experimentally; these mapped onto capsid and tail structure modules in the genome. These Ig-like domains on the cyanophages interact with carbohydrates on the cell surface and thus facilitate phage adsorption, these Ig-like domain appear to be one of a number of conserved domains displayed on phage surfaces that serve to increase infectivity by binding or degrading polysaccharides [7].

Conclusion

Generally, on the basis of the obtained results it can be concluded that, the purification procedure applied for cyanophage Egyption isolates is satisfactory where virus particles suspended in 0.2 M phosphate buffer pH 7.2 had absorption ratio A260/A280 of 1.4 and 1.1 for virulent and temperate isolates respectively**.** The titers of the purified cyanophages isolates using the produced polyclonal antibodies for each isolate in microprecipitin and Ouchterlony tests were found to be 1/32 and 1/16 for the virulent and temperate isolate, respectively. The presence of 14 bands for the antiserum belonging to each isolate; mainly in beta-globulin, post-transferen, transferen alpha-globulin, albumin and pre-albumin. It is clear that the normal serum does not contain three bands that belongs to post -transferen (with RF: 0.2,0.3 & 0.38) and bands with RF 0.41, 0.43, 0.46 and 0.49 have lower intensity in comparison with the two antisera. RNA folding of cp-genes for both cyanophage isolates are stable; with hairpin, internal, stability, exterior and multi branch loops, however the virulent cp-DNA shows higher stability than that of temperate isolate. Protein folding of amount tryptophan by weight is 10.92 and 6.59 for virulent and temperate isolates, respectively.

Conflicts of Interest: None declared.

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