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Isolation and Virulence Evaluation of Currently Circulating *Avibacterium paragallinarum* in Poultry

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Abstract **Original Research Article**

Avibacterium paragallinarum is a Gram-negative bacterium that causes infectious coryza (IC), a respiratory disease that is highly contagious among chickens. Through PCR testing and sequence analysis of the FlfA gene, a total of 10 *Av. paragallinarum* field isolates were identified, accounting for 5.7% of the samples. In this study, we assessed the virulence of field isolates by utilizing a rapid artificial intrasinus injection route model. Upon observation, we have found that all 10 field isolates from Egypt that were identified as type B strains possess virulent properties and have the ability to cause Infectious Coryza (IC) disease in chickens. The current study aimed to sequence the FlfA gene and conduct phylogenetic analyses to better understand the *Avibacterium paragallinarum* field isolates that have been circulating in Egypt in recent years. This research will provide valuable insights into the genetic makeup and evolution of this pathogen, which can inform future efforts to control and prevent its spread. The amino acid sequences of *Avibacterium paragallinarum* from Egypt and the reference sequence exhibit a remarkable degree of homology, ranging from 97.96% to 100%. This finding underscores the close relationship between the two sequences and highlights the potential for further research into the genetic makeup of this pathogen. Furthermore, the discovery of a fimbrial cluster that is identical to the Galli bacterium FlfA in the *Av. paragallinarum* genome indicates that these two species may have recently exchanged this fimbriae. This exchange could have occurred during the natural co-colonization of the chicken's upper respiratory tract. It is possible that this exchange could have implications for the pathogenicity and virulence of these bacteria. The FlfA protein has unique characteristics and was subjected to bioinformatics analysis to predict its properties and protein interactions. The FlfA protein structure is stable with many coiled regions. Analysis shows the FlfA protein lacks any transmembrane helix and has a function in carbohydrate binding and cell adhesion. Linear B epitope prediction found 7 epitopes that can induce a robust immune response and may be good candidates for further research. In conclusion the virulence of the field isolates was consistent and that they were all capable of causing disease in chickens. The fact that they all formed a single disease peak during the study period indicates that there may be a common mechanism or pathway by which these isolates cause disease. FlfA is a protein that plays a crucial role in the virulence of certain *avibacterium paragallinarum*, allowing them to evade the host immune system and cause disease. By understanding how FlfA works and how it contributes to bacterial pathogenesis, scientists can design drugs or vaccines that specifically target this protein and prevent its harmful effects.

Keywords: *Avibacterium paragallinarum*, FlfA protein, B epitope, Bioinformatics.

INTRODUCTIN

Worldwide, *Avibacterium paragallinarum* is the etiological agent of infectious coryza in poultry. Infectious coryza, caused by *Avibacterium paragallinarum*, is an acute respiratory disease that affects poultry worldwide Blackall *et al.*, (2020). The bacterium was initially categorized into three serogroups by Page, based on the plate agglutination test. However, further research led to the identification of nine Kume serovars, which were distinguished using hemagglutination inhibition tests. These serovars include A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4, as outlined by Blackall *et al.*, (1990). Infectious coryza is a respiratory disease that causes inflammation of the upper respiratory tract, facial edema,

conjunctivitis, nasal discharge, diarrhea, and anorexia. In some cases, wattles may also be affected Blackall *et al.,* (2020). This disease is caused by the bacterium *Avibacterium paragallinarum* and can affect chickens, pheasants, and other birds. It is highly contagious and can spread rapidly through a flock, especially in crowded or unsanitary conditions. The symptoms of infectious coryza can be severe and can lead to decreased egg production, weight loss, and even death. Treatment typically involves antibiotics and supportive care, but prevention through good biosecurity practices is the best approach. Vaccines are also available for some strains of *Avibacterium paragallinarum*.It is crucial for poultry farmers to be vigilant for signs of infectious coryza and to take appropriate measures to prevent its spread within their flocks. This includes maintaining good hygiene practices, such as regularly cleaning and disinfecting equipment and facilities, limiting visitors to the farm, and isolating sick birds. By implementing these measures, farmers can protect their flocks and ensure the health and productivity of their birds.

There is no public health significance to *Av. paragallinarum,* and it is mainly reported in layers with a reduction of up to 40% in egg production, but it can get complicated when other pathogens are associated, increasing these losses up to 85% with considerable mortality rates Blackall *et al.,* (2020). Clinical signs and a reduction in egg production typically resolve within 20 days, although more severe cases may persist for up to two months (Blackall *et al.,* 2013). It is worth noting that infectious coryza has also been observed in broilers, often in conjunction with other bacterial or viral agents (Khatun *et al.,* 2016). In some cases, the infection can lead to arthritis and septicaemia (Gallardo *et al*., 2020). Furthermore, a recent study found that co-infection with Fowl Adenovirus increased mortality rates in an experimental inoculation with a serogroup A field strain of *Av. Paragallinarum* (Mei *et al.,* 2020). Fimbriae, which are proteinaceous filaments, are expressed on the surfaces of numerous pathogenic bacteria. These filaments play a crucial role in the virulence of these bacteria by allowing them to adhere to host cells and tissues. As a result, they are essential for the bacteria's ability to cause disease.

Liu *et al.,* (2016) have highlighted the importance of fimbriae in bacterial adhesion to host cells. Fimbriae are highly specific, with different types of fimbriae binding to different receptors on host cells. This specificity enables bacteria to target specific tissues and organs, increasing their ability to cause disease. Therefore, understanding the role of fimbriae in bacterial pathogenesis is crucial for developing effective treatments and preventive measures against bacterial infections. Fimbriae, in addition to their adhesive function, also play a crucial role in the formation of biofilms. Biofilms are clusters of bacteria that grow on surfaces and are known for their resistance to antibiotics and immune responses. Fimbriae facilitate the attachment of bacteria to surfaces and to each other, enabling the formation of these protective communities. Understanding the structure and function of fimbriae is important for developing new treatments for bacterial infections. By targeting fimbriae, researchers may be able to prevent bacterial adhesion and biofilm formation, making it easier to treat infections with antibiotics or other therapies. Fimbriae are thin, hair-like structures that protrude from the surface of bacteria. They play a crucial role in the adhesion of bacteria to host cells. F-17 fimbriae, a type of fimbriae, belong to a group that binds to receptors containing N-acetyl-D-glucosamine (Glc-NAc) on the surface of host cells. This binding mechanism is believed to facilitate the adhesion of bacteria to the mucosal surfaces within the host. This information was first reported by Klemm *et al.,* (2000). *Avibacterium paragallinarum* is a bacterium that causes respiratory infections in chickens. One of the key factors that contribute to its pathogenicity is the F17-like fimbria, which is a hair-like structure that protrudes from the bacterial surface. This structure is encoded by a four-gene cluster, which includes flfD, flfC, flfG, and flfA Bager *et al.,* (2013).

These genes encode for a chaperone, an usher protein, an adhesion protein, and a structural subunit protein, respectively. The chaperone and the usher protein work together to facilitate the folding, assembly, and secretion of the structural subunit protein, which forms the stem of the fimbriae. The adhesin, located at the tip of the fimbrial structure, is responsible for recognizing and binding to specific receptors on host cells. This interaction is crucial for the attachment and colonization of host tissues, which is a key step in the establishment of infection.The chaperone and usher proteins act as molecular chaperones, guiding the structural subunit protein through the periplasmic space and facilitating its correct folding and assembly into the fimbrial stem. Once assembled, the fimbriae are transported to the outer membrane where they are anchored by their C-terminal domains. At this point, the adhesin becomes exposed and is able to recognize and bind to specific receptors on host cells leiu *et al.,* (2016). Understanding the mechanisms underlying fimbrial biogenesis and adhesion is critical for developing new strategies to prevent or treat bacterial infections. By targeting these processes, it may be possible to disrupt the ability of bacteria to attach to host tissues and establish infection. This could lead to the development of new treatments for bacterial infections, which could have a significant impact on public health.

The FlfA fimbrial protein is a critical virulence factor and vaccine candidate of *Avibacterium paragallinarum*. Research on virulence factors is crucial to gain a better understanding of the mechanisms underlying the pathogenicity of *Av. paragallinarum*. In 2014, Bager *et al.,* (2014)

demonstrated that the FlfA fimbria is essential for virulence in vivo, as a knockout mutant of flfA was attenuated. This finding suggests that the FlfA fimbria plays a crucial role in the pathogen's ability to cause disease in a living host. Without this virulence factor, the pathogen is less capable of establishing an infection and causing damage to host tissues. Understanding the function of FlfA and other virulence factors can help researchers develop new strategies for preventing and treating infections caused by these pathogens. Additionally, studying the mechanisms by which these factors contribute to virulence can provide insights into basic biological processes that are relevant to many different types of organisms. The FlfA fimbrial protein is a vital component of *A. paragallinarum's* virulence, and its study can lead to the development of new treatments and prevention strategies for infections caused by this pathogen. By understanding the mechanisms underlying pathogenicity, researchers can gain insights into basic biological processes that have implications for a wide range of organisms.

For that purpose, the classification of local Egyptian stains of *Av. paragallinarum* was performed. This classification was important in order to understand the virulence of different strains of *Av. paragallinarum* and their potential impact on poultry health. By studying the capacity of these stains to cause clinical signs, horizontal infection, and septicaemia in chickens, researchers can better understand how to prevent and treat outbreaks of this disease. This information can also be used to develop more effective vaccines and control measures for *Av. paragallinarum* infections in poultry populations. Ultimately, this research could help to improve the overall health and productivity of the poultry industry in Egypt. Applying phylogenetics and Gene sequence analyses of The FlfA fimbrial protein of *Avibacterium paragallinarum* to understand of the mechanisms underlying pathogenicity of *Av. paragallinarum*. By analyzing the gene sequences of FlfA from different strains of *Av. paragallinarum*, researchers can gain insights into the genetic diversity and evolutionary history of this important protein. This information can then be used to develop more effective diagnostic tools and vaccines for controlling infectious coryza. Furthermore, by studying the molecular mechanisms underlying FlfA-mediated pathogenicity, researchers can identify potential targets for therapeutic intervention. For example, if it is found that FlfA interacts with specific host cell receptors to facilitate bacterial attachment and invasion, drugs or antibodies that block these interactions could be developed as novel treatments for infectious coryza.

By conducting an immunoinformatics approach based on FlfA fimbrial protein, we can identify the most promising vaccine candidates for *Avibacterium paragallinarum* disease in Egyptian poultry farms. This will allow us to develop a multiepitope vaccine that can be used universally to protect against this devastating disease. The FlfA fimbrial protein is a key component of *Avibacterium paragallinarum*, and by analyzing its structure and function, we can gain valuable insights into how the pathogen interacts with the host immune system. Using this information, we can identify specific epitopes that are likely to elicit a strong immune response in chickens, which will be critical for developing an effective vaccine. Once we have identified the most promising vaccine candidates, we can begin designing a multiepitope vaccine that incorporates multiple epitopes from different regions of the FlfA protein. This will increase the likelihood of generating a robust immune response and provide broad protection against *Avibacterium paragallinarum*.

MATERIAL AND METHODS

Ethical approval

All procedures related to animal handling, including the collection and disposal of samples, were conducted in strict adherence to the guidelines and recommendations outlined in the European Communities Council Directive 1986 (86/609/EEC). This directive serves as a comprehensive framework for the ethical treatment of animals used in scientific research, ensuring that their welfare is prioritized at all times. By following these established protocols, we were able to ensure the safety and well-being of the animals involved in our study, while also maintaining the highest standards of scientific rigor and integrity.

Samples collection

A total of 175 suspected cases, exhibiting symptoms such as nasal discharges, lacrimation, and head swelling, were identified in both layer and broiler chickens. Samples were carefully collected and transported to the laboratory for further analysis. Our team will conduct a series of tests to confirm the presence of *avibacterium paragallinarum* in the samples. These tests will include bacterial culture, biochemical tests, and molecular identification techniques. By utilizing these methods, we aim to provide accurate and reliable results to aid in the diagnosis and treatment of affected birds.

Isolation and identification of *Avibacterium paragallinarum*

The swabs from the infraorbital sinus are the most suitable samples for the isolation of *Avibacterium Paragallinarum* as it is the primary site of infection in chickens. The brain heart infusion agar media and chocolate agar both supplemented with NADH (V-factor) 25µg/ml are used for the growth and isolation of *Avibacterium Paragallinarum*. These media provide essential nutrients required for bacterial growth, and NADH acts as a cofactor for many enzymatic reactions. After streaking the swabs onto these media, they are incubated at an optimal temperature of 37°C for 24 hours under anaerobic conditions in the CO2 incubator

and then sub- cultured for purification. The colonies that grow on these media are then subjected to further tests to confirm their identity as *Avibacterium Paragallinarum*, according to procedures described by (Calderón *et al.,* 2010). Plates were incubated at 37 ºC, and suspected colonies were selected and examined under a microscope after being stained with Gram's stain. All isolates with phenotypic characteristic suggestive of *Av. paragallinarum* were characterized biochemically by using Vitek2 compact system. The use of Vitek2 compact system allows for rapid and accurate identification, reducing the time and resources required for traditional culture-based methods. Overall, this approach is a valuable tool in diagnosing and managing *Av. paragallinarum* infections in poultry populations.

Pathogenicity Tests

A total of 110 SPF chicks, 8–9 weeks old, were randomly assigned to 11 groups of 10 birds, each housed in an isolator. Ten bacterial isolates were used. The first 10 groups were challenged by inoculation of the infraorbital sinus with 0.2 ml of the inoculum (1 x 10^9 colony forming units (CFU) per ml), from the 10 field isolates grown in tryptic soya broth for 8 hours. Group 11 was the control group, which was also inoculated with 0.2 ml of sterilized of sterilized saline. From the second to the eleventh day following vaccination, clinical signs of IC were noted. A standardized and impartial evaluation of the severity of the infection in the chickens is possible thanks to this scoring system; specifically, 0: no clinical signs; 1: mild signs (slight facial swelling and nasal discharge); 2: moderate signs (moderate facial swelling and nasal discharge); and 3: severe signs (severe facial swelling, abundant nasal discharge, and lacrimation) according to (Bragg, 2002). By dividing the mean daily disease score by the number of observation days, the total disease score for each group was determined. At 10 D post challenge (dpc), infraorbital sinus swabs from all chickens were streaked on blood agar and crossed with an *S. aureus* feeder strain. The satellite growth in the plates was evaluated after 24 hours of incubation at 37°C with 5 percent CO2.

DNA extraction and PCR

DNA extraction was performed using the QIAamp DNA Mini kit from Qiagen, Germany, GmbH, following the manufacturer's instructions. To detect *Avibacterium paragallinarum*, a set of primers was used from the conserved DNA region specific to this bacterium. The universal *A. paragallinarum* primers consisted of Universal F: 5'-TGA GGG TAG TCT TGC ACG CGA AT 3' and R: 5'-CAA GGT ATC GAT CGT CTC TCT ACT 3', which encoded a 500 bp amplicon. Additionally, common primers (Chen *et al*., 1996) were used, including F: 5'-GGCTCACAGCTTTATGCAACGAA-3'A and R: 5'-CGCGGGATTGTTGATTTTGTT-3', which encoded a 0.8 kbp amplicon, B: R: 5'-GGTGAATTTCACCACACCAC-3' encoded a 1.1 kbp amplicon, and C: R: 5'-TAATTTTCTTATTCCCAGCATCAATACCAT-3' encoded a 500 1.6 kbp amplicon (Sakamoto *et al.,* 2012). PCR reactions were conducted using a 25-µl master mix consisting of 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of forward and reverse primers (10 pmol conc.), and 3 µl of purified DNA. The reaction volume was completed to 25 µl with 7.5 µl of PCR grade water. The PCR conditions included an initial denaturation at 95ºC for 10 minutes, followed by 35 cycles of denaturation at 95ºC for 1 minute, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 1 minute. The final extension was set at 72°C for 7 minutes.

To ensure accurate and efficient PCR amplification, a precise and standardized protocol was followed. The master mix was carefully prepared with the appropriate amounts of reagents, including the Emerald Amp Max PCR Master Mix and primers. The purified DNA was added to the mix, and the reaction volume was completed with PCR grade water. The PCR conditions were optimized for maximum yield and specificity, with an initial denaturation step to separate the DNA strands, followed by cycles of denaturation, annealing, and extension. The final extension step ensured complete amplification of the target DNA sequence. Overall, this PCR protocol was designed to produce reliable and reproducible results for downstream applications. The PCR reaction was conducted using Applied Biosystem 2720 thermal cyclers from Thermo-Fisher Scientific in Germany. The PCR products were then cycled and electrophoresed in a 1% agarose gel from Abgene, also from Thermo-Fisher Scientific in Germany. To ensure the accuracy of the PCR product, a Generuler 100 bp ladder from Fermentas in Germany and a 100 bp DNA ladder H3 RTU from Genedirex in Taiwan were used. The gel images were captured using a gel documentation system from Alpha Innotech and Biometra, and electronic software from Automatic Image Capture in the USA.

Sequence Analysis of the FlfA fimbria Gene

The *Av. paragallinarum* FlfA fimbria gene was successfully amplified using a PCR assay. To achieve this, a primer pair consisting of a forward primer (5'-cca tgg ACC AAA CAA ACT CAG GAA CT-3') and a reverse primer (5'-gtc gac TTC GTA TGC GAT GGT ATA ATT-3') was utilized to amplify the FlfA gene sequences from Av. paragallinarum isolates. The PCR products were then directly sequenced and purified using the QIAquick PCR Product extraction kit from Valencia, Qiagen. For the sequence reaction, the Bigdye Terminator V3.1 cycle sequencing kit from Perkin-Elmer was used, and the Centrisep spin column was used to purify the product. To obtain the DNA sequences, a BLAST® (Basic Local Alignment Search Tool) analysis was applied.To construct a phylogenetic tree of the FlfA gene sequences, the Maximum

likelihood method in Tree Dyn web tool (Chevenet *et al.,* 2006) was used. This was done using the 107 sequences already published in GenBank (https:/ / www.ncbi.nlm.nih.gov/ genbank/). Overall, this study successfully amplified and sequenced the *Av. paragallinarum* FlfA fimbria gene, and constructed a phylogenetic tree to better understand its evolutionary history.

Immunoinformatics and machine learning based approaches

Multiple sequence alignment analysis and conserved domain analyses

The sequence similarity search tool, BLASTP from the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find homologs for FlfA fimbrial protein. BLASTP is a widely used bioinformatics tool that compares protein sequences to identify homologs, or proteins that share a common ancestor. By using BLASTP to search the NCBI database, researchers can quickly and easily identify potential homologs for a given protein of interest, such as FlfA fimbrial protein. Once potential homologs have been identified, researchers can use this information to gain insights into the function and evolution of the protein. For example, by comparing the sequences of different homologs, researchers can identify conserved regions that may be important for the protein's function. They can also use phylogenetic analysis to reconstruct the evolutionary history of the protein and its relatives. A multiple sequence analysis (MSA) was carried out using MAFFT Multiple Sequence Alignment Software Version 7 to examine the similarity between FlfA fimbrial protein in *avibacterium paragallinarum* and other organisms. Uncertain regions (i.e. e. containing gaps and/or improperly aligned) were eliminated using BMGE (Block Mapping and Gathering with Entropy).

The physicochemical properties of FlfA fimbrial protein

Expasy ProtParam tool (https://web.expasy.org/protparam/) was used to analyze the chemical formula, number of amino acids, molecular weight, theoretical pI, number of charged residues, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY).

The 2ndry and tertiary structure of FlfA protein of *Avibacterium paragallinarum*

The SOPMA tool https:// npsa-prabi.ibcp.fr/cgi-bin/ npsa_automat.pl?page=/ NPSA/npsa_sopma.html was firstly applied to analyze the secondary structure of FlfA fimbrial protein. Swiss –Model server (https://swissmodel.expasy.org/) was used for homology modeling of FlfA fimbrial protein. Swiss –Model is a fully structural bioinformatics web-server dedicated to homology modeling of 3D protein structures; it provides several levels of user interaction accessible. The Swiss-Model server is one of the most widely used tools for homology modeling, as it provides an automated pipeline that can generate high-quality models in a matter of minutes. Once the best templates have been predicted by Swiss Model, they can be used in Deep View to create a 3D model of the protein structure. The project mode in Deep View allows for multiple models to be created and compared, making it easier to determine which model is the most accurate.

Additionally, Deep View offers a range of tools for analyzing and manipulating the protein structure. Overall, using Swiss Model and Deep View (Swiss-PdbViewer) together can greatly simplify the process of creating accurate protein models for research purposes. We select the best one with high GMQE score to build the 3D model. The Sterochemical quality of the built models was evaluated by conducting Ramachandran plots using structural assessment tool**.** Ramachandran plots are a commonly used tool in structural biology to assess the quality of protein models. They provide a visual representation of the distribution of phi and psi angles for each residue in a protein structure, allowing researchers to identify regions of the structure that may be incorrectly modeled or have poor stereochemistry. By conducting Ramachandran plots using a structural assessment tool, we can gain insight into the quality of our built models. If the majority of residues fall within the allowed regions of the plot, it suggests that our model has good stereochemistry and is likely to be accurate. However, if there are many residues outside of these regions, it may indicate that our model is incorrect or has poor stereochemistry. Overall, Ramachandran plots are an important tool for evaluating the quality of protein models and ensuring that they accurately represent the structure and function of biological molecules.

Prediction of membrane protein topology and signal peptides

For the prediction of transmembrane helix services, TMHMM - 2.0 server (https: // services. healthtech.dtu.dk/ service.php? TMHMM-2.0) was used. Aaaitionally Boctopus server https: // b2.topcons.net/pred/result/ rst_dk75czgq/ seq_0/ was applied for determining membrane protein topology. In order to identify the presence of transmembrane helices and signal peptides, SignalP-6.0 (available at dtu .dk/ services/ SignalP-6.0/) was used.

Prediction of eukaryotic protein sub cellular localization

Deep learning methodology. Eukaryotic proteins' subcellular localization is predicted by DeepLoc-1.0. It is capable of distinguishing between ten different localizations, including those in the nucleus, cytoplasm, extracellular, mitochondria, cell membrane, endoplasmic reticulum, chloroplast,

golgi apparatus, lysosome/vacuole, and peroxisome. PSORT, a computer program for predicting protein localization sites in cells (https://www.psort.org/), was also used to forecast the sub cellular localization of eukaryotic proteins.

The gene ontology Prediction

By accurately predicting the gene ontology of FlfA protein, we can gain insights into its molecular function and biological processes. This information can be used to better understand the role of FlfA in various cellular pathways and to identify potential drug targets for diseases associated with its dysfunction. The use of PFP (Protein function prediction) https:// kiharalab.org/web/results.php?job_id=54777 and Argot2 (Annotation Retrieval of Genel Ontology Terms) http:// www. medcomp.medicina.unipd.it / Argot2/form.php tools allows us to efficiently annotate protein function and retrieve relevant gene ontology terms, which is crucial for advancing our understanding of the complex molecular mechanisms underlying life processes. Overall, our study highlights the importance of accurate protein function prediction in advancing biomedical research and improving human health.

In silico prediction of Virulence factors, Information molecule, Cellular process and Metabolism molecule in the Bacterial proteins

By predicting bacterial virulent protein sequences, researchers can gain a better understanding of the molecular mechanisms underlying pathogenicity. This knowledge can be used to identify and characterize novel virulence-associated factors, which may be targeted for drug or vaccine development. Additionally, identifying proteins that are indispensable to pathogenicity can provide new targets for therapeutic intervention. Finally, understanding the complex virulence mechanism in pathogens can help researchers develop more effective strategies for preventing and treating infectious diseases. Overall, prediction of bacterial virulent protein sequences has far-reaching implications for improving public health and reducing the global burden of infectious diseases. VICMpred (Saha and Raghava, 2006) is a powerful tool for researchers and scientists who are interested in studying the functions of bacterial proteins. By using this webserver, we can quickly and accurately classify proteins into different categories based on their roles in virulence, information processing, cellular processes, and metabolism. This information can be incredibly valuable for understanding how bacteria function and interact with their environments. The SVM-based method used by VICMpred is particularly effective because it takes into account not only the amino acid sequence of the protein but also its dipeptide composition. This allows for a more nuanced analysis of the protein's structure and function, which can lead to more accurate predictions about its role in the cell. Overall, VICMpred is an important tool for anyone studying bacterial proteins and their functions. Its ability to quickly and accurately classify proteins into different categories makes it an invaluable resource for researchers looking to better understand these complex organisms.

VirulentPred is a tool that uses machine learning algorithms to predict whether a given protein is virulent or not. The bi-layer cascade SVM approach used by VirulentPred involves multiple layers of SVM models, each trained on different features of the protein sequence and structure. This allows for more accurate predictions and reduces the risk of false positives or false negatives. By accurately predicting which proteins are virulent, VirulentPred can help researchers and clinicians better understand the mechanisms of bacterial infections and develop more effective treatments. VirulentPred is available as a freely accessible World Wide Web server – VirulentPred, at http://bioinfo.icgeb.res.in/virulent/.

Functional domains Analyses and predicting protein-protein interaction

Functional domains analyses and predicting protein-protein interaction are two important aspects of understanding the function of proteins in biological systems. Functional domains refer to specific regions within a protein that are responsible for carrying out a particular function. By analyzing these domains, researchers can gain insight into the overall function of the protein and how it interacts with other molecules in the cell. Predicting protein-protein interactions is also crucial for understanding cellular processes. Proteins rarely work alone, and instead often interact with other proteins to carry out their functions. By predicting which proteins interact with each other, researchers can begin to unravel complex signaling pathways and identify potential drug and vaccine targets. Together, functional domain analyses and predicting protein-protein interactions provide a powerful toolset for understanding the role of proteins in biological systems. These techniques can be used to study a wide range of diseases, from cancer to neurodegenerative disorders, and may ultimately lead to new treatments and therapies. The STRING (https://string-db.org/) database was utilized to find out with which proteins FlfA Iinteracts. The FlfA protein was searched by its name and the search was run with the highest confidence score. The FlfA protein was examined to identify its precise functional domains using Pfam (https:/ /www.ebi.ac.uk/interpro), MOTIF(https://www.genome.jp/tools/motif/)), Hmm(Scanhttps://www.ebi.ac.uk/Tools/hmmer/search/ hmmscan), Scanprosite (https://prosite.expasy.org/scanprosite/), and InterProScan https:// www.ebi.ac.uk/interpro/ result/ InterProScan/ iprscan5-R20230406-125255-0456-50302609-p1m/

Linear B cell Epitope Prediction

Various techniques will be utilized to predict epitopes for the B cell receptor (BCR). These techniques will result in protein fragments that will be analyzed in this study. One of the methods used is SVMTriP, which predicts linear B cell epitopes. Another online epitope prediction server, BepiPred, uses a hidden Markov model and a propensity measure to predict epitopes. The IEDB server employs different techniques to determine various attributes, including ABCpred, which predicts epitopes for 14-mers using a default recurrent neural network threshold of 0.51. Additionally, a novel approach utilizing kernel approaches, the BCPreds server, will be used to predict linear B-cell epitopes. By utilizing these various techniques, we aim to accurately predict epitopes for the BCR. This information can be used to develop vaccines and therapies for *Avibacterium paragallinarum.* Our study will contribute to the advancement of immunology research and aid in the development of new vaccines for *Avibacterium paragallinarum.*

Antigenicity, Allergenicity analysis and Toxicity analysis

In order to ensure the safety and efficacy of our research, we will be predicting the antigenicity, allergenicity, and toxicity of each B-cell epitope. To determine the antigenicity of the peptides, we will be utilizing two web-servers: VaxiJen v2.0 and ANTIGENpro. These tools will provide us with valuable insights into the potential immunogenicity of our chosen epitopes. To assess the potential allergenicity of our epitopes, we will be using the AllergenFP v.1.0 and AllerTOP v2.0 web servers. These tools will allow us to identify any potential allergens that may be present in our peptides, ensuring that our research is safe for all individuals. Finally, we will be utilizing the Toxin Pred web server to calculate the potential toxicity of our chosen epitopes for the host. This will allow us to ensure that our research is not only effective, but also safe for all individuals involved. By utilizing these powerful web servers, we can ensure that our research is of the highest quality and safety standards. We are committed to providing groundbreaking research that is both effective and safe for all individuals involved.

RESULTS

Isolation Identification and Prevalence

During 2021 to 2022, 175 samples from suspected outbreaks of infectious coryza (IC) were evaluated by PCR testing for birds that showed signs of acute upper respiratory tract infections like coughing, nasal discharge and facial edema. From the samples, 10/175 (5.7%) were positive for *Av. paragallinarum*. From the 10 PCR-positive samples, 10 *Av. paragallinarum* strains were isolated using BHI and chocolate agar media. The *Av. paragallinarum* isolates produced smooth colonies, as characterized by tiny dewdrops in the media.

Pathogenicity Tests

Clinical Signs

This study highlights the virulence of the 10 field strains of IC in causing clinical manifestations in chickens. The presence of typical signs such as nasal discharge and facial swelling suggests that these strains are capable of causing respiratory infections in birds. The observation of mild to moderate signs such as edema and nasal discharge indicates that the severity of infection can vary depending on the strain. However, the presence of severe signs such as hematoma, temporary blindness, and conjunctivitis suggests that some strains may be more pathogenic than others. Further research is needed to understand the mechanisms behind these varying degrees of virulence and to develop effective strategies for controlling IC infections in poultry.

Average Scores for the Clinical Signs during this study.

To identify any trends, we plotted the scores of the clinical signs for each isolate over the 7-day observation period. Our analysis revealed that the various isolates exhibited differences in their virulence towards 9-week-old chickens. It is worth noting that the uninfected negative control group did not display any clinical signs.

Re-isolation Rates

The bacteria re-isolated from a few chickens exposed to four field strains on 10 dpc using media formed typical satellite growth patterns on blood agar. The re-isolation rates for *Av. paragallinarum* A,B,C,D,E,F,G,H,I and J isolates were 40, 30, 20, 20 , 15, 10 ,20 ,30 , 20 and 15%, respectively. Strains were not isolated from the negative control group.

Sequence Analysis of the FlfA fimbria Gene

Maximum likelihood Phylogenetic Tree of based on the deduced Amino acids sequences of the FlfA protein was conducted as shown in (figure 9). The phlogenetic tree Showed genetic relationship between the Egyptian *Avibacterium paragallinarum* isolate obtained in this study and other selected *Avibacterium paragallinarum* isolates. The field *Avibacterium paragallinarum* isolate was more closely related to MBF4102349.1_type_1_fimbrial_protein_Gallibacterium_anatis(Russia),WP_136712021.1_fimbrial_protein_*Avibacterium_paragallinarum*,APF30613.1_F17like_fimbrial_subunit_Gallibacterium_anatis(China),APF30618.1_F17like_fimbrial_subunit_Gallibacterium_anatis(china),WP_013745626.1_fimbrial_protein_Gallibacterium_anatis,WP_035684837.1_fimbrial_protein_*Avibacterium_paragallinarum*,WP_017806535.1_MULTISPECIES_fimbrial_protein_Pasteurellaceae.The Amino acids sequence homology between *Avibacterium paragallinarum* isolate from Egypt and *Avibacterium*

paragallinarum reference sequence was in the range of 97.96%−100%.

The multiple sequence alignment of the FlfA protein as shown in (figure 8) showed Amino acids sequence substitutions between the field isolates and other representative *Avibacterium paragallinarum* sequences retrieved from GenBank at positions: 98 S, L 99 S, F 100 D, S101 A, N 115 G, K 116G, P 117 S, G118 P, S120D, D123Q, Q144D and Y 141Q.

Functional domains Analyses and predicting protein-protein interaction

As shown in (figure 6) A STRING database search was carried out to identify a possible functional interaction network of the virulence FlfA protein AOGF01000022 _gene 425. The identified functional protein partners with the corresponding scores were as follows: AOGF01000022 _ gene428 AOGF01000022 _gene426 (0.916), AOGF01000022_gene427 (0.838),AOGF01000012_gene2724(0.829), AOGF01000017_gene784 (0.829),AOGF01000012_gene2723, htrE_1 (0.828), AOGF01000017_gene785, htrE_2 (0.828).

Prediction of membrane protein topology and signal peptides

Membrane protein topology was analyzed using TMHMM - 2.0 server and Boctopus server. The FlfA protein was predicted to have no transmembrane helix as shown in figures (10 and 11). As shown in (figure 12) the existence and position of cleavage sites in FlfA protein were predicted using the SignalP-6.0 server. Prediction: Signal Peptide (Sec/SPI): Cleavage site between pos. 22 and 23. Probability 0.979036

The gene ontology Prediction

Molecular function and Biological process of FlfA protein was predicted to be carbohydrate binding and cell adhesion binding respectively

In silico prediction of Virulence factors, Information molecule, Cellular process and Metabolism molecule in the Bacterial proteins

The FlfA protein was analyzed using the VICMpred and Virulent Pred to understand their functional attributes. The FlfA protein was found to be virulent protein at scores 0.212 and 1.0140 respectively.

The prediction of sub- cellular localization of the FlfA protein

The prediction of sub -cellular localization of using PSORT, CELLO v2.5 and DeepLoc-1.0 indicated that the FlfA protein is Extracellular and Soluble protein as shown in (figure 13).

Determination of physical parameters of the proteins

The physicochemical features of FlfA protein sequences obtaining from ExPASy ProtParam were summarized. The molecular weight of the protein was predicted to be 20.191 KDa. The pI value (PI value 7.65) proving the acidic nature of FlfA protein. The total number of negatively charged residues (Asp + Glu) are 19 and total number of positively charged residues (Arg + Lys) are 20. Expasy Prot Param classifies the FlfA protein as stable on the basis of instability index (instability index,13.15). Extinction coefficient of FlfA protein at 280 nm was 11920 /M/cm. The biocomputed half-life was 30 hours (mammalian reticulocytes, in vitro), 20 hours (yeast, in vivo) and 10 hours (*Escherichia coli*, in vivo). Aliphatic index and Grand average of hydropathicity (GRAVY) of FlfA protein were 85.05 and - 0.195 respectively. The aliphatic index of FlfA protein revealed that it is thermostable. This estimated low range GRAVY values of FlfA protein were predicting that FlfA is hydrophilic, possibility of better interaction with water.

The 2ndry and tertiary structure and Model validation of FlfA protein of *Avibacterium paragallinarum*

As shown in (figure 7) Graphical results for FlfA protein secondary structure prediction was carried out by using SOPMA secondary structure prediction server. The secondary structure prediction of the FlfA protein was 20.10% alpha helix, 28.87% extended strand, beta turn (4.64%) and 46.39% random coil. Swiss model was recruited for homology modeling. FlfA protein 3D structure revealed a protein with full stability based on Ramachandran plot predictions as shown in figure 1. The 3D model validity was evaluated by Ramachandran blot where it was seen 88.9 % of the residues are in favored region as shown in figure 2. The more number of residues in favored region the more stable is the protein. Model validations, as shown in figure Local model evaluation: The "Local Quality" plot in the figure 3 above shows, for each residue of the model (reported on the x-axis), the expected similarity to the native structure (y-axis). QMEAN Z-Scores, QMEAN are a composite scoring function for the estimation of the global and local model quality. The score of a model is also shown in relation to a set of high-resolution PDB structures (Z-score). The FlfA protein model has a Z-score of -3.54, this value denote higher quality of the model as shown in figure 4.

Linear B cell Epitope Prediction

Seven epitopes passed the three B-cell prediction tools SVMTriP, ABCpred and BCPreds (as shown in tables1,2 and3 respectively). The most promising and top lineaer B epitopes passed the antigencity and allergencity and toxicity tests are illustrated in table 4.

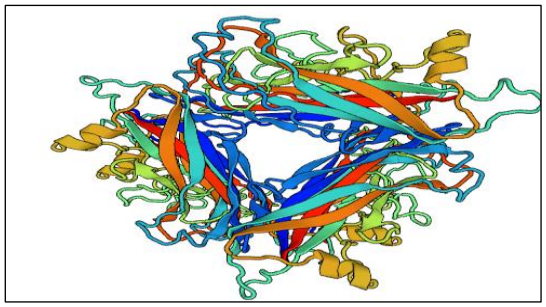

Figure 1: The final 3D structure model of FlfA protein obtained by Swiss model

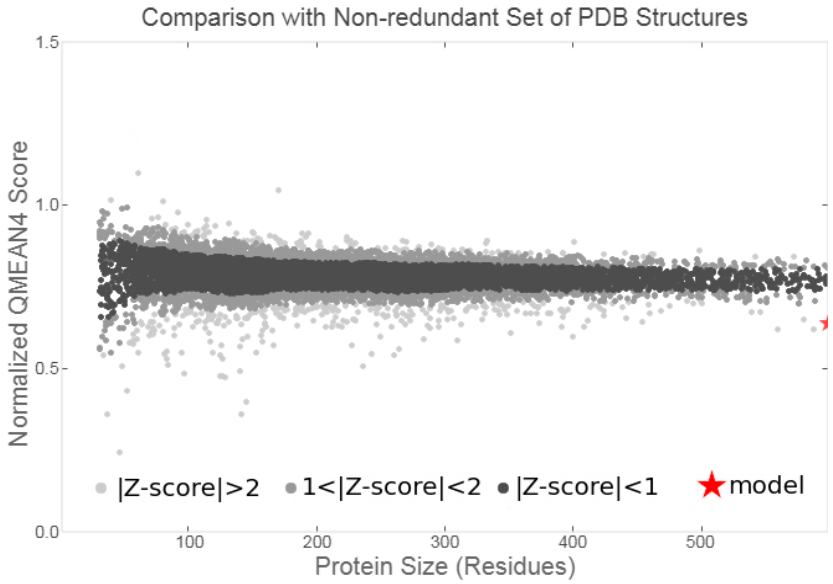

Figure 2: QMEAN Z-score analysis. The x-axis shows protein length (number of residues). The y-axis is the "QMEAN" score. Every dot represents one experimental protein structure. Black dots are experimental structures with a "QMEAN" score within 1 standard devation of the mean (|Z-score| between 0 and 1), experimental structures with a |Z-score| between 1 and 2 are grey. Experimental structure that are even further from the mean are light grey. The actual model is represented as a red star.

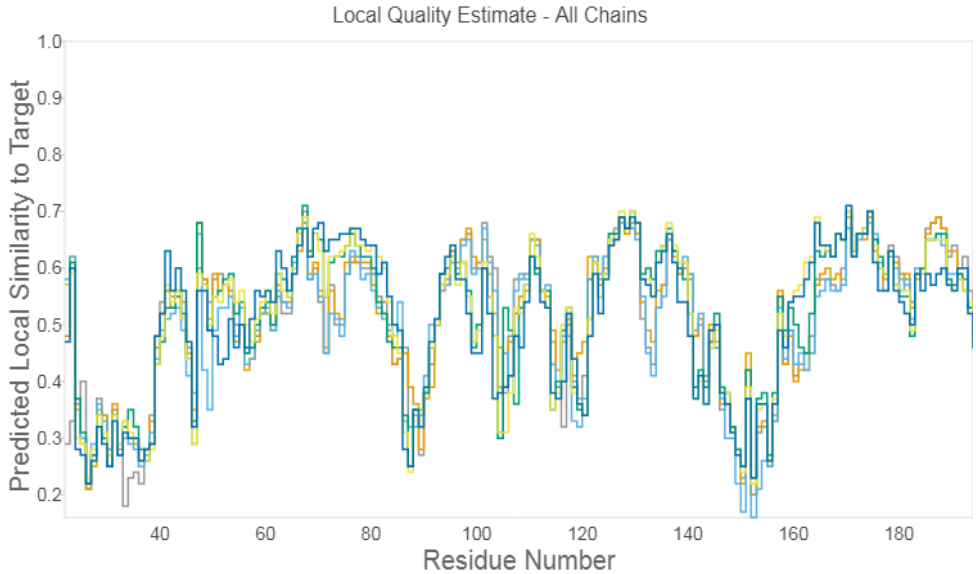

Figure 3: Local model evaluation: The "Local Quality" plot in the figure above shows, for each residue of the model (reported on the x-axis), the expected similarity to the native structure (y-axis).

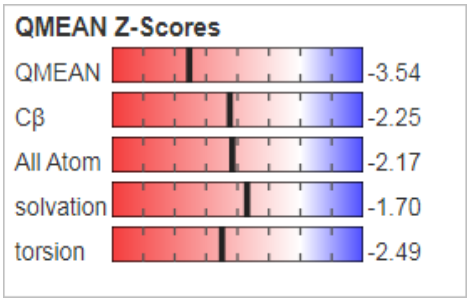

Figure 4: Model validations: QMEAN Z-Scores, QMEAN are a composite scoring function for the estimation of the global and local model quality. The score of a model is also shown in relation to a set of high-resolution PDB structures (Z-score). The FlfA protein model has a Z-score of -3.54

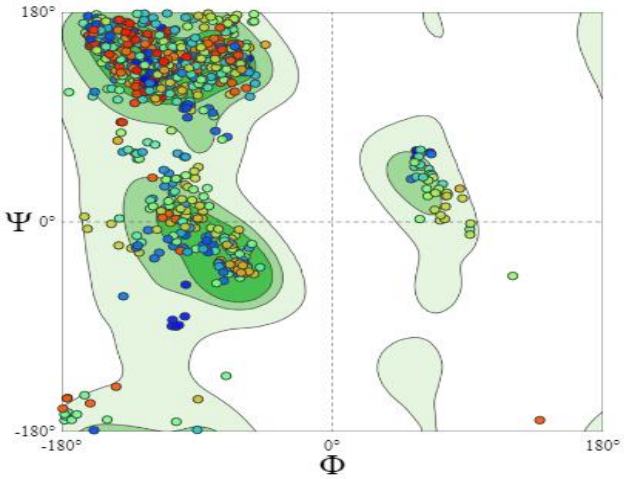

Figure 5: Validation of FlfA protein structure using Ramachandran plot. Ramachandran plot shows that 88.9 % of the residues are in favored region

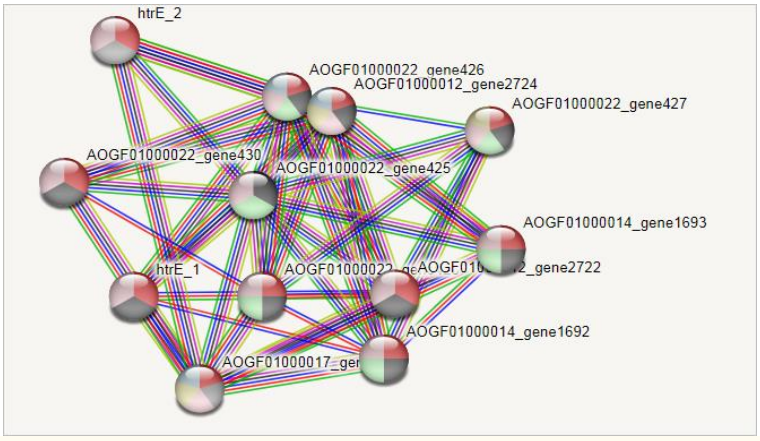

Figure 6: A STRING database search was carried out to identify a possible functional interaction network of the virulence FlfA protein AOGF01000022 _gene 425. The identified functional protein partners with the corresponding scores were as follows: AOGF01000022 _gene428(0.914) AOGF01000022 _gene426 (0.916), AOGF01000022_gene427 (0.838), AOGF01000012_gene2724 (0.829), AOGF01000017_gene784 (0.829), AOGF01000012_gene2723, htrE_1 (0.828), AOGF01000017_gene785, htrE_2 (0.828)

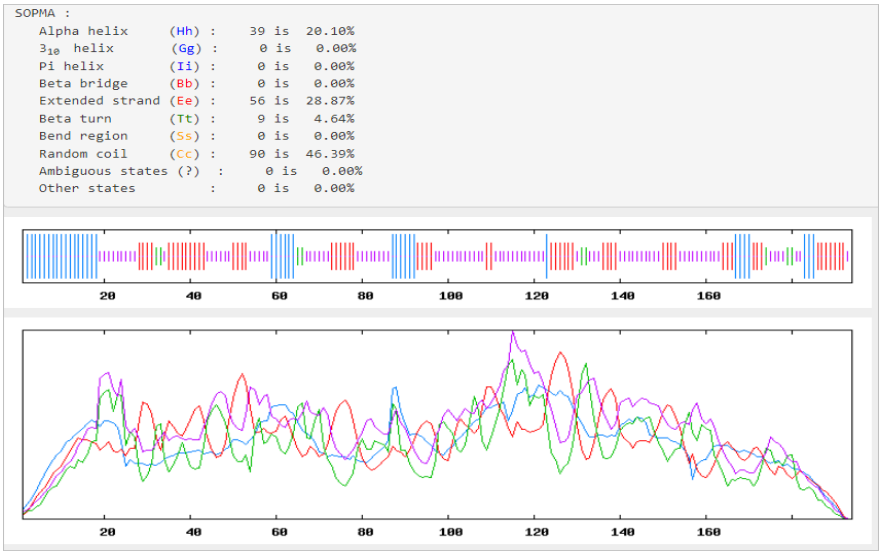

Figure 7: Graphical results for FlfA protein secondary structure prediction was carried out by using SOPMA secondary structure prediction server. The secondary structure prediction of the FlfA protein was 20.10% alpha helix, 28.87% extended strand, beta turn (4.64%) and 46.39% random coil

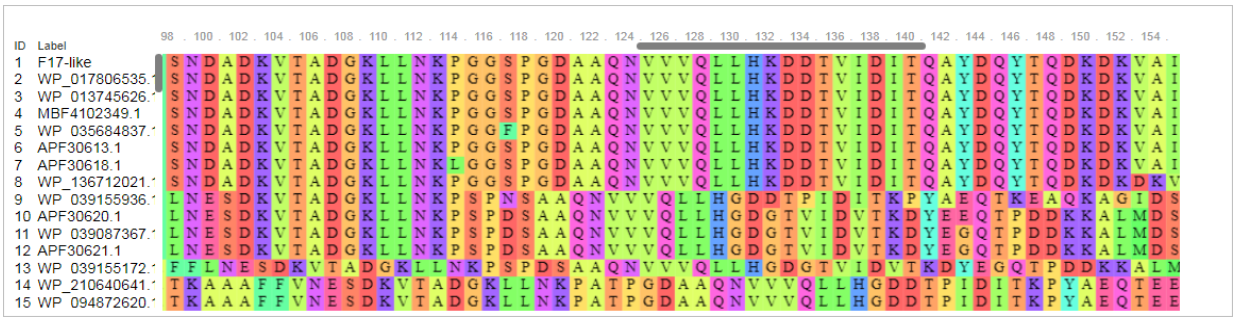

Figure 8: Deduced Aminoacids multiple sequence alignment of FlfA protein. Alignment Sequences were aligned with MAFFT Multiple Sequence Alignment Software Version 7. After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with BMGE (Block Mapping and Gathering with Entropy)

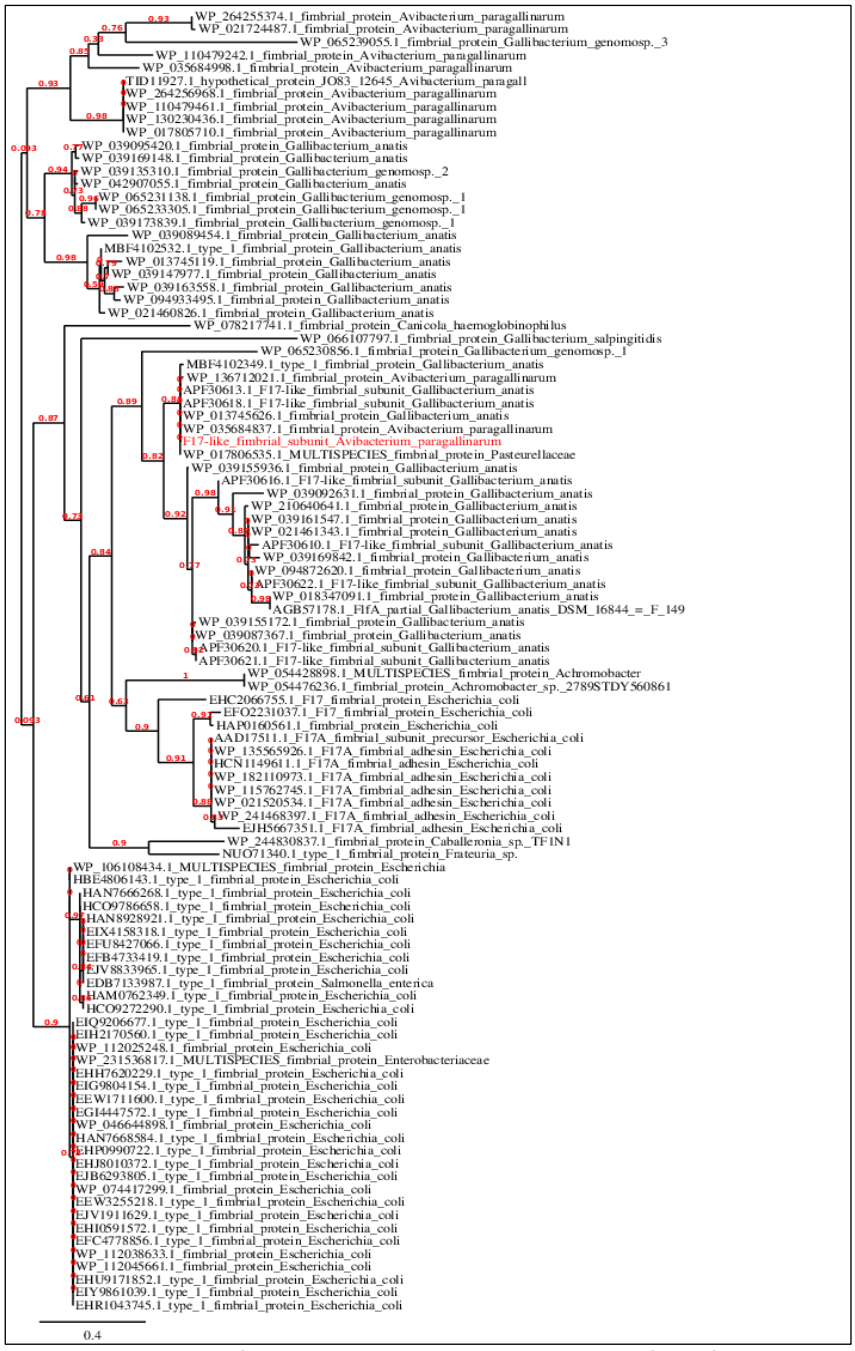

Figure 9: Maximum likelihood Phylogenetic Tree of based on the deduced Amino acids sequences of the FlfA protein. Showing genetic relationship between the Egyptian *Avibacterium paragallinarum* isolate obtained in this study and other selected *Avibacterium paragallinarum* isolates

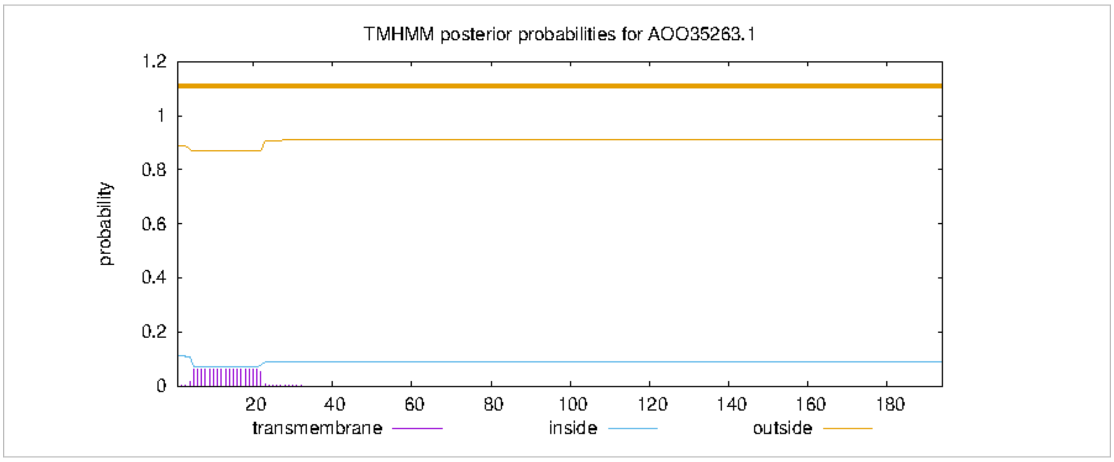

Figure 10: TMHMM - 2.0 server was applied for determining membrane protein topology. No transmembrane helix found in FlfA protein selected sequence

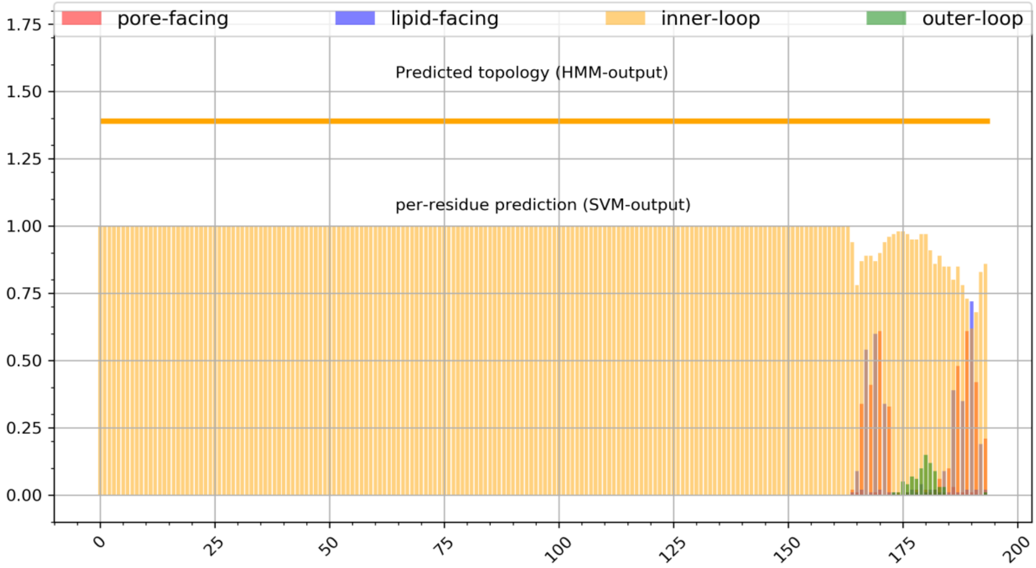

Figure 11: Boctopus server https://b2.topcons.net/pred/result/rst_dk75czgq/seq_0/ was applied for determining membrane protein topology. No transmembrane helix found in FlfA protein selected sequence

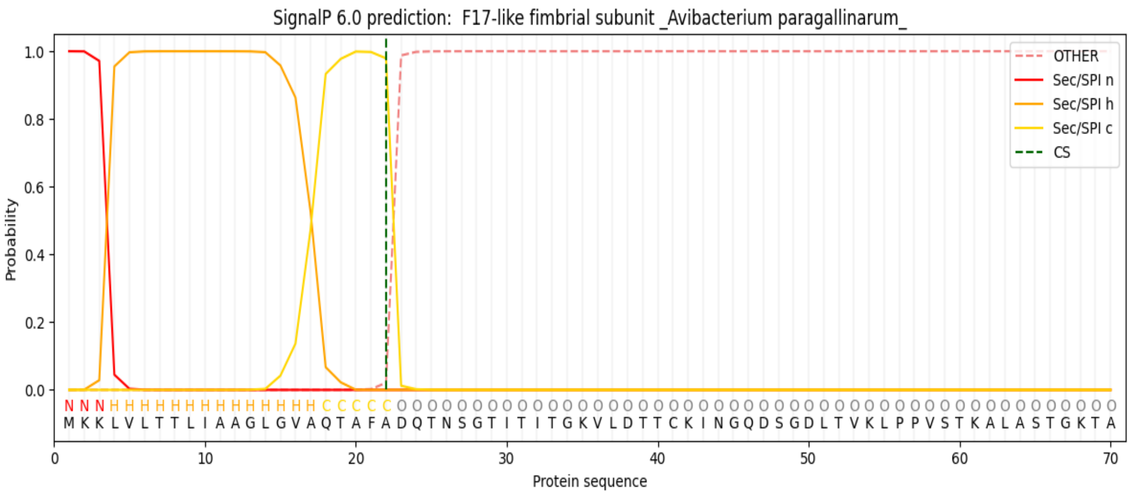

Figure 12: **The existence and position of cleavage sites in FlfA protein were predicted using the SignalP-6.0 server. Prediction: Signal Peptide (Sec/SPI): Cleavage site between pos. 22 and 23. Probability 0.979036**

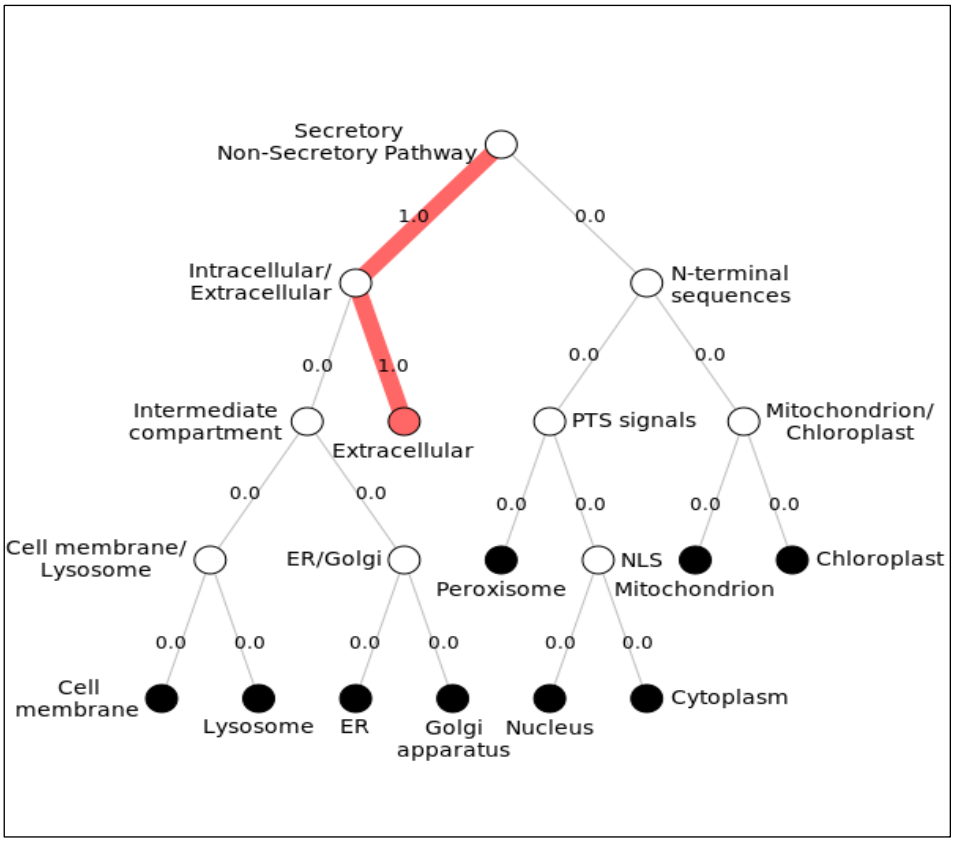

Figure 13: Prediction of eukaryotic protein sub cellular localization using deep learning approach. DeepLoc-1.0 predicts the sub cellular localization of eukaryotic proteins. It can differentiate between 10 different localizations: Nucleus, Cytoplasm, Extracellular, Mitochondrion, Cell membrane, and Endoplasmic reticulum, Chloroplast, Golgi apparatus, Lysosome/Vacuole and Peroxisome. FlfA is predicted to be Extracellular, Soluble

Table 1: Predicted Linear B Epitopes predicted from FlfA Protein using SVMTriP server

Position	Linear B Epitope	Score
126-145	VVQLLHKDDTVIDITQAYDQ	1.000
47-66	DSGDLTVKLPPVSTKALAST	0.914

Table 2: Predicted Linear B Epitopes predicted from FlfA Protein using ABCpred Prediction Server The predicted B cell epitopes are ranked according to their score obtained by trained recurrent neural network. Higher score of the peptide means the higher probability to be as epitope. All the peptides shown here are above the threshold value chosen.

Start Position	Linear B Epitope	Score
33	TGKVLDTTCKINGQDS	0.88
25	TNSGTITITGKVLDTT	0.88
42	KINGQDSGDLTVKLPP	0.85
103	KVTADGKLLNKPGGSP	0.85
88	KLASKAAVYFSNDADK	0.84
17	AQTAFADQTNSGTITI	0.84
134	DTVIDITQAYDQYTQD	0.84
50	DLTVKLPPVSTKALAS	0.82
169	KARYYATDVAGAGEVK	0.82
161	NGKAKLQYKARYYATD	0.82

Table 3: Predicted Linear B Epitopes predicted from FlfA Protein using BCPRED server

Item	Position	Epitope
Flexibility	20	AFADQTNS
	41	CKINGQDS
	61	KALASTGK
	79	LSECSSAS
	95	VYFSNDA
	109	KLINKPGGSP
	144	DQYTQDK
	177	VAITGNSPNG
	178	AGAEVK
Antigenic Propensity	1	KLVLTTLI
	35	KVLDTTCK
	50	DLTVKLPPVSTK
	123	QNVVVQLLHKD
Antigenic Propensity	96	YFSNDAD
Turns	156	TGNSPNGK

Table 4: Antigencity, Allergencity and Toxicity of Predicted Linear B Epitopes predicted from FlfA Protein

Linear B epitope	Antigencity	Allergencity	Toxicity
DSGDLTVKLPPVSTKALAST	0.905	Non Allergen	Non Toxin
TNSGTITITGKVLDTT	0.7373	Non Allergen	Non Toxin
KVTADGKLLNKPGGSP	0.9374	Non Allergen	Non Toxin
DLTVKLPPVSTKALAS	0.9328	Non Allergen	Non Toxin
NGKAKLQYKARYYATD	0.8558	Non Allergen	Non Toxin
DLTVKLPPVSTK	1.3009	Non Allergen	Non Toxin

DISCUSSION

175 samples from suspected outbreaks of infectious coryza (IC) were evaluated by PCR testing for birds that showed signs of acute upper respiratory tract infections like coughing, nasal discharge and facial edema. From the samples, 10/175 (5.7%) were positive for *Av.paragallinarum.* From the 10 PCR-positive samples, 10 *Av. paragallinarum* strains identified to be strains of sero group B were isolated using BHI and chocolate agar media. The *Av. paragallinarum* isolates produced smooth colonies, as characterized by tiny dewdrops in the media. This finding suggests that *Av. paragallinarum* is present in a relatively small proportion of the overall population of gallinaceous birds in the area studied. However, it is important to note that these results only reflect the prevalence of this particular strain of *Av. paragallinarum* and do not necessarily indicate the overall prevalence of infectious coryza in the population. Further research is needed to determine the full extent of this disease and its impact on poultry production in the region. Additionally, these findings highlight the importance of using molecular methods such as PCR and sequence analysis for accurate identification and characterization of bacterial pathogens in animal populations.

In this study, we evaluated virulence in the field isolates using a rapid artificial intrasinus-injection-route model. We observed that all 10 field isolates were virulent and able to cause IC disease in chickens. This suggests that the virulence of the field isolates was consistent and that they were all capable of causing disease in chickens. The fact that they all formed a single disease peak during the study period indicates that there may be a common mechanism or pathway by which these isolates cause disease. Further investigation into this mechanism could lead to the development of more effective treatments or preventative measures for IC disease in chickens. Additionally, it is important to continue monitoring these field isolates and their virulence in order to stay ahead of any potential outbreaks and protect poultry populations. We found that the 10 isolates displayed relatively high pathogenicity towards chickens and high re-isolation rates also, indicating that its pathogenicity may be correlated with its reproductive capacity. *Av. paragallinarum*, the causative agent of infectious coryza in chickens, is known to have high genetic variability and can rapidly evolve to evade immune responses. Therefore, it is crucial to identify the virulence factors and pathogenicity of field isolates to ensure that vaccines are effective against circulating strains. Failure to do so can result in outbreaks of infectious coryza and economic losses for poultry farmers. Additionally, understanding the prevalence patterns of *Av. paragallinarum* can aid in developing targeted control measures and reducing the use of antibiotics in poultry production. Overall, determining the pathogenicity of field isolates is a critical step towards ensuring the health and welfare of poultry

populations and maintaining sustainable farming practices. In the present study, virulence in the field isolates was responsible for substantial clinical illness in the flocks.

Phylogenetics and gene sequence analyses have become powerful tools in understanding the evolution and pathogenicity of bacterial pathogens. The FlfA fimbrial protein is an important virulence factor of *A. paragallinarum*, which plays a crucial role in adhesion to host cells and colonization. By analyzing the gene sequences of FlfA from different strains of *A. paragallinarum*, researchers can identify the genetic variations that contribute to differences in virulence and pathogenicity. Phylogenetic analysis can also help to reconstruct the evolutionary history of *A. paragallinarum* and its relationship with other related bacteria. Understanding the mechanisms underlying the pathogenicity of *A. paragallinarum* is essential for developing effective strategies for disease control and prevention. By identifying key virulence factors such as FlfA, researchers can develop targeted therapies to combat *A. paragallinarum* infections.

In the present study, FlfA gene was sequenced and phylogenetic analyses was conducted to characterize the *Avibacterium paragallinarum* field isolates circulating in Egypt over recent years. Results showed that genetic relationship between the Egyptian *Avibacterium paragallinarum* isolate obtained in this study and other selected *Avibacterium paragallinarum* isolates. The field *Avibacterium paragallinarum* isolate was more closely related to MBF4102349.1 _type_1_ fimbrial_ protein_ *Gallibacterium*_ *anatis*(Russia), WP_136712021.1_ fimbrial_ protein_*Avibacterium*_*paragallinarum*,APF30613.1_F17like_fimbrial_subunit_*Gallibacterium_anatis*(China), APF30618.1_F17like_fimbrial_subunit_*Gallibacterium_anatis*(china),WP_013745626.1_fimbrial_protein_Gallibacterium_anatis,WP_035684837.1_fimbrial_protein_*Avibacterium_paragallinarum*,WP_017806535.1_MULTISPECIES_fimbrial_protein_Pasteurellaceae.The Amino acids sequence homology between *Avibacterium paragallinarum* isolate from Egypt and *Avibacterium paragallinarum* reference sequence was in the range of 97.96%−100%.Moreover, the presence of a fimbrial cluster identical to the *Gallibacterium* FlfA in the genome of *A. paragallinarum* suggest that this fimbriae may have been recently exchanged between these two species, which could theoretically occur during natural co-colonization of the chicken upper respiratory tract. Our results agreed with Kudirkienė *et al.,* (2014). This finding raises questions about the potential for horizontal gene transfer between bacterial species in the chicken microbiome. It also highlights the importance of understanding the dynamics of bacterial communities in this environment, as well as the potential implications for poultry health and production. Further research is needed to investigate the mechanisms and frequency of such exchanges, as well as their impact on host-pathogen interactions and disease outcomes. Ultimately, a better understanding of these processes could inform strategies for controlling and preventing bacterial infections in poultry populations.

The multiple sequence alignment of the FlfA protein showed Amino acids sequence substitutions between the field isolates and other representative *Avibacterium paragallinarum* sequences retrieved from GenBank at positions:V 98 S, L 99 S , F 100 D, S101 A, N 115 G, K 116G, P 117 S, G118 P, S120D, D123Q, Q144D and Y 141Q. These amino acid sequence substitutions could potentially affect the function of the FlfA protein in the field isolates of *Avibacterium paragallinarum*. Further studies are needed to determine if these substitutions have any impact on the virulence or pathogenicity of the bacteria. Additionally, understanding the genetic diversity of *Avibacterium paragallinarum* can aid in developing effective vaccines and treatments for this important poultry pathogen.

The FlfA protein sequence underwent a physicochemical analysis using the ProtParam tool on the Expasy server. The results showed that the FlfA protein has negative GRAVY scores, indicating its solubility in hydrophilic solvents, which is consistent with previous studies by Mushtaq *et al.,* (2020). Additionally, the average extinction coefficient of 11920 suggests that the protein can absorb a significant amount of light at 280 nm. The pI value of the FlfA protein was found to be 7.65, indicating an alkaline nature. This information can be useful in developing buffer systems for the purification of recombinant proteins using the isoelectric focusing method. The aliphatic index is important in determining the protein's stability and function. Proteins with a higher aliphatic index tend to be more stable, as the aliphatic side chains are less reactive and more hydrophobic, which can help to prevent denaturation. Additionally, proteins with a high aliphatic index may have specific functions related to their hydrophobicity, such as membrane binding or enzymatic activity. On the other hand, proteins with a low aliphatic index may be more flexible and able to undergo conformational changes necessary for their function. Overall, understanding the aliphatic index of a protein can provide insight into its structure and function, which can be useful in fields such as biochemistry and biotechnology. The FlfA protein sequence showed a high aliphatic index (Ai) of 85.05, indicating its thermostability.

Finally, the instability index (II) of the FlfA protein was calculated using the ProtParam tool, and the result of 13.15 classified the protein as stable. The instability index (II) is a measure of a protein's stability under both in-vivo and in-vitro conditions. A protein with an instability index (II) of less than 40 is predicted to be stable, while a value above 40 suggests that the protein may be unstable. When examining the amino

acid distribution of the FlfA protein, it was found that the most abundant amino acid was Ala, accounting for 12.4% of the protein's primary structure. Conversely, histidine was the least common amino acid. The presence of a significant amount of hydrophilic amino acids, such as Ser, Thr, and Asp, suggests that the protein is extracellular in nature. As Asp is a charged and polar amino acid, it is likely to be located on the surface of the protein and involved in salt bridge formation. On the other hand, Lys, which is positively charged, is more likely to be found in the side chain of proteins and also involved in salt bridge formation.

Results generated by secondary structure prediction tool SOPMA showed the abundance of coiled region (46.39%) indicated higher conservation and stability of the model. This is because the secondary structure of a protein, which includes alpha helices and beta sheets, provides important information about the local folding patterns of the protein. These folding patterns can then be used to predict how different regions of the protein will interact with each other in order to form the final 3D structure. By understanding these interactions, scientists can make more accurate predictions about the overall shape and function of a protein, which can be useful for drug design and other applications. Additionally, knowing the secondary structure arrangement can help identify potential binding sites or active sites on the protein surface, which can be targeted by drugs or other molecules. Overall, understanding the secondary structure arrangement is an important step in predicting and understanding the tertiary structure of a protein. This is because X-ray crystallography and NMR are both experimental techniques that require the protein to be in a stable, crystalline form or solution, respectively. However, many proteins do not readily form crystals or may be unstable in solution, making it difficult to obtain high-resolution structures using these methods.

Secondary structural elements prediction, on the other hand, uses computational algorithms to predict the location of alpha helices and beta sheets within a protein sequence. This information can then be used to model the overall tertiary structure of the protein. While these models were accurate as those obtained through X-ray crystallography or NMR, they can provide valuable insights into protein function and interactions. Furthermore, secondary structural elements prediction can also be used in conjunction with experimental techniques to refine and validate protein structures. By combining experimental and computational approaches, researchers can gain a more comprehensive understanding of the structure-function relationship of proteins and how they respond to different environmental stimuli or interactions with other molecules. Any conformational changes within the protein can lead to alterations in these secondary structures, which can have significant functional consequences. Therefore, accurate prediction of these secondary structural elements is essential for detecting any conformational changes within the protein of interest.

The protein 3D model gained from SWISS-MODEL workspace was evaluated by both QMEAN. QMEAN, which stands for Qualitative Model Energy Analysis, is a composite scoring function describing the major geometrical aspects of protein structures. This scoring function is widely used in the field of protein structure prediction and refinement. It takes into account various factors such as bond lengths, angles, torsion angles, hydrogen bonding, and non-bonded interactions to evaluate the quality of a given protein model. QMEAN scores are particularly useful for comparing different models of the same protein or for assessing the quality of a model relative to experimental data. The use of QMEAN has greatly improved our ability to predict and refine protein structures, which has important implications for drug discovery and other areas of biotechnology. The z-scores of the QMEAN terms of the protein model were − 2.25, − 2.17, − 1.7, and -2.49 for Cβ interaction energy, all atom energy, salvation energy, and torsion angle energy, respectively. These scores implied that the predicted protein model could be considered a quality model. For high-resolution models, the average z-score is '0'. Here, QMEAN z-score for the query model was − 3.54, which was lower than the standard deviation '1' from the mean value '0' of good models, so This suggests that the estimated model was of good quality and could be used for further analysis. However, it is important to note that the QMEAN z-score is a useful metric for evaluating protein models.

According to the Ramachandran plot 88.9% of residues are found in the favored region, thus, a good stereo-chemical quality of the model was ensured by the Ramachandran plot. The Ramachandran backbone φ,ψ plot remains the simplest, most sensitive tool for assessing the stereochemical quality of a protein model. This analysis displays the main-chain ψ vs. φ torsion angles for each residue. Residues tend to cluster in certain favored regions, and are excluded from certain disallowed regions primarily because of steric hindrance between the side-chain Cβ and main-chain atoms. Moreover, it can be used to identify potential structural problems in proteins that may affect their function or interactions with other molecules. Overall, the Ramachandran plot remains a fundamental tool for understanding and improving our knowledge of protein structure and function. Membrane protein topology was analyzed using TMHMM - 2.0 server and Boctopus server. The FlfA protein was predicted to have no transmembrane helix, confirming the extracellular production nature of The FlfA protein.

A protein's optimum performance depends on the regional environment which dictates its interaction patterns and biological networks. Therefore, predicting

the subcellular localization is one of the important steps in specifying the cellular function of a hypothetical or uncharacterized protein. The subcellular localization prediction of a protein involves finding out where the protein actually resides within a cell. This is because the subcellular localization of a protein can provide important clues about its function within the cell. Knowing the subcellular localization of a protein can also help researchers design experiments to study its function and interactions with other proteins. Additionally, predicting subcellular localization can aid in drug discovery by identifying potential targets for drugs that need to enter specific cellular compartments. Overall, predicting subcellular localization is an essential step in understanding the role of a protein within a cell and its potential impact on animal health and disease. Subcellular localization predicted by the PSORT, DeepLoc-1.0 and CELLO v2.5 indicated that the FlfA protein is Extracellular, Soluble protein. The FlfA fimbrial protein is a key component of the bacterium's ability to cause disease by allowing it to attach to and invade host cells. As such, targeting this protein with a vaccine could be an effective way to prevent or reduce the severity of infectious coryza in poultry populations. Overall, understanding the role of virulence factors like FlfA in bacterial infections is crucial for developing targeted treatments and preventative measures. By targeting these proteins, we can potentially reduce the impact of infectious diseases on both poultry sector health.

The FlfA protein was analyzed using the VICMpred and Virulent Pred to understand their functional attributes. The FlfA protein was found to be virulent protein at scores 0.212 and 1.0140 respectively. Virulence factors are molecules or proteins produced by pathogens that contribute to their ability to cause disease. These factors can be targeted by drugs or vaccines to prevent or treat infections. By understanding the specific virulence factors involved in a particular infection, researchers can identify potential drug targets and design vaccines that elicit an immune response against these factors. Targeting virulence factors can help reduce the likelihood of antibiotic resistance developing, as it is less likely for pathogens to develop resistance against molecules that are essential for their survival and pathogenicity. Therefore, understanding and targeting virulence factors is crucial in the development of effective treatments and prevention strategies for infectious diseases.

MOTIF, Pfam and interproscan servers were used to predict the conserved domain and motifs regions in the FlfA sequence. Three domains were found in the FlfA gene sequence. FimA PF16970, Type-1 fimbrial protein, A :(55...194 bp), FimA is a family of Gram-negative fimbrial component A proteins that form part of the pili. There are usually up to 1000 copies of this subunit in one pilus that form a helically wound rod onto which the tip fibrillum (FimF.FimG, FimH) is attached. Pilus subunits are translocated from the cytoplasm to the periplasm via the general secretory pathway SecYEG. DUF2574 (1.44 bp) This family of proteins is functionally uncharacterized, Fimbrial-type adhesion domain (31-193 bp) This domain is found in bacterial proteins that are involved in regulation of length and mediation of adhesion of fimbriae. Fimbriae are hair-like structures found on the surface of many bacteria that allow them to attach to surfaces and other cells. The fimbrial-type adhesion domain is a crucial component in the regulation of fimbriae length and adhesion. It is responsible for mediating the attachment of bacteria to host cells, which can lead to infection and disease. Understanding the function of this domain is important for developing strategies to prevent bacterial infections. By targeting this domain, researchers may be able to disrupt the ability of bacteria to attach to host cells, preventing infection from occurring. Additionally, understanding how this domain regulates fimbriae length could provide insights into how bacteria adapt and evolve in response to changing environments. Overall, the fimbrial-type adhesion domain plays a critical role in bacterial pathogenesis and represents an important target for developing new treatments for bacterial infections. Fimbriae (also called pili), are polar filaments radiating from the surface of the bacterium to a length of 0.5-1.5 micrometers, that enable bacteria to colonize the epithelium of specific host organs. Fimbriae are also responsible to promote virulence.

A STRING database search was carried out to identify a possible functional interaction network of the virulence FlfA protein AOGF01000022 _ gene 425. The identified functional protein partners with the corresponding scores were as follows: AOGF01000022 _ gene428 AOGF01000022 _ gene426 (0.916), AOGF01000022 _ gene427 (0.838), AOGF01000012_gene2724(0.829), AOGF01000017_gene784 (0.829), AOGF01000012_gene2723, htrE_1 (0.828), AOGF01000017_gene785, htrE_ 2 (0.828).

Molecular function and Biological process of FlfA protein was predicted to be carbohydrate binding and cell adhesion binding respectively. Carbohydrate binding proteins (CBPs) play a crucial role in the survival and virulence of bacteria. These proteins are involved in various cellular processes, including adhesion, colonization, and nutrient acquisition. CBPs recognize and bind to specific carbohydrates on the surface of host cells or other bacteria, allowing the bacteria to attach and invade host tissues. In addition to their role in pathogenesis, CBPs also play a critical role in bacterial metabolism. Many bacteria use CBPs to transport and metabolize complex carbohydrates such as starch, cellulose, and chitin. These proteins are essential for breaking down these complex molecules into simpler sugars that can be used as an energy source by the bacteria. CBPs are also important targets for the development of new antibiotics. By disrupting the function of these proteins, it may be possible to prevent

bacterial adhesion and colonization or inhibit carbohydrate metabolism, leading to bacterial death. Overall, carbohydrate binding proteins are essential components of bacterial physiology and pathogenesis. The adhesion protein is thought to be involved in binding to host cells or extracellular matrix components, allowing *Av. paragallinarum* to establish infections in its avian hosts. Understanding the genetic basis of fimbria production in this bacterium could lead to new strategies for preventing or treating infections caused by *Av. paragallinarum* or related pathogens.

One of the most important goals of these research is Conducting immunoinformatics approach based on FlfA fimbrial protein to map the plausible vaccine candidates of *Avibacterium paragallinarum* disease in the Egyptian poultry farms, which will lead to universal blueprints for multiepitope *Avibacterium paragallinarum* vaccine design. Peptide vaccines, which are based on immunoinformatics, have emerged as a highly promising alternative to traditional vaccines. This innovative approach involves the use of computer algorithms to identify specific peptides that can stimulate an immune response against a particular pathogen. By targeting these peptides, peptide vaccines can provide a more targeted and effective immune response, while also reducing the risk of adverse reactions. This exciting development in vaccine technology has the potential to revolutionize the way we prevent and treat infectious diseases, and represents a major step forward in the fight against global health threats. Unlike traditional vaccines, which use weakened or dead pathogens to stimulate an immune response, peptide vaccines are designed to target specific parts of the pathogen that are recognized by the immune system. This targeted approach reduces the risk of side effects associated with conventional vaccines, such as fever, fatigue, and soreness at the injection site.

Immunoinformatics is a field that combines computer science and biology to identify potential vaccine targets based on genomic and proteomic data. By analyzing the genetic sequence of a pathogen, researchers can identify specific peptides that are likely to elicit an immune response. These peptides can then be synthesized and used as the basis for a vaccine. One of the key advantages of peptide vaccines is their ability to induce a strong and specific immune response without triggering unwanted side effects. Seven epitopes passed the three B-cell prediction tools BCPreds, ABCpred and SVMTriP. The use of peptide vaccines targeting specific epitopes of the *Avibacterium paragallinarum* can provide a more targeted and effective approach to controlling the infection. These identified peptides can be used as antigens to stimulate an immune response in poultry, leading to the production of antibodies that can recognize and neutralize the *Avibacterium paragallinarum*. These antibodies can confer protection against future infections, reducing the incidence and severity of infectious coryza outbreaks. Furthermore, peptide vaccines offer several advantages over traditional vaccines, including reduced risk of adverse reactions, improved stability and shelf-life, and potential for multivalent formulations targeting multiple pathogens. As such, peptide-based approaches hold great promise for controlling *avibacterium paragallinarum* infection in poultry populations and improving overall animal health.

CONCLUSION

FlfA is a protein that plays a crucial role in the virulence of certain *avibacterium paragallinarum*, allowing them to evade the host immune system and cause disease. By understanding how FlfA works and how it contributes to bacterial pathogenesis, scientists can design drugs or vaccines that specifically target this protein and prevent its harmful effects. This approach has the potential to be more effective than broad-spectrum antibiotics, which can also harm beneficial bacteria in the body and contribute to antibiotic resistance. Targeted therapies based on virulence factors like FlfA could help reduce the burden of *avibacterium paragallinarum* infection on poultry populations. Peptide vaccines have the potential to be more effective than traditional vaccines because they target specific epitopes of the pathogen. This means that the immune response is focused on the most important parts of the pathogen, which can lead to a stronger and more targeted immune response. In the case of *Avibacterium paragallinarum*, a bacterium that causes respiratory disease in chickens, peptide vaccines could be particularly useful because they could help to control the spread of the infection more effectively. By targeting specific epitopes of the bacterium, peptide vaccines could help to reduce the severity and duration of infections, as well as prevent new infections from occurring.

REFERNCES

- Bager, R. J., Kudirkiene, E., da Piedade, I., Seemann, T., Nielsen, T. K., Pors, S. E., ... & Bojesen, A. M. (2014). In silico prediction of Gallibacterium anatis pan-immunogens. *Veterinary Research*, *45*, 1-12.
- Bager, R. J., Nesta, B., Pors, S. E., Soriani, M., Serino, L., Boyce, J. D., ... & Bojesen, A. M. (2013). The fimbrial protein FlfA from Gallibacterium anatis is a virulence factor and vaccine candidate. *Infection and immunity*, *81*(6), 1964-1973.
- Blackall, P. J., & Soriano-Vargas, E. (2020). Infectious coryza and related bacterial infections. *Diseases of poultry*, 890-906.
- Blackall, P. J., & Soriano-Vargas, E. (2020). Infectious coryza and related bacterial infections. *Diseases of poultry*, 890-906.
- Blackall, P. J., Eaves, L. E., & Rogers, D. G. (1990). Proposal of a new serovar and altered

nomenclature for Haemophilus paragallinarum in the Kume hemagglutinin scheme. *Journal of Clinical Microbiology*, *28*(6), 1185-1187.

- Bragg, R. R. (2002). Virulence of South African isolates of Haemophilus paragallinarum. Part 1: NAD-dependent field isolates. Onderstepoort J. Vet. Res., 69. 163-169.
- Calderón, E. N., Thomas, K., Morales-Erasto, V., Salgado-Miranda, C., & Soriano-Vargas, E. (2010). Identification of Avibacterium paragallinarum serovar B-1 from severe infectious coryza outbreaks in Panama. *Avian diseases*, *54*(3), 1095-1097.
- Chen, X., Miflin, J. K., Zhang, P., & Blackall, P. J. (1996). Development and application of DNA probes and PCR tests for Haemophilus paragallinarum. *Avian diseases*, 398-407.
- Chevenet, F., Brun, C., Bañuls, A. L., Jacq, B., & Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC bioinformatics*, *7*, 1-9.
- Gallardo, R. A., Da Silva, A. P., Egaña-Labrin, S., Stoute, S., Kern, C., Zhou, H., ... & Corsiglia, C. (2020). Infectious coryza: Persistence, genotyping, and vaccine testing. *Avian Diseases*, *64*(2), 157-165.
- Khatun, M.M., Lijon, M.B., Islam, M.A., & Sultana, N. (2016). Detection of Antibiotic Resistant Avibacterium paragallinarum from Broiler Chickens in Bangladesh. *J. Adv. Vet. Anim. Res., 3,* 173–177.
- Klemm, P., & Schembri, M. A. (2000). Bacterial adhesins: function and structure. *International journal of medical microbiology*, *290*(1), 27-35.
- Kudirkienė, E., Bager, R. J., Johnson, T. J., & Bojesen, A. M. (2014). Chaperone-usher fimbriae in a diverse selection of Gallibacterium genomes. *BMC genomics*, *15*, 1-13.
- Liu, C. C., Ou, S. C., Tan, D. H., Hsieh, M. K., Shien, J. H., & Chang, P. C. (2016). The fimbrial protein is a virulence factor and potential vaccine antigen of Avibacterium paragallinarum. *Avian Diseases*, *60*(3), 649-655.
- Liu, C. C., Ou, S. C., Tan, D. H., Hsieh, M. K., Shien, J. H., & Chang, P. C. (2016). The fimbrial protein is a virulence factor and potential vaccine antigen of Avibacterium paragallinarum. *Avian Diseases*, *60*(3), 649-655.
- Mei, C., Xian, H., Blackall, P. J., Hu, W., Zhang, X., & Wang, H. (2020). Concurrent infection of Avibacterium paragallinarum and fowl adenovirus in layer chickens. *Poultry science*, *99*(12), 6525-6532.
- Mushtaq, A., Ansari, T. M., Mustafa, G., Shad, M. A., Cruz-Reyes, J., & Jamil, A. (2020). Isolation and characterization of nprB, a novel protease from Streptomyces thermovulgaris. *Pakistan Journal of Pharmaceutical Sciences*, *33*.
- Saha, S., & Raghava, G. P. S. (2006). VICMpred: an SVM-based method for the prediction of functional proteins of Gram-negative bacteria using amino acid patterns and composition. *Genomics, proteomics & bioinformatics*, *4*(1), 42-47.
- Sakamoto, R., Kino, Y., & Sakaguchi, M. (2012). Development of a multiplex PCR and PCR-RFLP method for serotyping of Avibacterium paragallinarum. *Journal of Veterinary Medical Science*, *74*(2), 271-273.