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DETECTION AND CHARACTERIZATION OF HEMOLYSIN (hlyA) GENE *Aeromonas hydrophila* AND PREDICTION OF T AND B CELL EPITOPE IN GOURAMI FISH (*Osphronemus gouramy*)

Deteksi dan Karakterisasi Gen Hemolisin (hlyA) *Aeromonas hydrophila* dan Prediksi Epitop Sel T dan B Pada Ikan Gurami (*Osphronemus gouramy*)

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ABSTRACT

In 2050, fish consumption in Indonesia will continue to increase, with a target of 59 kg/capita by 2024. Gourami fish is one of the main commodities, but the problem faced is that it is susceptible to disease, especially due to Aeromonas hydrophila, the cause of Motile Aeromonas Septicemia (MAS). This bacteria is a toxin pathogen that damages fish tissue. The pathogenesis of A. hydrophila involves enzymes and toxins such as hemolysine (hlyA). Hemolysin can lyse red blood cells, cause necrosis and increase bacterial virulence. Control can be carried out organically or inorganically. Bioinformatics-based vaccines are a potential solution by targeting the hemolysin gene, which plays a role in bacterial virulence. This research is a descriptive laboratory and observational research. Samples were taken from fish suspected of being infected with MAS and analyzed through bacterial isolation, purification, and biochemical tests. After that, the bacterial DNA is extracted, then the hemolysin gene is amplified using PCR with a specific primer. PCR results were analyzed through electrophoresis, then sequenced using the Sanger method. Homological analysis was performed with BLAST, while phylogenetic relationships were mapped using MEGA11. Prediction of immunogenic epitopes is carried out with bioinformatics devices such as IEDB. The results showed the detection of the hlyA gene through the PCR method resulted is 592 bp. Homology analysis of isolate GGBS3 A. hydrophila has a similarity rate of 98%-100% with 7 strains from GenBank. Phylogenetic analysis showed a close relationship with the GenBank database of strains from India and China with a genetic distance of 0.98 (98%). Epitope prediction resulted in seven best candidates for B cells, as well as 30 epitopes for MHC II and 10 epitopes for MHC I that are non-allergenic, antigenic, non-toxic, and water-soluble.

Keywords*:* Aeromonas hydrophila, PCR, T and B cell epitope prediction, Bioinformatics

ABSTRAK

Konsumsi ikan di Indonesia terus mengalami peningkatan seiring dengan target nasional mencapai 59 kg/kapita, menjadikan ikan gurami sebagai salah satu komoditas budidaya penting. Namun, produksi gurami menghadapi tantangan serius akibat tingginya kerentanan terhadap penyakit, khususnya *Motile Aeromonas Septicemia* (MAS) yang disebabkan oleh Aeromonas hydrophila. Bakteri ini memiliki tingkat virulensi tinggi yang dipengaruhi oleh berbagai faktor patogenik, salah satunya enzim dan toksin hemolisin (hlyA) yang berperan dalam melisis sel darah merah, memicu nekrosis jaringan, dan memperparah infeksi. Upaya pengendalian penyakit dapat dilakukan secara organik maupun anorganik, namun pengembangan vaksin berbasis bioinformatika menjadi pendekatan yang menjanjikan dengan menargetkan gen virulensi seperti hemolisin. Penelitian ini merupakan penelitian laboratorium dengan pendekatan observasional deskriptif. Sampel diperoleh dari ikan gurami yang diduga terinfeksi MAS dan dianalisis melalui tahapan isolasi bakteri, pemurnian, serta uji biokimia. DNA bakteri kemudian diekstraksi dan gen hemolisin diamplifikasi menggunakan metode PCR dengan primer spesifik. Produk PCR dianalisis melalui elektroforesis dan disekuensing menggunakan metode Sanger. Analisis homologi dilakukan menggunakan BLAST, sementara hubungan filogenetik dianalisis dengan perangkat lunak MEGA11. Selain itu, prediksi epitop imunogenik dilakukan menggunakan perangkat bioinformatika berbasis IEDB. Hasil penelitian menunjukkan keberadaan gen hlyA dengan ukuran 592 bp pada isolat A. hydrophila. Analisis homologi menunjukkan tingkat kesamaan 98–100% dengan tujuh strain dari GenBank, serta hubungan filogenetik yang erat dengan strain asal India dan Cina. Prediksi epitop menghasilkan tujuh kandidat terbaik untuk sel B, serta 30 epitop MHC II dan 10 epitop MHC I yang bersifat antigenik, non-alergenik, tidak toksik, dan larut air. Temuan ini menunjukkan potensi gen hemolisin sebagai kandidat vaksin berbasis bioinformatika untuk pengendalian MAS pada ikan gurami.

Kata Kunci: Aeromonas hydrophila, PCR, Prediksi epitop sel T dan B, Bioinformatika

INTRODUCTION

By 2050, the world population is projected to reach 9.7 billion, leading to an increased demand for protein, including fish. Gourami is the second-largest fishery commodity, with a production reaching 1,135,069 tons and boasting a high protein content. However, this increased production carries the risk of disease, one of which is infection with the bacteria Aeromonas hydrophila, which causes Motile Aeromonas Septicemia (MAS). This bacterium is a gram-negative pathogen that can cause wounds, bleeding, and even tissue necrosis in fish (Rozi et al., 2017). Its virulence is determined by its ability to produce enzymes and toxins such as aerolysin, hemolysin, protease, enterotoxin, chitinase, and deoxyribonuclease (Sadique et al., 2022).

Solutions to control A. hydrophila infections utilize both organic and inorganic approaches. Organic approaches include the use of probiotics, herbal plants such as Moringa leaves, and immunostimulants (Pandiyan et al., 2013). Meanwhile, inorganic approaches involve the use of antibiotics such as oxytetracycline, which are effective but carry the risk of developing bacterial resistance. Vaccines are a better solution for preventing this infection by inducing long-term immunity in fish. Several types of vaccines have been developed, such as inactivated vaccines, attenuated vaccines, protein subunit vaccines, and DNA/RNA vaccines. Reverse vaccinology is a new technology that utilizes genomic and bioinformatics data to predict antigens suitable for vaccination (Mailani et al., 2020).

Previous research has shown that inactivated vaccines still have a relatively high mortality rate. Another study suggests that the hemolysin gene (hlyA) could be a potential candidate for vaccine development due to its role in the pathogenicity of A. hydrophila. This

study aims to detect the presence of the hemolysin gene in A. hydrophila from gourami fish in Surabaya using PCR and analyze the gene's phylogenetic relationship with data available in GenBank. Furthermore, this study will also analyze T and B cell epitope predictions to determine their potential immunogenicity as vaccine candidates.

RESEARCH METHODS

This research was conducted in October 2024 – January 2025. The process of fish preparation, bacterial isolation, extraction, and DNA amplification were carried out at the Microbiology Laboratory of the Faculty of Fisheries and Marine Sciences, Airlangga University, bacterial biochemical tests were carried out at the Fish Quarantine Center for Quality Control and Safety of Fishery Products Surabaya II, and the process of purification and sequencing of Aeromonas hydrophilla DNA was carried out by a third party, PT. Genetica Science. This research used various laboratory equipment, including bioinformatics software, incubators, micropipettes, PCR thermal cyclers, UV trans illuminator, and electrophoresis systems. The materials used included sick gourami fish, culture media (Triptic Soy Broth and Agar), as well as various reagents and DNA extraction kits such as the G-Spin Genomic DNA Extraction Kit, hemolysin gene primers, and markers.

RESULT

Morphological Characterization and Biochemical Tests of A. hydrophila Isolates

Based on the observation results of the identification of bacterial isolation from gourami fish that the colony morphology originated from two bacterial isolates, GGBS3 which showed a circular shape with an average diameter of 2-3 mm and appeared yellowish on nutrient agar. Based on the comparison and characteristics, the two isolates were identified as A. hydrophila, following Popoff and Veron (1976). These characteristics include cream colony color, convex elevation, smooth inner structure, gas and H2S production, fermentation, positive results (+) on catalase and oxidase, oxidative, motility, indole, novobiocin susceptibility, arginine, Voges Proskauer (VP), aesculin, growth at 30°C, salt tolerance of 6.5% NaCl, and Simmons Citrate test. However, it produced negative results on urea, lysine and ornithine decarboxylation, O/129 disc susceptibility, and methyl red (MR) test. The glucose, sucrose, lactose, and maltose tests yielded positive results, while the mannitol, inositol, and sorbitol tests yielded negative results.

Molecular Identification of Hemolysin Genes Using PCR

Detection of DNA sequence fragments of the hemolysin gene (hlyA) from a pure culture of A. hydrophila isolates was successfully electrophoresed using oligonucleotide primers that were detected positively, showing 592 bp. The following Figure 1 is the PCR amplification of hemolysin (hlyA).

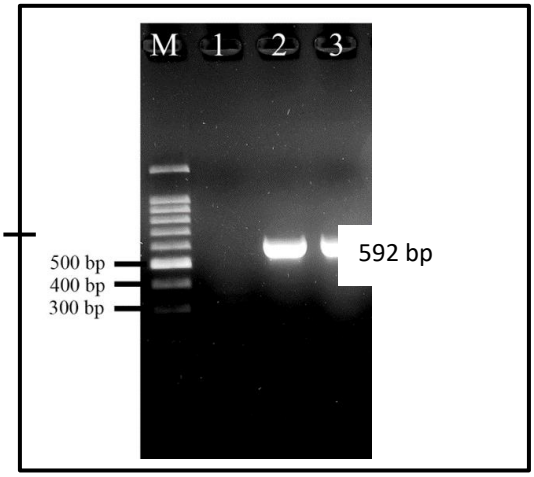


Figure 1. PCR results of Hemolysin (hlyA) A. hydrophila.

The results of PCR amplification of the hemolysin gene (hlyA) showed that the GGBS3 isolate showed 592 bp. This is slightly different from the reference of Hota et al. (2024), namely the target hemolysin gene (hlyA) of A. hydrophila was detected with a length of 600 bp using PCR. The success of DNA amplification with PCR is determined by several factors, namely, the purity and concentration of components in the PCR premix solution, gene oligonucleotide primers, the amount and purity of sample DNA, technical and non-technical factors such as contamination (Sjafaraenan et al., 2018).

Hemolysin (hlyA) Gene Sequencing Results of Aeromonas hydrophila

DNA sequencing results aim to determine the nucleotide sequence contained in DNA. The electropherogram results can be seen in Figure... An electropherogram is a diagram of the results of nucleotide sequencing with a peak graph indicating a nucleotide base. The following is the sequencing result of the DNA amplification of Hemolysin (hlyA) A. hydrophila.

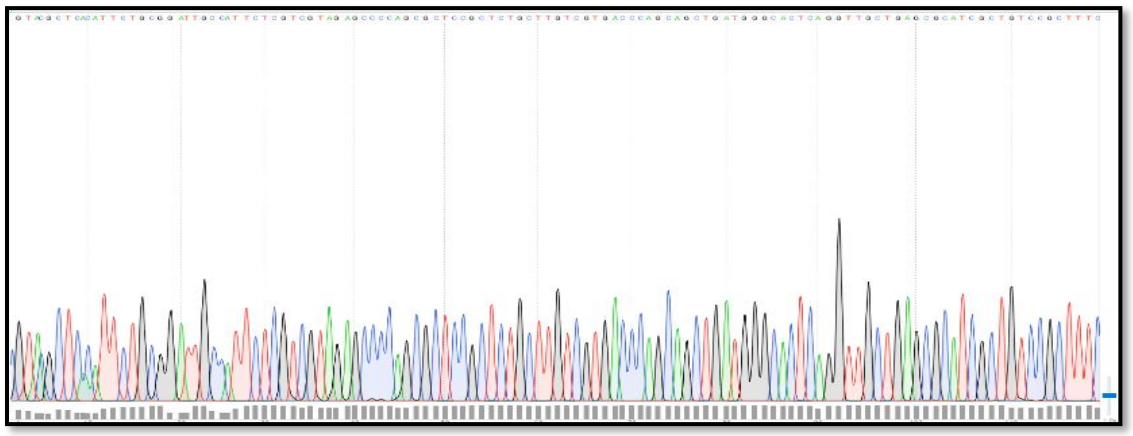


Figure 2. Electropherogram of Hemolysin A. Hydrophila Gene Sequencing.

DNA sequencing results aim to determine the nucleotide sequence contained in DNA. This DNA sequence will be related to hereditary genetic information in the nucleus, plasmids, mitochondria, and chloroplasts, which will form the basis of gourami development (Aisyah et al., 2020). A good electropherogram is characterized by the absence of noise and non-overlapping signal peaks, as can be seen in Figure 2. This is evidenced by the quality of the peaks in the electropherogram graph, which can be seen clearly without any overlapping or double peaks. Factors that affect the quality of the results are the amount of DNA template or

primer that is too little or insufficient, which will result in the primer not interacting with the template efficiently (Guspratiwi, et al., 2019).

Results of Nucleotide Sequence Homology of Hemolysin Gene (hlyA) Aeromonas hydrophila

Homology results on the nucleotide sequence of the hemolysin gene (hlyA) of A. hydrophila isolate Surabaya (GGBS3) were carried out using the BLAST program on the National Center for Biotechnology Information (NCBI) web server.

No.	Closest identity to GenBank reference	Accession Number	% Identity	Location
1.	Aeromonas sp. MTHA (hlyA)	JN602736.1	98.56%	India
2.	Aeromonas hydrophila strain BM178 haemolysin (hly)	MN587716.1	98.53%	China
3.	Aeromonas sp. MCCB 139 hemolysin (hlyA)	JN602738.1	98.0%	India
4.	Aeromonas hydrophila strain: AH201 (hlyA)	AB206041.1	97.83%	Jepang
5.	Aeromonas hydrophila strain JNC1007 (hlyA)	JQ003206.1	96.93%	China
6.	Aeromonas hydrophila strain JNC304 (hlyA)	JF738032.1	96.76%	China

The results of the DNA sequence of the hemolysin gene (hlyA) on the NCBI site showed that the hemolysin gene (hlyA) of the GGBS3 isolate (sample 3) had a kinship relationship of 98%-98.56% with isolates JN602736.1, MN587716.1 and JN602738.1 originating from India and China. Analysis of the kinship of the hlyA gene of the GGBS3 isolate with other reference A. hydrophila species had a kinship level of 96.76%-97.83% with A. hydrophila originating from Japan (AB206041.1) and in China (JQ003206.1 and JF738032.1). The most similar GenBank database sequences were characterized by the same Max Score and Total Score values, Query Coverage approaching 100%, E-value approaching 0.2 and Max Indent approaching 100%. Query coverage is the percentage of nucleotide lengths that match the database sequences found in BLAST. The acceptable percentage value is at least 95%. The closer the E-Value is to zero, the more reliable the results will be. If the value is 1, then it should not be used (Narita et al., 2012).

Phylogenetic Tree Results of Hemolysin Gene (hlyA) Aeromonas hydrophila

Phylogenetic tree analysis using the neighbor-joining method, followed by Maximum Composite Likelihood (MCL), and validation with 1000x bootstrap replicates will be displayed on the phylogenetic tree. The results of the kinship analysis can be seen in Figure 3.

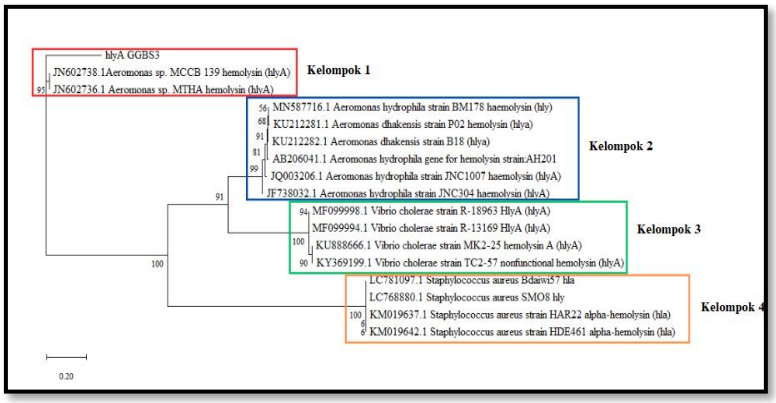


Figure 3. Results of kinship analysis with a Phylogenetic Tree based on the nucleotide sequence of the hemolysin gene (hlyA) of A. hydrophila isolate from Surabaya, Indonesia, matched with the nucleotide sequence in the GenBank database as a comparison isolate.

The results of the phylogenetic tree analysis show three subgroups (in-groups) and one group as an out-group. Out-group taxa are needed to demonstrate character polarization, known as the outgrouping method, to produce a tree with a root (rooted tree) as a clear boundary. This is also explained in Pangestika et al. (2015), that out-groups are necessary in phylogenetic tree reconstruction. This is because out-groups are useful for identifying primitive and derived characters from in-groups and as a starting point for forming a phylogenetic tree. Bootstrap analysis is a method that will test the phylogenetic tree to determine how well the model dataset is modeled. A bootstrap analysis with a value of 70% or more indicates that the grouping formed from the phylogenetic tree is reliable (Suherman and Arsad 2020).

DISCUSSION

Results of Epitope Prediction of T and B Cell Immune Hemolysin Gene of Aeromonas hyidrophila

a. Results of Chemical and Physical Properties

Protein analysis aims to determine the chemical and physical properties of the target protein. Protein analysis uses the ProtParam tools web server. The results of the ProtParam analysis of the hemolysin (hlyA) gene sequencing of A. hydrophila are shown in Table 5.

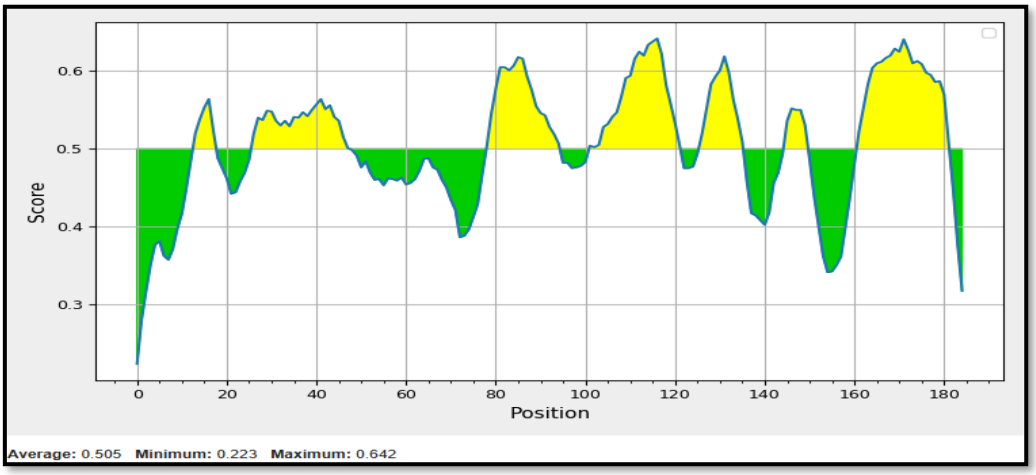
Parameter	Value
Number of Amino Acids	2785
Molecular weight	19253.43
Isoelectric point (pI)	7.26
Part time	2.8 hours (mammalia, in vitro). 10 minutes (ragi, in vivo). 2 menutes (Escherichia coli, in vivo).
Index stability (II)	31.40
aliphatic index	128.11
Grand average of hydropathicity (GRAVY)	-0.423

The physicochemical and solubility characteristics of the designed GGBS3 isolate were assessed using the ProtParam web server. The molecular weight of the designed protein construct was calculated to be 19253.4 kDa, indicating significant antigenic potential. With a theoretical isoelectric point (pI) of 7.26, it is characterized as an essential immunogenic protein

in the body (Gasteriger et al., 2005). The stability index of the target protein was 31.40. Based on the data obtained, it indicates that the target protein is a stable protein. An instability index exceeding 40 will indicate protein instability (Guruprasad et al., 1990). The aliphatic index of 128.11 represents the relative volume occupied by amino acids (alanine, valine, isoleucine, and leucine) that have aliphatic side chains and can be considered an important factor for increasing the thermostability of globular proteins. The estimated half-life is 2.8 hours, in yeast in vivo 10 minutes, and in Escherichia coli bacteria in vivo 2 minutes. In addition, the GRAVY value of -0.423 indicates hydrophilic (Kyte et al., 1982).

b. B Cell Epitope Prediction Results

The bioinformatics approach used to predict immunogenic epitopes for B cells using the IEDB software using the Bepipred Liner Epitope Prediction 2.0 method revealed that there are 7 immunogenic epitope candidates for the hlyA protein-coding gene. The electrophoregram results for the prediction of immunogenic epitopes for the hemolysin gene (hlyA) sample of A. hydrophila isolate Surabaya can be seen in Figure 6. The number of immunogenic epitope prediction results for the hemolysin gene (hlyA) of A. hydrophila isolate GGBS3 can be seen in Table 6.



No.	Start	Finished	Peptide	Long
1	14	18	PQRSA	5
2	27	48	MGTQVAERIAVRFPLPALTQAQ	22
3	79	95	EGLSRRGLVTGHRAQTV	17
4	102	122	AAVVKAPQLQVDRATAGKHRV	21
5	127	136	LEAGGGRHPS	10
6	146	150	EGDMG	5
7	162	182	VDGADVDRGVDLELGAARLVP	21
PQRSAMGTQVAERIAVRFPLPALTQAQEGLSRRGLVTGHRAQTVAAVVKAPQLQV DRATAGKHRVLEAGGGRHPSEGDMGVDGADVDRGVDLELGAARLVP				

c. T cell epitope prediction

The results of the T epitope prediction in MHC II predicted 2,349 epitopes, then ranked 10th, resulting in 227 epitopes. This was then screened to remove duplicate epitopes, resulting in 54 epitopes. The results were then selected with the highest antigenicity score, resulting in 30 epitopes. The results from MHC I predicted 4,995 epitopes, then ranked 1%, resulting in 50

epitopes. This was then screened to remove duplicate epitopes, resulting in 27 epitopes, and the highest antigenicity score was selected, resulting in 10 epitopes.

Epitope prioritization was carried out by testing the requirements for good epitope prediction, namely antigenicity, allergenicity, non-toxicity, and water solubility. This epitope prediction testing was carried out by entering the predicted epitopes from MHC I and MHC II one by one using a web server. The T cell epitope on MHC II, namely GVDGADVDRGVDLEL, has the highest antigenicity value of 1.63, while the T cell epitope VAAVVKAPQLQVDRA has the lowest antigenicity value of 0.53. The T cell epitopes on MHC I, namely QVDRATAGK and VAERIAVRF, have the highest antigenicity value of 1.42, and MGTQVAERI has the lowest antigenicity value of 0.70.

MHC II				
MHC II Results	**Antigen Value**	**Water Solubility**	**Toxicity**	**Allergenicity**
AAVVKAPQLQVDRAT	0,77	Good	Non-toxic	Non-allergenic
ADVDRGVDLELGAAR	0,97			
AERIAVRFPLPALTQ	1			
AMGTQVAERIAVRFP	1,25			
APQLQVDRATAGKHR	1,37			
DGADVDRGVDLELGA	1,36			
DMGVDGADVDRGVDL	1,12			
DRGVDLELGAARLVP	1,22			
DVDRGVDLELGAARL	0,93			
EGDMGVDGADVDRGV	1,01			
ERIAVRFPLPALTQA	0,94			
GADVDRGVDLELGAA	1,3			
GDMGVDGADVDRGVD	1,01			
GTQVAERIAVRFPLP	1,4			
GVDGADVDRGVDLEL	1,63			
KAPQLQVDRATAGKH	1,33			
LPALTQAQEGLSRRG	0,81			
MGVDGADVDRGVDLE	1,25			
PALTQAQEGLSRRGL	0,75			
PLPALTQAQEGLSRR	0,78			
PQLQVDRATAGKHRV	1,19			
PQRSAMGTQVAERIA	0,79			
PSEGDMGVDGADVDR	1,16			
RSAMGTQVAERIAVR	1,16			
SAMGTQVAERIAVRF	1,27			
SEGDMGVDGADVDRG	1,17			
TVAAVVKAPQLQVDR	0,63			
VAAVVKAPQLQVDRA	0,53			
VDGADVDRGVDLELG	1,5			
VKAPQLQVDRATAGK	1,13			
MHC I				
HPSEGDMGV	1.25	Good	Non-toxic	Non-allergenic
LELGAARLV	0.80			
MGTQVAERI	0.70			
QEGLSRRGL	1.15			
QVAERIAVR	1.24			
QVAERIAVRF	1.41			
QVDRATAGK	1.42			
RATAGKHRVL	0.90			
TQVAERIAVR	1.18			
VAERIAVRF	1.42			

The goal of the highly specific and actively acquired adaptive immune response is to eliminate or halt the growth of infection. Memory B cells generated by adaptive immunity

recognize the organism upon subsequent contact after initial recognition. The immunological memory of adaptive immunity forms the basis for vaccination. In the adaptive immune system, the primary task of B and T lymphocytes is to generate antibody-dependent cellular immunity against foreign pathogens. Hemolysin sequencing is used to identify MHC-I, MHC-II, and B cell epitopes (Alawam and Alwethaynani MS, 2024). Hemolysin was found to have 7 B cell epitopes of varying lengths, which were then processed to predict MHC-I and MHC-II epitopes. Epitopes with the lowest percentile scores were prioritized and selected to ensure maximum binding to immune receptors.

CONCLUSION

Based on the research that has been done, regarding the detection and characterization of the hemolysin gene (hlyA) of Aeromonas hydrophila and the prediction of T and B cell epitopes, namely This study detected and characterized the hemolysin gene (hlyA) in Aeromonas hydrophila from gourami fish in Surabaya. PCR results showed the presence of the hlyA gene with a size of 592 bp. Phylogenetic analysis showed that the GGBS3 isolate had a genetic similarity of 98%-98.56% with strains from India and China. Prediction of T and B cell epitopes using the EIDB web identified 7 candidate B cell epitopes as well as 30 MHC II epitopes and 10 MHC I epitopes, which were non-allergenic, antigenic, non-toxic, and water-soluble.

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