RESEARCH ARTICLE

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In silico recombinant plasmid design of pHA171 with *phdABCD* insertion for ethidium bromide degradation



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ABSTRACT

Background: Ethidium bromide is a common reagent that is used in nucleic acid staining. However, ethidium bromide has toxic and carcinogenic properties that are harmful to the environment. Phenanthrene dioxygenase (encoded by *phdA*, *phdB*, *phdC*, and *phdD* genes) in *Nocardioides sp.* KP7 can oxidize the phenanthridine structure aim to eliminate carcinogenic properties.

Objective: This study aims to visualize and predict the structure, active site, and characteristics of the phenanthrene dioxygenase using bioinformatics tools.

Methods: Plasmid design were prepared by inserting genes of interest *phdA*, *phdB*, *phdC*, and *phdD* from the NCBI database. Furthermore, several protein analysis tools were used for structure visualization, active site enzyme improvement, and protein characteristic of phenanthrene dioxygenase.

Results: The prediction results found that phenanthrene dioxygenase reacts with the ethidium bromide substrate through the interaction of Fe^{3*} ions with water. The solubility level of phenanthrene dioxygenase protein is 0.404, suggesting that the protein has low solubility. The protein isoelectric point (pl) is between 5.17 to 5.36, and the protein molecular weight is 121.143 kDa.

Conclusion: *In silico* analysis has supported that recombinant plasmid met characteristics for the construct which consists of gene interest and protein library.

Keywords: bioinformatic, bioremediation, escherichia coli, ethidium bromide, phenanthridine

Introduction

Ethidium bromide reagent used for nucleic acid staining in specimen testing for primary confirmation using gel electrophoresis purposes. Many molecular biology researchers consider this system as the highly sensitive and low-cost method to visualize DNA in agarose gel after electrophoresis [1]. However, ethidium bromide is considered a serious biohazard due to its high potential for mutagenicity, carcinogenicity, and teratogenicity [2]. According to Regulation (EU) No. 1907/2006, this material and its container must be disposed of as hazardous waste. Improper disposal of ethidium bromide has the potential

to contaminate groundwater which may be used for parks, agriculture, washing or even drinking purposes. Because ethidium bromide is a potent mutagen, high priority should be put to manage the waste containing that compound [3]. Therefore, one alternatif to overcome the problem is through a bioremediation strategy. Youssef *et al.* (2020) reported a photocatalytic degradation strategy for ethidium bromide and has successfully degraded 60% of the dye within one hour [4]. Furthermore, another bioremediation strategy should be implemented for rapid ethidium bromide degradation, such as technology with enzymes that have a highly specific compound targeted.

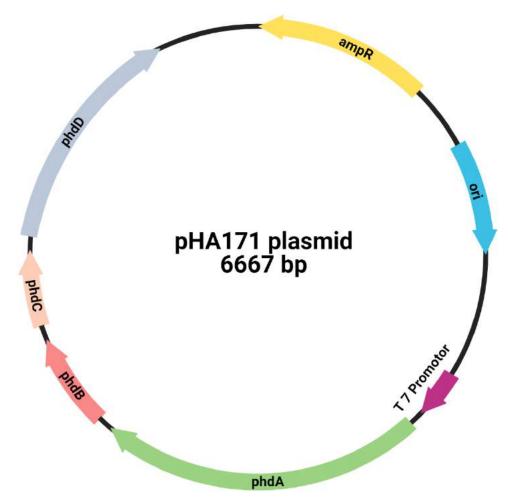


Figure 1. Recombinant plasmid design created by using Benchling. Gene in plasmid rearranges with some tools of gene interest such as phenanthrene dioxygenase encoded by *phdA*, *phdB*, *phdC*, *phdD* gene, and protein marker library to simplify purification process. Images created with <u>biorender.com</u>

The use of enzymes to degrade a compound has been used in various applications. Major biodegradative enzymes are over-expressed using recombinant DNA technology [5]. Meanwhile, ethidium bromide has a phenanthridine structure which causes carcinogenic properties in ethidium bromide. Phenanthridine belongs to polycyclic aromatic hydrocarbon groups, consist of carbon and hydrogen atoms with two or more fused aromatic rings arranged in varying structural forms that make it has environmental persistence [6]. The breakdown of phenanthridine structure into other forms can be used as a form of ethidium bromide waste treatment so that it can be safer for the environment.

One of the enzymes that have the ability to change the form of phenanthridine is phenanthrene dioxygenase. Phenanthrene dioxygenase enzyme activity in *Nocardioides sp.* KP7 has the potential to oxidize the phenanthridine structure thereby reducing carcinogenic properties [7]. However, this ability still needs to be optimized. This research aims to provide a bioinformatics analysis to visualize and predict the structure, active side, and characteristics of the phenanthrene dioxygenase enzyme in *Escherichia coli* BL21 (DE3) to degrade ethidium bromide in its phenanthridine structure. Ultimately, it can be a solution for handling ethidium bromide waste through strategies bioremediation.

Methods

Bioinformatic searching database

This research was carried out within *in silico* studies using bioinformatic databases such as National Center for Biotechnology Information

Recombinant Plasmid pHA171 6667 bp Ndel (2400) T 7 Promoter phdB phdC ampR ori phdA phdD source

Figure 2. *In silico* recombinant plasmid design with restriction site. The purple arrow is a source of the whole plasmid within 6667 bp that have *Nde*I restriction site on 2400 bp and *Hind*III restriction site on 2876 bp, both are cutting position for *phdABCD* gene as the insert. Image created with biorender.com

(NCBI) and Snapgene. To construct the plasmid model, we choosed Nocardoides sp. KP7 as bacterial strain that has a phenanthrene degradation gene sequence such as *phdA*, *phdB*, *phdC*, and *phdD* genes. Genes were constructed in Escherichia coli BL21 (DE3) with T7 as main promoter which is found in snapgene database. E. coli BL21 (DE3) can be used as a host due to growth fast and cultivate easily for gene expression [8]. DNA recombinant model consists of restriction enzymes, T4 DNA ligase, purification tags such as three base ATG start codon and three base TGA stop codon, FNDG Tat signal peptide, three bases GSG for the linker, 6-His affinity tag, Flag peptide tag, and TEV protease tag [9]. All of these components were designed at Benchling as a bioinformatics tool.

Plasmid recombinant construction

Recombinant plasmids were prepared by inserting genes of interest, phdA, phdB, phdC, and phdD genes are collected from the NCBI database. These sequences were converted to a txt file before exporting into Benchling to reconstruct the recombinant plasmid. Restriction enzyme was chosen at the sites of 2400 bp (NdeI) and 6630 bp (HindIII). The gene clusters were located between NdeI and HindIII to help to find the restricted site when an experiment in vitro is carried out. T7 promoter and Ribosome Binding Site (RBS) sequence were designed before gene clusters (Figure 1). The ATG start codon was added after the promoter for initiating translation

and also FNDG tat, 6 his-tags, and a linker were added before *phdA*. Plasmid expression vector pHA171 has several features including *ampR* gene for the screening process and RBS as a ribosome attachment site in the translation process. The reconstructed plasmid may be transformed into *E. coli* BL21 (DE3) as host for screening process using blue-white screening techniques.

Protein prediction analysis

Amino acid sequences of phenanthrene dioxygenase were analyzed using in silico approaches. Several protein analysis tools were used for structure visualization, active site enzyme improvement, and protein characteristic determination: i) Open Predict Protein platform to predict secondary structure, ii) ExPaSy and Swiss model to predict tertiary structure, iii) PROTTER platform to predict transmembrane protein, iv) Kyte-Doolittle Hydropathy Plot to analyze protein hydrophobicity, v) Protein-SOL to analyze protein solubility, vi) ExPaSy tool to characterize physicochemical characteristics such as isoelectric point and molecule weight of the protein. In each platform, amino acid sequences of phenanthrene dioxygenase from NCBI were entered into the platform.

Results phdABCD plasmid construction

A secondary database was used to simulate dry laboratory genetic engineering constructs

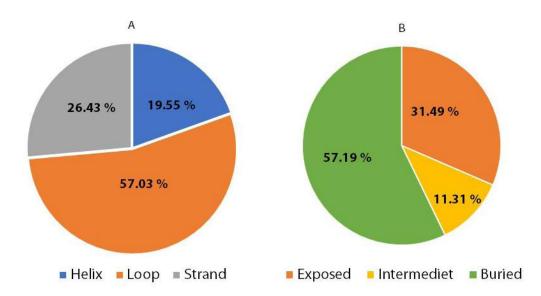


Figure 3. Prediction of secondary structure of proteins. A). Composition of secondary structure. B). Solvent accessibility of protein. Images created from <u>predictprotein.org</u>

Plasmid (Vector) Genes Source **Enzyme** Phenanthrene pHA171 (pT7-7) phdABCD Nacordiodes sp. KP7 dioxygenase pJHF3051 (pUC119) todC1C2BA Pseudomonas putida F1 Toluene dioxygenase Pseudomonas Pseudoalcaligenes pKF6622 (pUC1118) bphA1A2A3A4 Biphenyl dioxygenase KF707 P. putida F1 Toluene/biphenyl pKF6256 (pUC118) todC1::bphA2A3A4 P. pseudoalcaligenes KF707 dioxygenase

Table 1. Suitable plasmid for phenanthrene dioxygenase [10]

in determining the design of plasmids, bacterial cells, restriction enzymes, and ligase enzymes. Secondary data was obtained by utilizing gene bank platform/software such as NCBI, AddGene, ecoCYC database, as well as some literature studies. Shindo et al., (2001) summarized the potential of degrading enzymes with a suitable plasmid for the biohazard remediation process (Table 1) [10].

Plasmid pHA171 can be used as a vector for the *phdABCD* gene that obtained from *Nocardoioides sp.* KP7. Saito *et.al.* (2000) described that plasmid pHA171 was transformed into *Escherichia coli* BL21 (DE3) to observe tricyclic-fused aromatic heterocycles [11]. The results indicate the phenanthrene dioxygenase can convert phenantherene/phenanthridine into compounds

that less toxic-carcinogenic. Thus, *E. coli* BL21 (DE3) is suitable for host transformant and pHA171 as a plasmid for the genetic engineering process. In the plasmid design, *phdA* is located at 2456-3805 bp, *phdB* is located at 3843-4364 bp, *phdC* is located at 7275-7484 bp, *phdD* is located at 7481-8725 bp (Figure 2).

The plasmid pHA171 have been met characters for gene recombination. The characters were modeled in Benchling by assembling the marker *ampR*, promoter, gene of interest, and site of restriction. *ampR* was found in plasmid as a selectable marker to help gene selection during the screening process. This plasmid has a suitable T7 promoter for the host *E. coli* BL21 (DE3) and a Ribosome Binding Site (RBS) as a place for attaching ribosomes to initiate the translation of the *phdABCD* gene. Restriction sites

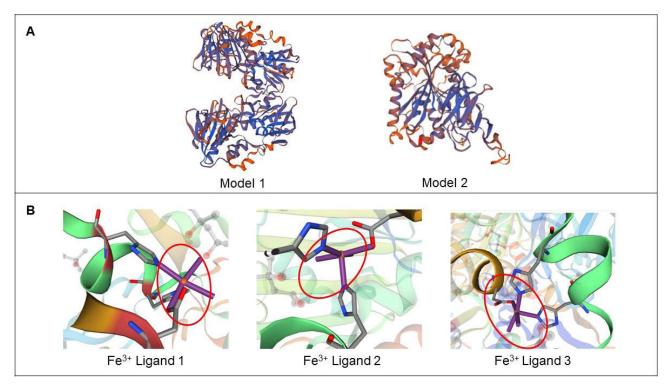


Figure 4. ExPaSy tertiary structure prediction. A). Potential model prediction of tertiary structure. B). Ion Fe3⁺ ligand on model 2 phenanthrene dioxygenase enzyme.

were introduced to insert the *phdABCD* gene into the plasmid pHA171. In this mapping, the location of the restriction sites known is *NdeI* at 2400 bp and *HindIII* at 3876 bp. The *phdABCD* insert region is the most suitable site for gene insertion because it is located after the T7 and RBS promoter. After the *phdABCD* was inserted into the plasmid, the restriction site must be reattached to T4 ligase. The final result of the plasmid design is pHA171 as a recombinant plasmid for ethidium bromide degradation.

Recombinant plasmid mapping has ORF pHA171 (6667 bp) containing 2558-6088 sites that include cluster locations of the phenanthrene/phenanthridine (phdABCD) gene (Figure 2). A gray bar showed the length of the nucleotide base of the inserted gene. The arrows indicate the direction of translation. The orange represents the phdA gene, a subunit of the oxygenase component; blue indicates the phdB gene, a subunit of the oxygenase component; green indicates the phdC gene, a component of ferredoxin; and the purple indicates the phdD gene, a component of ferrodixin reductase [11].

Prediction of the protein secondary structure

According to Predict Protein tool, the phenanthrene dioxygenase has 1105 amino acids length. The secondary structure consists of 26.43% beta-strand, 19.55% alpha-helix, and 54.03% loops (Figure 3A). The solvent accessibility was 57.19% buried, 31.49% exposed, and 11.31% intermediate (Figure 3B). From this analysis, the buried condition is more dominant than exposed condition, suggesting that phenanthrene dioxygenase has low solubility. Amino acids that have hydrophobic properties are buried, while amino acids that have hydrophilic properties are protrude to the surface and can be easily accessed by solvents [12]. Table 2 shows amino acids composition of phenanthrene dioxygenase.

Prediction of the protein tertiary structure

Three-dimensional (3D) visualization of the tertiary structure of phenanthrene dioxygenase protein was carried out using ExPaSy. Visualization of the tertiary structure of the protein can be used to find the location of the protein ligands.

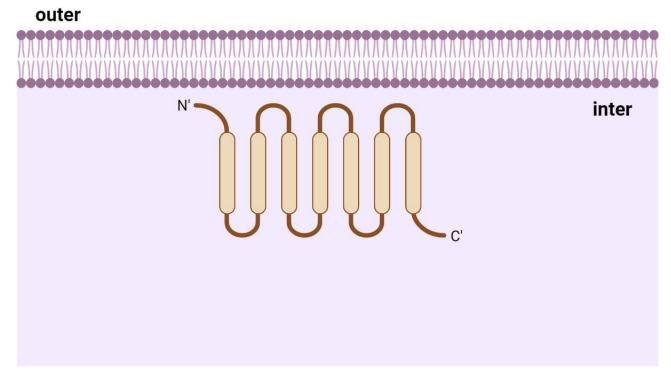


Figure 5. Transmembrane was visualized using PROTTER. It shows that protein localize intermembrane of bacteria cell. Depicted protein is just an illustration and does not represent real structure. Images created with <u>Biorender.com</u>

Table 2. Amino acids composition of phenanthrene dioxygenase

Amino acid	Percentage	Amino acid	Percentage
L (Leucine)	10.05%	N (Asparagine)	2.35%
V (Valine)	9.5%	Q (Glutamine)	2.62%
G (Glycine)	9.5%	H (Histidine)	2.44%
A (Alanine)	9.86%	M (Methionine)	2.35%
P (Proline)	5.07%	Y (Tyrosine)	2.26%
E (Glutamic acid)	6.24%	F (Phenylalanine)	3.8%
R (Arginine)	8,6%	W (Tryptophan)	1.99%
D (Aspartic acid)	7.24%	I (Isoleucine)	2.53%
T (Threonine)	5.43%	K (Lysine)	1.27%
S (Serine)	5.61%	C (Cysteine)	1.27%

(Source: predictprotein.org)

This ligand functions as the active site of the phenanthrene dioxygenase to bind to the ethidium bromide substrate. Figure 4A shows two prediction models, that model 1 has an indication rate of 37.31%, while model 2 is 58.19% towards the target amino acid sequence phenanthrene dioxygenase. In the structure of model 1, the location of the ligands is not found, so that protein interactions can be identified, while model 2 has two types of

ligands that cannot be determined as the active site of the enzyme.

In model 2, the ligands are the Fe³⁺ ion and the FES ion (Fe²⁺/S), which each ligand has a different location and type of bonding interactions. From the tertiary structure visualization, phenanthrene dioxygenase may require O_2 to break the ethidium bromide aromatic chain (Figure 4B). Therefore, the interaction of Fe³⁺ ion ligands with water is a

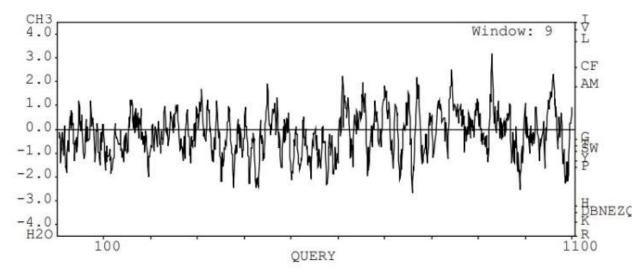


Figure 6. Kyte-Doolittle Hydropathy Plot

ligand that has the potential to bind phenanthridine substrates to ethidium bromide [13].

Transmembrane protein prediction

PROTTER visualization showed that there was no alpha-helix protein phenanthrene dioxygenase at the transmembrane location. By this prediction model, protein is located in the intracellular (Figure 5), implying that the phenanthrene dioxygenase interact with ethidium bromide as an enzyme inside the cell. PROTTER analysis might be representative of proteomic data and integrated experimentally identified of the protein [14].

Analysis of hydrophobicity and solubility of proteins

The Kyte-Doolittle scale was used to detect hydrophobic areas in proteins. The greater the number (positive) on the plot scale, the more hydrophobic the amino acid is. Figure 6 shows a graph of the hydrophobicity value of each amino acid. Based on the amino acid composition calculation, the hydrophobicity value of phenanthrene was negative. It is predicted that the phenanthrene dioxygenase protein tends to be hydrophobic.

Protein solubility is an important characteristic in order to optimize protein performance. The increase in solubility is related to a decrease in molecular weight and an increase in the number of polar groups. Protein-Sol free website was used to predict protein solubility based on protein sequence (https://protein-sol.manchester.ac.uk/). The scaled solubility value (QuerySol) is the predicted solubility. The population average for the experimental dataset (PopAvrSol) is above 0.45, and therefore any scaled solubility value greater than 0.45 is predicted to have a higher solubility than the average soluble E. coli protein from the experimental solubility dataset Niwa et al. (2009), and any protein with a lower scaled solubility value is predicted to be less soluble [15]. The prediction was displayed that the protein solubility of protein is 0.36, while the pI is 5.28. KmR means K (lysine) minus R (arginine), the score was used as another option to know the solubility of the protein (Figure 7). In this result, KmR score is negative, therefore the protein tends to have low solubility.

Prediction of isoelectric point and molecular weight

Based on the pI/MW prediction using ExPASy, it was found that the estimated isoelectric point of the protein was 5.17, while the molecular weight of the protein was 121143.89 grams/mol or equal to 121.143 kDa (Table 3). Based on pI prediction using Protein Calculator, isoelectric point estimation of phenanthrene dioxygenase was 5.36. The protein charge at pH = pI is 0, at pH> pI is negative, and at pH <pI is positive. Solution of SDS-PAGE

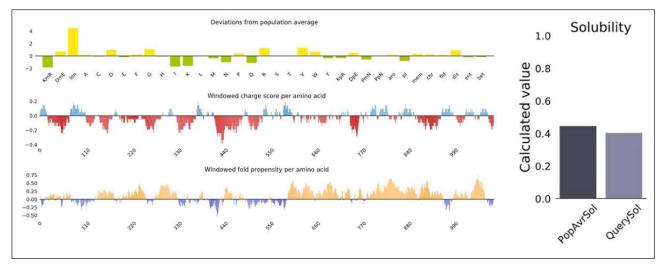


Figure 7. Protein-SOL Chart. The scaled solubility value (QuerySol) is the predicted solubility. PopAvrSol is the population average for the experimental dataset. There are seven amino acid composite scores: KmR = K-R, DmE=D-E, KpR = K+R, DpE = D+E, PmN = K+R-D-E, PmN = K+R+D+E, PmN = K+R+D+E,

that contains different pH than protein pI may cause changes in protein charge that can affect the protein separation process during electrophoresis. Therefore, the use of a solution with a pH that corresponds to the protein isoelectric point is an important thing to be considered. Based on the results of the two predictions, it can be concluded that in general the phenanthrene dioxygenase protein is suitable at pH 5.17-5.36.

Table 3. pl/Mw value by using ExPASy

рН	Protein calculator tool	ExPaSy	
	Charge	pl/MW	
4.00	93.6		
4.50	52.7		
5.00	16.1		
5.50	-4.5		
6.00	-15.9		
6.50	-25.5		
7.00	-33.7	5.17-5.36/121.143 kDa	
7.50	-39.1		
8.00	-43.4		
8.50	-48.7		
9.00	-55.1		
9.50	-63.3		
10.00	-75.0		

Discussion

Phenanthridine is a compound that is a derivative of phenanthrene. Hence, phenanthridine has a similar structure to phenanthrene. The difference between these two molecules is the presence of an N atom in the phenanthridine [16]. Phenanthrene degradation pathways using bacterial metabolism have been studied previously.

In general, phenanthrene will be oxidized to 3,4-dihydroxyphenanthrene which is then converted into 1-hydroxy-2-naptholic acid. Furthermore, 1-hydroxy-2-naptholic acid undergoes two degradation pathways, namely the phthalic acid pathway and the naphthalene pathway. In the phthalic acid pathway, 1-hydroxy-2-naptholic acid undergo ring degradation to form o-phthalic acid and protocatechuic acid then form pyruvic acid which eventually enters the tricarboxylic acid (TCA) cycle. On the other hand, in the naphthalene pathway, 1-hydroxy-2-naptholic acid will undergo oxidative decarboxylation to form 1,2-dihydroxynaphthalene, then undergo meta-degradation to form salicylic acid. Salicylic acid then becomes catechol or gentisic acid. Both catechol and gentisic acid can undergo ring degradation to form intermediate compounds in the TCA cycle [17].

Bacterial cells carrying the *phdABCD* gene are able to efficiently change the substrate of phenanthrene

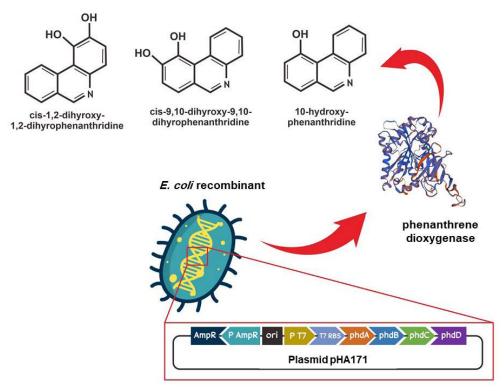


Figure 8. Mechanism of ethidium bromide degradation. *E. coli* recombinant carries on a plasmid expression gene code of phenanthrene dioxygenase enzyme. This enzyme work as a catalytic to convert ethidium bromide into three potential chemicals that less harmful

and phenanthridine [10]. *E. coli* which carries the *phdABCD* gene can degrade phenanthridine and convert it into three main products, namely cis-1,2-dihydroxy-1,2-dihydrophenanthridine, cis-9,10-dihydroxy-9,10-dihydrophenanthridine, and 10-hydroxy-phenanthridine (Figure 8). That study showed that recombinant cells are able to convert phenanthridine into an oxygenated form which allow to further degradation reactions. Therefore, these recombinant cells function as bioremediation agents for ethidium bromide which contains phenanthridine structure.

The study of the designed plasmid and phenanthrene dioxygenase protein analysis impact future research by providing the knowledge about databases that are able to use in development bioremediation, especially ethidium bromide degradation. This study contributes to give a model to improve bioremediation application and to solve toxic chemical waste that release after laboratory works. For the first place, this research aims to apply *in silico* study by using some prediction tools to simulate, visualize, and

measure physicochemical characteristics from an amino acid sequence [18]. The important point is using computations to make it easier to better knowing about molecular properties and biological systems [19].

Conclusion

The prediction analysis phenanthrene dioxygenase reacts with the ethidium bromide substrate through the interaction of Fe³⁺ ions with water. The solubility level is 0.404 which indicates that the protein has low solubility. Protein isoelectric point (pI) is between 5.17 - 5.36, and molecular weight is 121.143 kDa. Visualization of the protein structure could be a benchmark for understanding physicochemical within wet experiments to improve *in silico* study and obtain an ideal expectation in a real laboratory.

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Declaration of interest

The authors declare no conflict of interest.

Author contribution

Conceptualization, M.I.F., R.M.A., and A.N.M.; Methodology, M.I.F and R.M.A.; Investigation, M.I.F., R.M.A., S.I.Y., I.P., and A.N.M.; Writing – Original Draft, M.I.F. and A.N.M.; Writing – Review & Editing, S.I.Y., R.M.A., and I.P.; Resources, M.I.F., R.M.A., S.I.Y., I.P., and A.N.M.; Supervision, F.N.E. and M.N.

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