INTRODUCTION

Non-O157 Verotoxin producing *E. coli* (VTEC) is gradually recognised as a critical enteric foodborne pathogen which associates with life threatening complications (Colell et al., 2016; Khan et al., 2020). The production of verotoxin related virulence properties causes mild diarrhea, hemorrhagic colitis (HC), and fatal hemolytic uremic syndrome (HUS) across the worldwide. Generally, *E. coli* O157; H7 serotypes associated with foodborne illness and non-O157 serotypes have been accused of gastroenteritis and HUS outbreaks (Public Health England, 2018). Furthermore, VTEC has become a major public health issue in combination with serious food-borne disease and diseases (Mei et al., 2015; Khan et al., 2020).

In eukaryotic cells, Vtx is one of the virulent factors which produces one or more toxin which, inhibits protein synthesis (Cheung & Teachman, 2014). These are known as Shiga toxin-producing *E. coli* (STEC) or Verotoxin producing *E. coli* (VTEC) (Nazmul et al., 2012; Kaper & O’Brien, 2014) . The Vtx genes (Vtx1 and Vtx2) have the same structure and variation of the Vtx 1 and Vtx 2 sequences and variants has been defined (Hazards, 2013; Khan et al., 2020). Moreover, Vtx is the usual member of the bacteria of type 2 ribosome-inactivating proteins (RIPs) (Zhu et al., 2018). In laboratory conditions, the determination of the protein structure requires time and energy and is not an economic process. (Hai-You et al., 2016). In knowledge-based material, structural analysis of proteins is crucial while 3D (3-D) arrangement of amino acid atoms could provide the best solution for homology or comparative modelling. The simulation of molecular dynamics can also improve structure prediction with the lowest error and loss of data (Geng et al., 2019). X-ray crystallography and NMR is typically the most effective approach to solve protein structures across various steps. (Su et al., 2015). Protein structures of the same molecule have four distinct stages, referring to the prediction of secondary protein structure, modelling homology, molecular docking and dynamic molecular simulations. (Muhammed & Aki-Yalein, 2019).

Pathogenic associations with host cells also affect infectious diseases in practise (Fazlul et al., 2011; Fazlul et al., 2018). Thus, we attempt to generate multi-templates of plasmid-mediated verotoxin models. The truth is that a single template cannot cover the entire verotoxin sequence and thus multiple templates should be implemented to cover the entire sequence of the modelled verotoxin. In addition, a multiple templates combination may also cover one template's weakness by another template (Chakravarty et al., 2008). The models created allows the analysis of interactions in plasmid-mediated non-O157 *E. coli* verotoxin gene sequences. The predicted verotoxin structures were also simulated to show conformation changes in the height temperature. This provides explicit structural information to the verotoxin gene that is essential for better enzyme control.

METHODS AND MATERIALS

Model development

The plasmid mediated verotoxin producing amino acid sequences of MN696158 (Vtx1-1) and MN688720 (Vtx2) were obtained from NCBI Genbank and BLAST for finding acceptable models against the PDB database. Single and multiple models that have been chosen for Modelling was based on the coverage length, identity of the sequence and differences between the target and the template. Plasmid mediated verotoxin gene via MODELLER version 9.20 (Heo & Feig, 2018), models were produced. A hundred suitable models based on the lowest discrete optimised protein energy (DOPE) scores were chosen for the best one. The appropriate model was then subjected to a molecular dynamics simulation model refinement process.

Refinement process

The refinement approach was implemented with some alterations (Heo & Feig, 2018). First of all, the local stereochemistry of the chosen component models of verotoxin were refined using IoclPREFMD server before simulation of molecular dynamics using 5.1.4 GROMACS (Heo & Feig, 2018). The simulation was done by in a simple cubic box with a minimum of 10 Å from the edge of the box with

**Keywords**: Verotoxin gene, GROMACS, Molecular dynamic simulations, Non-O157 *E. coli*
a force field of CHARMM36m (Huang et al., 2017). With TIP3P water molecules, the mechanism was solved, and a sufficient number of sodium ions was neutralized. The ensemble was after that, energy reduced by 5000 steps of the steepest descent algorithm and balanced at NVT phase 298 K (constant number of particles, volume and temperature) and 1 bar pressure at 1 bar pressure NPT process for 100 ps, respectively (constant number of particles, pressure and temperature). Ultimately, the simulation was performed for 50 ns at 298 K and 1 bar pressure. To constrain the bond, LINear Constraint Solver (LINCQ) was used, whereas the electrostatic interactions were assessed by both Coulomb and van der Walls interactions, the particle mesh Ewald (PME) with a 12 Å cut-off. The integration time stage was 2 fs and every 1 ps during the simulation, a snapshot of the structures was taken. The stability of the trajectory was assessed using the GROMACS functionality by root-mean-square deviation (RMSD) and radius of gyration (Rg). For the final round of local stereochemistry refinement, the stabilized structure was extracted and introduced to locPREFMD.

Model evaluations

The stereochromatic content of the VTX models was determined and evaluated by Verify 3D (Chakkyanath & Natarajan, 2019), ERRAT (Souyriya et al., 2019) and PROCHECK (Chakryarat & Natarajan, 2019) via structure analysis and verification server version 5.0 (SAVES 5.0). The energy level of the VTX models was calculated using Swiss-PdbViewer (Amir et al., 2019). The molecular visualization of the protein-protein interactions was demonstrated in the PyMOL 2.2.2 edition (Fauré et al., 2019).

Thermal study for protein deterioration

Protein deterioration analysis plays an essential role in the process of thermal stability determination with exponential growth or decay with time. The Vtx sample was prepared using proteolytic activity assays as described by Duanis-Assaf (Duanis-Assaf et al., 2020) to determine the optimum temperature and allowed to hydrolyse casein for 10 minutes at 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. The reaction was terminated by trichloroacetic acid (TCA) (concentration of 110 mM), and the reaction mixture was centrifuged, and the supernatant was mixed with NaCO3 (concentration of 500 mM) while F-C phenol reagent (concentration of 0.5 M) for colour development. In the preparation of (TCA) and Folin and Ciocalteu’s (F-C) phenol, both the reagents were diluted to a final concentration of 110 mM and 0.5 M, respectively from their stock solution. Sodium carbonate (Na2CO3) buffer was prepared by adding 53 g of anhydrous Na2CO3 in 1 L of distilled water to a final concentration of 500 mM. After that, the crystals samples were incubated at five elevated temperatures of 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C without substrate for 1 hour. Similarly, the hydrolysis was conducted at 37 °C, as described above. A plot of Vtx activity against elevated temperatures was generated.

Thermal dynamic behaviours

The refined verotoxin models were set to the molecular dynamic simulation to examine dynamics and conformation variations at the specified temperature. Three separate systems have been developed for each verotoxin model, dissolved with TIP3P water molecules and neutralized by a sufficient number of sodium ions. After that, energy was minimized by 5000 steps of the steepest descent algorithm and balanced at 300 K, 313 K and 323 K, respectively, through NVT and NPT. Consequently, the simulation was done for 100 ns. LINCQ was used to constrain bond length, while electrostatic interactions were assessed by SMDs with 1 Å cut-off for both Coulomb and van der Walls correlations. The integration time stage was 2 fs and the structure snapshot was reported every 1 ps during the simulation. Besides, dynamic behavior and conformational changes of VTX models were investigated by RMSD analysis, root-mean-square fluctuation (RMSF), Rg, solvent-accessible surface area (SASA) and hydrogen bond number.

RESULTS AND DISCUSSION

Comparative modelling and model refinement

The similarities and differences among the Vtx1-land Vtx2 were determined while comparison studies disclosed important structural information of Vtx protein. The isolated Vtx gene sequences were BLAST against the PDB database for an appropriate Vtx protein model. The protein BLAST result revealed that Vtx1-1 are very identical to IA-IA with percent identity of 95.11 %, while 96.21 % identity to IA-IA was observed in Vtx2 (Table 1 and Table 2).

Table 1 Templates of protein revealed the maximum similarity with Vtx1-1 sequences based on various parameters

<table>
<thead>
<tr>
<th>Template</th>
<th>PDB report</th>
<th>Query analysis (%)</th>
<th>E-value</th>
<th>Identity (%)</th>
<th>Gaps (%)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M1U_A</td>
<td>Chain A, Shiga toxin</td>
<td>83</td>
<td>2e-77</td>
<td>55.61</td>
<td>10</td>
<td>209</td>
</tr>
<tr>
<td>1R4P_A</td>
<td>Chain A, Shiga toxin</td>
<td>83</td>
<td>2e-67</td>
<td>57.61</td>
<td>10</td>
<td>209</td>
</tr>
<tr>
<td>1DM0_A</td>
<td>Chain A, Shiga toxin</td>
<td>88</td>
<td>5e-118</td>
<td>95.05</td>
<td>10</td>
<td>337</td>
</tr>
<tr>
<td>1R4Q_A</td>
<td>Chain A, Shiga toxin</td>
<td>88</td>
<td>4e-118</td>
<td>95.11</td>
<td>10</td>
<td>337</td>
</tr>
<tr>
<td>4P2C_A</td>
<td>Chain A, Shiga toxin</td>
<td>83</td>
<td>3e-66</td>
<td>57.58</td>
<td>10</td>
<td>206</td>
</tr>
</tbody>
</table>

Table 2 Templates of protein revealed the maximum similarity with Vtx2 sequences based on various parameters

<table>
<thead>
<tr>
<th>Template</th>
<th>PDB report</th>
<th>Query analysis (%)</th>
<th>E-value</th>
<th>Identity (%)</th>
<th>Gaps (%)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M1U_A</td>
<td>Chain A, Shiga toxin</td>
<td>100</td>
<td>7e-147</td>
<td>96.21</td>
<td>3</td>
<td>400</td>
</tr>
<tr>
<td>1R4P_A</td>
<td>Chain A, Shiga toxin</td>
<td>100</td>
<td>7e-147</td>
<td>96.21</td>
<td>3</td>
<td>400</td>
</tr>
<tr>
<td>4P2C_A</td>
<td>Chain A, Shiga toxin</td>
<td>100</td>
<td>9e-135</td>
<td>91.03</td>
<td>4</td>
<td>255</td>
</tr>
<tr>
<td>1R4Q_A</td>
<td>Chain A, Shiga toxin</td>
<td>98</td>
<td>9e-90</td>
<td>61.06</td>
<td>4</td>
<td>255</td>
</tr>
<tr>
<td>1DM0_A</td>
<td>Chain A, Shiga toxin</td>
<td>11</td>
<td>1.6</td>
<td>26.09</td>
<td>0</td>
<td>16.9</td>
</tr>
</tbody>
</table>

However, the query coverage of Vtx1-1 by IA-IA was only 88 %, although Vtx2 exhibited the most extended query coverage of 100 %. Furthermore, the least E-value and gap with 4M1U_A, 1R4Q_A, 4P2C_A, 1DM0_A, and 1R4P_A was considered to finalize based on the recommendation by several studies (Kerfeld & Scott, 2011; Frith, 2019) for an appropriate alignment amongst the sequences. Primarily, IA-IA has appeared as the best template due to the most extended coverage (88 %) and sequence identity of 95.11 % for Vtx1-1 protein model. Besides, 4M1U_A has the highest coverage (100 %) with the sequence identity of 96.21 % and was selected for Vtx2. According to a recent study, protein modelled with a template of 30-50 % identity will not exceed 4 Å RMSD from its native structure (Monzon et al., 2017). Furthermore, the gap between gene sequences has to very low (1-2 %) to evade misalignment amongst the target and template sequence, which may mislead the structured protein (Dorn et al., 2014). The protein sequences of Vtx1, Vtx1-1, and Vtx2 was aligned with the available protein data structure in NCBI databases associated with the PDB database. The most compatible protein data was selected based on the various parameters to build up the perfect models.

In this study, a set of 100 templates for each Vtx sequences was generated to provide a pool of perfect results (Sefidbakht et al., 2014). The experiment was distinguished from the bad models using the MODELLER in-built assessment method based on DOPE score. DOPE is a potential statistical assessment that corresponds to non-interacting atoms in a homogeneous sphere with the radius dependent on a native sample structure (Jing & Dong, 2017). A lower DOPE score indicates the structure has a better packing of the atoms and is more accurate at its native conformation (Cloe et al., 2018). These scoring methods are precise sufficiently to select the most authentic model among the generated models using the MODELLER (Webb & Sai, 2017).

The stereochromatic consistency of the desired models must be carried out for further assessment. These selected models were investigated using the SAVES 5.0 server using various evaluation programs such as Verify 3D, which calculates the number of residues in an atomic model consistent with its amino acid sequence of 80 % (Tran et al., 2015). Meanwhile, ERRAT analysed the statistics of non-bonded interactions amongst various atom types (Wei et al., 2017) and PROCHECK assessed the protein stereochromatic quality of a protein models in favoured, allowed and outlier regions (Elengoe et al., 2014). These scoring methods are often satisfactorily adequate to select the most accurate generated models (Haddad et al., 2020).

Among the accurate generated models, 4M1U_A for Vtx1-1 protein was selected based on the scoring methods shown in Table 3. However, the Vtx1-1 protein structure passed the Verify 3D test with a score of 93.82 %, ERRAT (97.76) and PROCHECK (93.4 %). Meanwhile, 4P2C_A was selected template for Vtx2 protein with a Verify 3D score of 94.95 %, ERRAT (87.28) and PROCHECK (87.6 %) have satisfactory phi and psi dihedral angle allocations of amino acid residues in the modelled structures. Both Vtx1-1 (4M1U_A) and Vtx2 (4P2C_A) revealed an excellent quality of predicted protein model based on the evaluation tool ProQ server (Sajji et al., 2020).
The simulation time is sufficient based on the RMSD value to facilitate the Vtx structure to reach a stabilised state. Furthermore, the radius of gyration was evaluated for the stability of the Vtx models. The compactness of a protein structure was determined through the radius of gyration (Lobanov et al., 2008). Gyration values revealed that the compactness of Vtx1-1 (20. Å–21.2 Å) and Vtx2 (19.6 Å–21.2 Å) was maintained without substantial drift, signifying the predicted Vtx structure throughout the simulation (Figure 1(b)).

The quality of the initial Vtx models structure was refined by MODELLER prior to the looPREFMD refinement process to get the most suitable model for further analysis. Verify 3D showed that the percentages of amino acid with correct 3D conformation (Vtx1-1: 87.97 %; Vtx2: 80.00 %). Besides that, ERRAT analysis also indicted on nonbonded interactions within the Vtx model (Vtx1-1: 91.59 %; and Vtx2: 82.75 %). The PROCHECK Ramachandran plot indicated that 100 % and 99.3 % of the Vtx1 and Vtx2 amino acid residues are located in preferred, approved areas, respectively.

Table 3 Models assessment using Verify 3D, ERRAT and PROCHECK

<table>
<thead>
<tr>
<th>Model</th>
<th>Template</th>
<th>Verify (%)</th>
<th>3D (%)</th>
<th>ERRAT (%)</th>
<th>PROCHECK (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vtx1-1</td>
<td>4M1U_A</td>
<td>93.82</td>
<td>97.76</td>
<td>93.4</td>
<td>87.97</td>
</tr>
<tr>
<td></td>
<td>1R4P_A</td>
<td>93.98</td>
<td>95.29</td>
<td>92.6</td>
<td>87.97</td>
</tr>
<tr>
<td></td>
<td>1DM0_A</td>
<td>75.49</td>
<td>100</td>
<td>85.3</td>
<td>80.00</td>
</tr>
<tr>
<td></td>
<td>1R4Q_A</td>
<td>87.29</td>
<td>91.92</td>
<td>85.9</td>
<td>87.97</td>
</tr>
<tr>
<td></td>
<td>4P2C_A</td>
<td>94.95</td>
<td>87.28</td>
<td>87.6</td>
<td>87.97</td>
</tr>
<tr>
<td>Vtx1</td>
<td>4M1U_A</td>
<td>87.29</td>
<td>91.92</td>
<td>85.9</td>
<td>87.97</td>
</tr>
<tr>
<td></td>
<td>1R4P_A</td>
<td>92.15</td>
<td>83.18</td>
<td>92.6</td>
<td>87.97</td>
</tr>
<tr>
<td></td>
<td>4P2C_A</td>
<td>94.95</td>
<td>87.28</td>
<td>87.6</td>
<td>87.97</td>
</tr>
<tr>
<td></td>
<td>1R4Q_A</td>
<td>87.29</td>
<td>91.92</td>
<td>85.9</td>
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<tr>
<td></td>
<td>1DM0_A</td>
<td>75.49</td>
<td>100</td>
<td>85.3</td>
<td>80.00</td>
</tr>
</tbody>
</table>

Overall, the correctness of the Vtx1-1 protein structure globally was measured by LG score of 6.716 with a MaxSub score of 0.541 to assess the quality of the protein structure while Vtx2 obtained LG score of 5.794 and a MaxSub score of 0.525. Therefore, the final selected models are Vtx1-1 (4M1U_A) and Vtx2 (4P2C_A) based on their overall stability in Verify 3D, ERRAT, and PROCHECK, and other assessment tools. These final models were successively used in further analysis.

Model refinement

The initial protein structure model may not always be accurate (Ganugapati & Akash, 2017), and this comparative model has to go for further validation for better accuracy. In this study, the Vtx structure was improved using multiple similar known templates. The structure may still contain errors due to differences (insertions and gaps) in amino acids amongst target and templates during the absence of biomolecules (proteins, nucleic acid and ligands) interactions (Feig, 2017). In comparative modelling, the primary protein structure has to refine to achieve enhanced accuracy (Heo & Feig, 2018). In the current study, the Vtx structures were refined through molecular dynamics simulation. Molecular dynamics refine the structure of the proteins and shapes a group of conformities to their native state. (Raval et al., 2012). Hence, the stability of the Vtx structure was estimated as a function of simulation time. The value of RMSD for Vtx1-1 was consistently deviated at ~2.5 Å after 5 ns and fluctuated between 2 Å and 2.5 Å at 40 ns to 50 ns. Consequently, Vtx2 reached a plateau state at around 2.0 Å after 10 ns and consistently deviated between 2 Å–2.5 Å until the end of the simulation. Overall, RMSD values of Vtx1-1 and Vtx2 fluctuated and deviated between 2.0–2.5 Å after 5 ns (Figure 1(a)). Furthermore, the energy level of Vtx1-1 and Vtx2 was 18555.111 kJ/mol and 17386.025 kJ/mol, respectively after refinement. Native protein frequently folds into conformation with the lowest energy, which is the most constant form (Kazlauskas, 2018). These indicate that refined Vtx models via MODELLER (built-in Chimera 1.14) are closer and more related to the native models (Table 4). Vtx1-1 and Vtx2 protein were modelled with the best match selections from the PDB structure database. In this study, Vtx1-1 was modelled in accordance with 4m1u_A while Vtx2 with 4p2c_A. Alignment of the new modelled structure of Vtx1-1 with 57.07 % identity (Figure 2 (a)) while Vtx2 with 89.47 % identity (Figure 2 (b)).
Figure 2: Alignment of new modelled structure Vtx1-1 with the native structure 4M1U_A (a) and (b) new modelled structure Vtx2 with native structure 4P2C. Alignment of both Vtx1-1 and Vtx2 was assessed by Needleman-Wunsch using BLOSUM-62 (Figure 3). Evaluation superpositions across in the final alignment overall RMSD: 0.797 and Q-score: 0.924 at cutoff 5.0 while 279 residues pairs were aligned. This alignment defines the secondary structure of proteins with the 86.87% identity.

Figure 3: Alignment of the superimposed new structured model Vtx1-1 and Vtx2. Secondary structure prediction

All the modelled plasmid-mediated verotoxin gene of MN696158 (Vtx1-1) and MN688720 (Vtx2) contain 10 alpha-helices (H1-H10) and six beta-strands (E1-E10) as shown in Figure 4. Despite the variations in amino acid residues in each VTX sequences (MN696158 and MN688720), the formation of secondary structures revealed a consensus between each verotoxin models (Vtx1-1 and Vtx2). This is because the protein structure is well preserved compared to the amino acid sequence throughout evolution (Dong et al., 2018). In addition, there is a preference for the collection of amino acid residues within a secondary structure (Figure 5). It can be shown that the residues of amino acids T, I, V, E and F are extremely abundant in the alpha-helix regions. These residues are classified as Helix Formats due to their low energy cost for helix formation. In the other hand, the residues of amino acids Q, S, R, T, L, V, A and Y are prevalent in beta-strands due to the ability of their hydrophobic side chains to stabilize the beta structure (Merkel et al., 1999).

Figure 4: 3D structure superimposition of Vtx1-1 and Vtx2. The alpha-helices (H1-H10), beta-strands (E1-E10) and loops of Vtx are coloured in red, green, and yellow respectively

Figure 5: The legend of secondary structure (Vtx1-1 and Vtx2)

Root-mean-square-deviation

In this current analysis, RMSD values reflect VTEC’s different thermal motion at the temperature concerned. Vtx1-11 The molecular dynamics simulation review (Figure 6 (a)) showed that Vtx1-1 retained an RMSD value of 3 Å to 5 ns at 303 K and fluctuated from 1.5 Å to 2.5 Å to 30 ns and remained constant at 2 Å until the end of the simulation. The RMSD value of Vtx1-1 at 313 K reached a plateau state at 2 Å at 20 ns and maintained a constant deviation between 2.5 Å and 3 Å for the last 80 ns of the simulation. Vtx1-RMSD 1’s value has steadily deviated from 1 Å-2 Å to 65 ns at 323 K. Slightly inclined to 3 Å at 65 ns and minor variations between 1 Å and 2 Å during the simulation were also observed. At 303 K, on the other hand, the RMSD value of Vtx2 (Figure 6 (b)) fluctuated marginally between 1 Å-2.5 Å to 25 ns and held at 1.5 Å until 100 ns of simulation continuously deviated. While Vtx2 showed a fluctuation between 1Å-3.5 Å and 1. at 313 K, to 25 ns and steadily deviated to ~2 Å for the rest of the simulation process.

Figure 6: Root-mean-square-deviation (RMSD) of the protein backbone as a time function at 303 K, 313 K and 323 K of (a)Vtx1-1 and (b) Vtx2

Furthermore, after 5 ns, the RMSD value of Vtx2 entered a plateau state and continuously deviated from 2.5 Å-3 Å until 100 ns of simulation at 323 K. During MD simulation, RMSD modifications suggest that the protein backbone of all VTEC structures has moved to a new conformation to preserve stability and versatility at different temperatures (Fields et al., 2015). The overall changes in RMSD (1.5-2.0 Å) are considered minimal because the Vtx protein
conformations (with the exception of Vtx2 at 323 K) are depicted closely in line with their initial structures (Kato et al., 2017). The RMSD outcome therefore shows that the temperature has the least effect on Vtx1-1 and Vtx2, respectively. In addition, no significant deviation was observed at the time of the MD simulation of 100 ns after the RMSD plateau was reached by VTEC to allow VTEC to obtain a new constant conformation.

**Radius of gyrations**

Protein compactness is another way to calculate a protein’s stability (Paul et al., 2014). From a Rg study, the impact of temperature on the VTEC dimension was assembled. At all temperatures, Vtx1-1 displayed a similar dimension of ~19 Å~20 Å Meanwhile, the Rg value of Vtx1-1 was stretched between ~20 Å until 100 ns at 303 K, which corresponds to its RMSD changes that occurred at the same time frame. The Rg value was also held at 313 K and 323 K respectively at a steady value of 19.5-20.6 Å (Figure 7 (a)). At all temperatures, the Rg value of Vtx2 has decreased to 19.5 Å from 21.5 Å between 20-100 ns (Figure 7 (b)). Overall, during the simulation, the Rg of VTEC models remained stable, indicating that VTEC structures are successful in preserving their original compactness even when the temperature rises. Consequently, during the MD simulation study, the entire size of Vtx1-1 remained constant, although exceptions were observed on Vtx2. The shifts in Rg suggest that temperature plays a crucial role in the loosening of the protein system's molecular structural network and/or collapse at 323 K (Gu et al., 2019). The VTEC compactness analysis via Rg, similar to the RMSD analysis, also indicates that the Vtx1-1 model presents maximum stability at higher temperatures than Vtx2.

**Solvent accessible surface area**

The hydrophobic effect is driven by protein folding of the solvent accessible surface area and is temperature dependent (Pucci & Rooman, 2017). Hydrophilic residues are often present under normal conditions on the protein surface, while hydrophobic residues are often suppressed away from the aqueous environment within the protein (Ramli et al., 2018). The hydrophobic region is exposed to the solvent during the protein denaturation process (the enzyme loses its catalytic activity) (Paul et al., 2014). Soluble proteins, in interaction with the solvent, minimise the surface (Malleshappa et al., 2014).

The SASA study of VTEC models showed a pattern close to that presented during the simulation phase by RMSD and Rg. The SASA study of Vtx1-1 and Vtx2 showed stability at 303 K and 313 K respectively (native fold without disruption). The SASA study of Vtx1-1 and Vtx2 was, by comparison, expanded, killed or unfolded at 323 K. This enlargement of Rg showed that within the hydrophobic heart, hydrophobic molecules were distributed on the surface instead of suppressed. Furthermore, the decrease in SASA analysis is associated with the overall decrease in scale, resulting in high-temperature accumulation (Rosa et al., 2017). Residues congregate within the solvent protein, resulting in a decrease in SASA during protein aggregation (Mishra et al., 2018).

**Intramolecular hydrogen bonds**

The stability of the preservation of the protein structure is based on temperature-dependent interactions of the hydrogen bond (Pace et al., 2014). The hydrophobic effects, on the other hand, are the folding of the overall protein structure, since the hydrogen bond is directly connected to the protein structure (secondary and tertiary structure) and protein interaction selectivity (Gao et al., 2015). The Vtx1-1 and Vtx2 starting structures have intramolecular hydrogen bonds of 298 and 236, respectively (Figure 9). In comparison, the lowest number of intramolecular hydrogen bonds found in the Vtx2 models refers to distortion at high temperatures in RMSD, Rg and SASA.

**Figure 7** Radius of the gyration as a time function at 303 K, 313 K and 323 K of (a)Vtx1-1 and (b) Vtx2. A recent study shows that the hydrophobic effect and temperature play a major role in protein folding (Pucci & Rooman, 2017). Hydrophilic residues are often present under normal conditions on the protein surface, while hydrophobic residues are often suppressed away from the aqueous environment with the protein (Ramli et al., 2018). The hydrophobic region is exposed to the solvent during the protein denaturation process (the enzyme loses its catalytic activity) (Paul et al., 2014). Soluble proteins, in interaction with the solvent, minimise the surface (Malleshappa et al., 2014).

**Figure 8** Solvent accessible surface area (SASA) of (a) Vtx1-1 and (b) Vtx2 as a time function at 303 K, 313 K and 323 K.

**Figure 9** Number of intramolecular hydrogen bonds in the initial structure of Vtx1-1 and Vtx2. The simulation regardless of temperature plays an essential role in intramolecular hydrogen bonds. At 303 K, Vtx1-1 retained an average number of 120.97 intramolecular hydrogen bonds (Figure 10 (a)). This average number further decreased to 113.94 at 313 K and increased to 124.26 at 323 K respectively. In addition, Vtx2 has an average number of 137.37 intramolecular hydrogen bonds at 303 K, 138.30 at 313 K and 142.30 at 323 K, respectively (Figure 10 (b)).
At residue level, the stability of verotoxin (VTX) was also experienced. The flexibility and mobility of protein residues at various temperatures has been demonstrated by RMSF. In Vtx1-1, the fluctuation was recorded at residue position 4-8, 45-48, 121-124, and 190-192 at 303 K (Figure 11 (a)). These residues also fluctuated at 4-12, 43-52 at 313K and 44-51 at 323K, respectively. In the meantime, Vtx2 demonstrated versatility at different residue positions; 198-207 at 303 K, 5-12, 21-26, 40-47, 197-199, 313 K at 200-205 and 323 K at 9-11, 157-160, 161-165, 204-208 (Figure 11 (b)).

CONCLUSION
In this study, we reported structural analysis of two plasmid-mediated verotoxin gene sequences. Despite these plasmid-mediated verotoxin gene sequences showed slight differences in sequence level. The 3D verotoxin models produced demonstrated remarkable variations in their relative versatility of conformation and stability when the temperature increased. Protein loops on the L-subdomain are among the key fluctuated temperature-increasing regions because of the lack of electrostatic interactions and hydrophobicity in this region. It is predicted that a thermal, stable plasmid mediated verotoxin gene model can be best suitable model for the predictions of vaccine developments through the protein engineering process of structural information obtained.

REFERENCES