Protein Modelling Insight to the Poor Sensitivity of Chikungunya Diagnostics on Indonesia’s Chikungunya Virus

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Abstract: Sensitive detection of infectious diseases is crucial for effective clinical care. However, commercial rapid tests may be limited in their ability to detect pathogen variants across different countries. It was found that the sensitivity of a chikungunya rapid test on local strain was only 20.5% as compared to the East, Central, and South Africa (ECSA) phylogroup. Therefore, the development of geographically specific diagnostics is essential. Investigating the distinctive structural properties of a locally sourced antigenic protein is an important initiative for the development of a specific antibody. This study utilized structural bioinformatics and molecular dynamics simulations to investigate the differences between the E1-E2 antigenic proteins of the Indonesian chikungunya virus (Ind-CHIKV) and that of ECSA. The results showed that some of the mutation points are located at the antibody binding sites of Ind-CHIKV. G194S and V318R mutations were proposed as distinctive features of Ind-CHIKV, leading to weaker antibody binding compared to ECSA. It suggests that modifying the antibody to accommodate bulkier side chains at positions 194 and 318 could improve its effectiveness against Ind-CHIKV. These insights are valuable for developing a highly sensitive immunoassay for Ind-CHIKV and other regional pathogens, ultimately enhancing diagnostic capabilities in Indonesia.

Keywords: chikungunya; bioinformatics; diagnostics; Indonesia; molecular dynamics simulation

INTRODUCTION

Sensitive diagnosis of infectious diseases plays an important role in maintaining the quality of health services. Despite the availability of imported rapid tests in Indonesia, the genetic variation of pathogens amongst different countries has created some causes for concern. Kosasih and colleagues [1] found that the sensitivities of commercial chikungunya rapid tests in detecting the local strains were not satisfying. Two imported products
had sensitivities of only 20.5 and 50.8% on the local samples, as compared to 90.3% when tested on the East, Central, and South Africa (ECSA) phylogroup. A similar result was also reported by the Center for Diseases Control (CDC) in 2016, where at least five commercial chikungunya rapid test performance was unacceptable [2]. The genotype variation between Asian and ECSA strains was suggested as the main source of different results. Therefore, the development of a diagnostic test that is specific to local strains should become a priority. Moreover, chikungunya was recently found to be endemic in Bandung, one of the big cities in Indonesia and some of them belong to acute infection [3-5].

Chikungunya is a disease caused by chikungunya virus (CHIKV). This virus is transmitted to humans by two species of mosquito: Aedes albopictus and Aedes aegypti. CHIKV causes a range of clinical manifestations, including high fever, headache, erythematous skin rash, and incapacitating arthralgia [6]. CHIKV is an enveloped, positive-stranded RNA virus that belongs to the alphavirus genus of the Togaviridae family [7-8]. The genome of CHIKV is ~11.8 kb long and encodes nine viral proteins, five of which are structural proteins, i.e., capsid, E3, E2, E1, and 6K [9-10]. In the mature virion, two surface glycoproteins (E1 and E2) facilitate the viral binding and entry through receptor-mediated endocytosis and low-pH-mediated fusion within the endosomes [11-12]. Proteins E1 and E2 form a heterodimer that covers the viral surface. Kam and colleagues [13] showed that E2 is the primary target for the anti-CHIKV antibody response. Therefore, variations in E1-E2 sequences might lead to the different sensitivity of IgM-based rapid tests. Currently, there are 14 and 6 entries of E1-E2 and E1-only CHIKV sequences of the Bandung strain, respectively found in NCBI [3]. From those sequences, at least six amino acids were found to be different, indicating the polymorphism of CHIKV in Bandung itself. These variabilities also might contribute to the different sensitivity of the IgM-based rapid test.

In recent years, advances in molecular design have contributed to developing "tailored" molecules for diagnostics and therapeutics [14-16]. Recently, Holstein et al. [17] have developed an influenza-specific paper-based diagnostic test based on the computationally designed protein. The selection of this tailored protein could be an antibody fragment, such as a single-chain variable fragment (ScFv), or the other functional protein that could bind to the antigenic protein. The accessibility of genetic and structural information of pathogenic molecules, in addition to the sophisticated bioinformatics method, has provided a great opportunity to design molecules with desired properties. Structural information on ECSA chikungunya’s antigenic protein and its antibody is available in the Protein Data Bank [18].

This study aims to investigate the structural differences between the E1-E2 antigenic protein of Indonesia’s CHIKV (Ind-CHIKV) and that of ECSA using a bioinformatics approach. The protein sequence of Ind-CHIKV and the crystal structure of E1-E2 of ECSA CHIKV were used to construct the structural model of local antigens. Then, the behavior of both structures was investigated using molecular dynamics simulation. The affinity of the antibody towards E1-E2 CHIKV was computed using protein docking and molecular mechanics generalized Born surface area (MM/GBSA) methods.

**EXPERIMENTAL SECTION**

**Materials**

The GenBank number AHA87256.1 which consists of amino acid sequences of E1-E2 antigen of Ind-CHIKV, complex structure of E1-E2 ECSA CHIKV (PDB ID: 3J2W) and its antibody (anti-CHIKV CHK-152, PDB ID: 3J30), and a crystal structure of E1-E2 CHIKV with PDB ID of 3N44 were used in this work.

**Instrumentation**

This study was carried out on a computer running Ubuntu 20.04.2.0 LTS and equipped with a processor core i7 CPU 920@2.67 GHz, a GPU NVIDIA GeForce RTX 980 4 GB, RAM memory 8 GB, and a hard disk 2 TB.

**Procedure**

**Homology modelling of E1-E2 antigen of Ind-CHIKV**

The model structure of the E1-E2 antigen of Ind-CHIKV was constructed by homology modeling method.
using MODELLER9.17 in command line mode [19]. The amino acid sequences of the E1-E2 antigen of Ind-CHIKV were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) with GenBank number of AHA87256.1, which consist of E1 and E2 sequences [3]. The template for protein modeling was selected based on the high sequence similarity using BLASTP in NCBI, the quality of template structure, and also the condition of the experimental. The discrete optimized protein energy (DOPE) score, a statistical potential used to assess homology models in protein structure prediction, was calculated for the structure model. The structure with the lowest DOPE score was selected as the best model. The quality of the model was assessed by Ramachandran plot using the PROCHECK server, Z-score using ProSA-web, and VERIFY-3D method [20-22].

**Molecular dynamics simulation**

All the minimization and molecular dynamics (MD) simulations were performed using pmemd.cuda from AMBER14 [23]. The type of cysteine and protonation states of histidine were adjusted based on their specific chemical environment manually. A box of TIP3P water was added to the system, where the distance between the protein and the edge of the box was set to 10 Å. The system was neutralized by the addition of sodium ions.

The structure was optimized by using 1000 steps of steepest descent, which was followed by 2000 steps of conjugate gradient minimization with 500 kcal mol Å⁻² of harmonic restraints applied to the backbone atoms. A final 5000 steps of unrestrained conjugate gradient minimization were performed in order to remove any steric clashes.

The system was gradually heated to 300 K in the NVT ensemble using harmonic restraints on the backbone atoms under control by the Langevin thermostat. Then, 1000 ps of NPT equilibration was performed with a gradual decrease of harmonic restraints by 1 kcal mol Å⁻² until it reached zero. A 100 ns of the production run in the NPT ensemble was performed with all hydrogen atoms constrained using the SHAKE algorithm. The temperature was controlled with a Langevin thermostat with a collision frequency of 1 ps⁻¹, while pressure was controlled using a Berendsen barostat with a coupling constant of 2 ps and a target pressure of 1 bar. The time step used during the production stage was set to 2 fs. A non-bonded cut-off value of 10 Å was used, and the long-range electrostatics were treated using the Particle Mesh Ewald method. The MD trajectories were analyzed using the cpptraj module in AmberTools 15.

**Evaluation of binding energy**

Before computing the binding energy, a cryo-EM resolved the complex structure of E1-E2 ECSA CHIKV and its antibody was shortly minimized in vacuo to remove the major steric clashes at the interface region without affecting the core structure. Each of the 250 steps for steepest descent and conjugate gradient minimization was performed using the sander module in AMBER 14. The minimized complex was submitted to the FireDock server (http:/bioinfo3d.cs.tau.ac.il/FireDock) as well as Ind-CHIKV with CHK-152 and CHK-152 mutants [24-25].

FireDock is a docking program specifically built for refining and re-scoring the docked protein-protein interactions. In the program options, the type of system was selected for the antibody-antigen complex. One hundred cycles of docking were performed, with a full refinement option activated.

Furthermore, the docked complex by FireDock was minimized in the explicit solvent system. A 100 ns of MD simulation was performed using a similar parameter as described in the previous subsection. Finally, the binding energy and its decomposition analysis were done by the MMPBSA.py module in AmberTools 14.

**RESULTS AND DISCUSSION**

**Structural Model of E1-E2 of Ind-CHIKV**

The sequence of E1-E2 of Ind-CHIKV was retrieved from NCBI. This virus was isolated from the human serum of infected patients from Bandung, Indonesia. Twenty entries were found, which were composed of 14 complete sequences of E1-E2 and 6 sequences of E1 only. Six variations within 20 Ind-CHIKV sequences were detected, but they are not located at the antibody binding site (Fig. S1). For this
reason, the sequence with the GenBank number AHA87256.1 was chosen to represent E1-E2 of Ind-CHIKV [3]. Sequence alignment showed that E1 and E2 of Ind-CHIKV shared sequence similarities of 98 and 96%, respectively, with that of the ECSA virus.

The selection of a template is a crucial step in protein modeling [26]. The first attempt in template searching using NCBI resulted in the PDB ID of 2XFC, which is a cryo-electron microscopy (cryo-EM) structure of E1-E2 CHIKV from Semliki Forest (African). However, a cryo-EM structure usually has a low atomic resolution. Hence, it is not suitable to be used as a template for homology modeling. For this reason, further searching was done using sequence similarity with the PDB ID of 2XFC in the PDB server. As a result, a crystal structure of E1-E2 CHIKV with PDB ID of 3N44, with the best resolution of 2.35 Å, was selected as a template. This structure is a mature glycoprotein E1-E2 CHIKV which was soaked in osmium to improve the quality of X-ray structure determination [18,27]. The template covered 729 residues of E1-E2 Ind-CHIKV without any gaps.

After five models of E1-E2 Ind-CHIKV were built using MODELLER, the best model was chosen based on the lowest DOPE score of −74029. Furthermore, the model quality was assessed by the Ramachandran plot (Fig. 1). It is shown that 91.3% of residues were located in the most favored regions, including all mutations in Ind-CHIKV. As many as 8.1 and 0.5% of residues fall into the additional allowed regions and generously allowed regions, respectively. Only one residue falls in the disallowed regions. Upon visual inspection, this residue is located at the backbone of the secondary structure, i.e., beta-sheet. Therefore, a loop optimization step was not required. In general, a protein model with more than 90% residues in the allowed region is categorized as a good model [18,26]. Moreover, model assessment using ProSA-web showed a small difference in Z-score between the model and the template structure (Fig. 2). VERIFY-3D method showed that the score of 94.10% residues of the model is higher than 0.2 (Fig. 3), which considered as a high-quality structure [21,28].
Mapping of Mutations to the Structure of E1-E2 IndCHIKV

As many as 21 mutations were detected when the sequences of E1-E2 of Ind-CHIKV and ECSA were aligned (Fig. 4). These mutations were found in almost all E1-E2 Ind-CHIKV sequences (Fig. S1). These mutations were visually mapped to the model of E1-E2 Ind-CHIKV. By using a solvent probe with a radius of 1.4 Å, only 2 out of 21 mutations were found to be buried in the solvent, V255 and I317 (Fig. 5).

Due to their location, these 19 residues were predicted to be involved in the interactions with antibodies. Among the 19 mutations, a polymorphism of L248F was detected in 6 out of 14 sequences of E2. This mutation is known as part of the B-cell epitope [13].

Mutations at the Epitopes

In short, the epitope is part of an antigen (E1-E2 of CHIKV) that is recognized by the immune system, e.g., antibodies, and B-cells. Rapid diagnostic kits that were tested by Kosasih and colleagues [1] were based on the detection of anti-chikungunya IgM. In the serum, IgM was produced as a response to the presence of epitope in CHIKV. Therefore, different epitopes would produce different antibodies. In this study, the structural differences between Ind-CHIKV and ECSA were observed to investigate the possibilities of structural changes at the epitope regions.
In 2014, Kam et al. [13], have identified B-cell epitopes that were recognized by anti-CHIKV antibodies. There were seven linear epitopes located at the E2 protein of CHIKV. Upon comparison with the sequence of E1-E2 Ind-CHIKV, there were five mutations located in the epitope regions of E2, i.e., N5H, G194S, I255V, V318R, and L248F (Fig. 6). In V318R mutation, valine is a relatively small hydrophobic residue, while arginine is a bulky, positively charged amino acid. Therefore, the presence of arginine at position 318 would cause a steric hindrance to the antibodies. Whereas the changes from aliphatic to aromatic residues in N5H and L248F mutations would result in different flexibility to adopt the antibody binding.

**Molecular Dynamics Simulation**

The deviation and fluctuation of both E1-E2 from ECSA and that of Ind-CHIKV were evaluated by computing root mean square deviation (RMSD) and root mean square fluctuation (RMSF) values with respect to the initial and average conformations, respectively. Fig. 7(a) shows that, in general, both systems have reached equilibrium after 3000 ps. Therefore, fluctuations were calculated from 3000–10000 ps to avoid bias from the unequilibrated motions. It is shown that the deviation of E1-E2 of Ind-CHIKV was higher, especially after 65 ns and reached up to 5.5 Å compared to that of ECSA which showed more stability during the simulation. This slight difference was due to dissimilar residues at positions 248 and 255, as suggested by the higher RMSF around position 250 in Fig. 7(b). Nevertheless, the residual fluctuation of E1-E2 Ind-CHIKV was generally comparable to that of ECSA. For this reason, it can be suggested that the overall dynamic behavior of both systems was similar.

Furthermore, the observation was more focused on the antibody binding site. Sun et al. [29] have also identified the footprints of a neutralizing antibody using cryo-EM, i.e., CHK-9, CHK-152, m242, and m10. These structures provide an opportunity to explain the mutation effect at the atomic level. All the antibody structures bound to G194 (Fig. 8) thus, it was interesting to be investigated. In addition, the CHK-152 suggests more effective neutralization at a lower concentration than the others [28]. Hence, CHK-152 was used to study the effect of G194S. However, it is noted that the structure should be treated carefully due to the low resolution of cryo-EM structures in general.
The Effect of G194S in Ind-CHIKV on the Binding of CHK-152

Based on the FireDock calculation, the binding energy of CHK-152 to the ECSA CHIKV was $-81.76 \text{ kcal mol}^{-1}$. Furthermore, a mutant model of G194S (representing Ind-CHIKV) was built to observe the possible impact of this mutation on the binding of CHK-152. Interestingly, the computed affinity of CHK-152 to G194S ($-71.17 \text{ kcal mol}^{-1}$) was predicted to be weaker than the ECSA. At first sight, it can be deduced that the bulkier side chain of serine ($-\text{CH}_2\text{OH}$ group) resulted in an unfavorable binding to the CHK-152. A visual inspection showed that serine 8 was positioned closer to the Y244 of CHK-152, as compared to that of glycine (Fig. 9).

A short distance between S194 and Y244 might result in a repulsive force. This hypothesis was tested by modifying the structure of CHK-152 by Y244G mutation. It is noted that glycine is the smallest residue among 20 amino acids. It was expected that modification of Y244G in the CHK-152 structure would improve its binding to the G194S by removing sterical clashes between S194 and Y244. Table 1 shows that the affinity between G194S and Fab Y244G was increased to $-80.48 \text{ kcal mol}^{-1}$, similar to that of WT (ECSA). This result indicates that the mutation of G194S in Ind-CHIKV is one of the reasons behind the different affinities with CHK-152. Thus, it can be used as a distinctive feature in developing a specific antibody for Ind-CHIKV.

Since the FireDock refinement was done in the implicit solvent model system, a more realistic 100 ns of the explicit solvent simulation was performed using AMBER. The docked conformation resulting from FireDock was submitted to the MD preparation protocol as described in the method section. The binding energy from the explicit solvent simulation was computed by MM/GBSA method. As a result, a similar trend of binding energy between the two systems is shown in Table 2.
Table 1. The calculated binding energy between E1-E2 CHIKV and Fab CHK-152 using Firedock

<table>
<thead>
<tr>
<th>Energy component</th>
<th>Energy (kcal mol$^{-1}$)</th>
<th>ECSA and CHK-152</th>
<th>Ind-CHIKV and CHK-152</th>
<th>Ind-CHIKV and CHK-152 (Fab Y244G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global energy</td>
<td>−81.76</td>
<td>−71.17</td>
<td>−80.48</td>
<td></td>
</tr>
<tr>
<td>Attractive VdW</td>
<td>−55.97</td>
<td>−55.97</td>
<td>−55.44</td>
<td></td>
</tr>
<tr>
<td>Repulsive VdW</td>
<td>17.35</td>
<td>35.41</td>
<td>14.83</td>
<td></td>
</tr>
<tr>
<td>ACEa*</td>
<td>11.68</td>
<td>12.33</td>
<td>12.27</td>
<td></td>
</tr>
<tr>
<td>H-bond</td>
<td>−10.56</td>
<td>−11.51</td>
<td>−10.04</td>
<td></td>
</tr>
</tbody>
</table>

*aatomic contact energy

Table 2. The calculated binding energy between E1-E2 of ECSA and Ind-CHIKV with the Fab CHK-152 using the MM/GBSA method

<table>
<thead>
<tr>
<th>Binding energy (kcal mol$^{-1}$)</th>
<th>ECSA</th>
<th>Ind-CHIKV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decomposition of electrostatic energy between residue 194 and Y244 (kcal mol$^{-1}$)</td>
<td>−30.62</td>
<td>−24.48</td>
</tr>
<tr>
<td></td>
<td>−0.08</td>
<td>+0.37</td>
</tr>
</tbody>
</table>

The binding of ECSA to the CHK-152 (−30.62 kcal mol$^{-1}$) was better than that of Ind-CHIKV (−24.48 kcal mol$^{-1}$). Furthermore, the decomposition energy between residues at position 194 and the Y244 from the CHK-152 was analyzed. The result showed that S194 has a positive electrostatic energy (+0.37 kcal mol$^{-1}$) with the Y244, as compared to that of G194 in the ECSA (−0.08 kcal mol$^{-1}$).

**Structural Bioinformatics Study**

Similar clinical manifestations between CHIKV, DENV, and Zika virus represent a diagnostic challenge to differentiate itself. Hence a sensitive diagnosis is needed [30-31]. Some diagnostic methods that usually be used to differentiate amongst them are RT-PCR, ELISA, and indirect immunofluorescence (IFA). Unfortunately, these methods require special equipment, need more time, and technical skills from medium to high levels that may not be available in many laboratories, especially in rural areas. Diagnostic using rapid tests has many advantages, such as being highly cost-effective, easy to use, and the results can easily be evaluated and fast. Nevertheless, the commercial rapid test of CHIKV was reported poorly and needs major improvement [2]. This study proposes that the difference in sensitivity of chikungunya rapid tests on the Indonesian strain was due to the genetic variation between ECSA and Ind-CHIKV. Since the rapid tests were based on the detection of IgM anti-CHIKV, then the differences in sensitivity would derive from the distinctive features of epitopes on the surface of CHIKV. Several mutations were found on the surface of E1-E2 of Ind-CHIKV. Two of them were positioned at the antibody binding sites, i.e., G194S and V318R. In addition, these two mutations were found in all E1-E2 of Ind-CHIKV. These results can be used as a structural basis to develop a more specific molecule to detect antibodies or antigens to develop more sensitive rapid tests. Liu et al. [32] proposed that a single chain variable fragment (ScFv) of antibodies can also be engineered to be used for immunoassay. In addition, Holstein et al. [17] also suggested that a recombinant antibody fragment could be used as a functional protein for a paper-based diagnostic test. ScFv is widely used for diagnostic purposes in many fields, and there are to detect diseases in humans or plants [33-35].

MD simulation on E1-E2 of Ind-CHIKV and CHK-152 revealed that the binding affinity of this antigen-antibody was low compared to the ECSA. This approach can also be used to design a new antibody, especially ScFv, that has a high affinity to E1-E2 of Ind-CHIKV. Leong et al. [36] designed ScFv against *Salmonella typhi* TolC protein using MD simulation. This ScFv was designed de novo by evaluating the shape complementarity and calculating the binding affinity against TolC protein to 74 ScFv designs originating from
five crystal structures. The top five of ScFv designs have high binding affinity against TolC protein, and the top one originated from PDB ID of 3JUY, a variant of b12 antibody targeting HIV-1 gp120 envelope protein. These results can be used to develop the diagnosis for typhoid.

Another study of MD simulation reported for evaluating the binding of ScFv against ESAT-6 for TB diagnostic. In this study, two high affinities of ScFv were isolated and built a model structure. MD simulations revealed that the hydrogen bond between amino acid residues of the light chain and residue of ESAT-6 was more in ScFv-7 than in ScFv-3. Hence, joining the heavy chain of ScFv-3 to the light chain of ScFv-7 is good for making new ScFv which may have higher affinity [37,38]. From these cases, MD simulation can be used to design and evaluate the protein-protein interactions, especially antigen-antibody interaction [39-40].

**CONCLUSION**

A possible reason for the low sensitivity of the current diagnostic test to the Ind-CHIKV was due to the genotype variations among strains of viruses. Using a structural bioinformatics approach, this study was able to identify the mutation points at the E1-E2 protein surface of Ind-CHIKV, especially at the antibody binding sites. Furthermore, G194S and V318R mutations were proposed as distinctive features of Ind-CHIKV, different from that of ECSA’s. To develop a tailored antibody that would bind to the E1-E2 of Ind-CHIKV, one should accommodate a bulkier side chain at positions 194 and 318. It is expected that the results would provide valuable insight into developing an antibody-based functional protein for the further development of a highly sensitive immunoassay for the Ind-CHIKV or the other regional pathogens.

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**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: Bevi Lidya, Muhammad Yusuf, Umi Baroroh, Bachti Alisjahbana, Toto Subroto. Analyzed the data: Muhammad Yusuf, Umi Baroroh, Korry Novitriani, Bevi Lidya. Wrote the first draft of the manuscript: Bevi Lidya, Muhammad Yusuf, Umi Baroroh, Toto Subroto. Contributed to the writing of the manuscript: Bevi Lidya, Muhammad Yusuf, Umi Baroroh, Korry Novitriani, Iman Rahayu, Toto Subroto. Agree with manuscript results and conclusions: Bevi Lidya, Muhammad Yusuf, Umi Baroroh, Korry Novitriani, Bachti Alisjahbana, Iman Rahayu, Toto Subroto. Jointly developed the structure and arguments for the paper: Muhammad Yusuf, Umi Baroroh, Toto Subroto. Made critical revisions and approved final version: Bevi Lidya, Muhammad Yusuf, Umi Baroroh. All authors reviewed and approved the final manuscript.

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