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A phylogenetic analysis of Indonesian SARS-CoV-2 isolates from March to December 2020: Compared with Delta and Mu variant

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Abstract: Indonesia's economy and global health are endangered due to the SARS-CoV-2 outbreak. Our present study aims to examine the phylogenetic analysis of SARS-CoV-2 isolates in Indonesia and compare these isolates to other Southeast Asian countries. In the present study, we retrieved 105 isolates from GISAID EpiCoV and other isolates from GenBank, NCBI. Then, we extracted the full genome and focused on the spike (S) protein gene (3882 bp). We employed Molecular Evolutionary Genetics Analysis (MEGA) X software to construct a phylogenetic analysis using the maximum likelihood approach. Here, we demonstrated and revealed the relationship between Indonesian and other Southeast Asian SARS-CoV-2 isolates. In summary, our work presents the phylogenetic analysis of 105 isolates in Indonesia. Our study assists in monitoring the deployment of the disease spreading. Furthermore, we suggest that the genomics and epidemiological surveillance investigations on COVID-19 should be enhanced, especially in Indonesia.

Keywords: COVID-19, coronaviruses, SARS-CoV-2, phylogenetic analysis, spike protein

INTRODUCTION

The first case of SARS-CoV-2 emerged in Wuhan, China and it sporadically transmitted worldwide.[1] The WHO declared that the infection was considered as a pandemic in March 2020. The outbreak of SARS-CoV-2 endangered the economy and global health. The problem calls for large-scale scientific research to reveal more information related to SARS-CoV-2, for instance, various aspects of the genome.[2,3] In late December 2020, the virus had infected about 85 million people across the globe with more than 1.5 million global deaths. The data are supported by the CSSE, Johns Hopkins University, USA.[4] The symptoms of COVID-19 are not much different from the symptoms of infection caused by other types of Coronaviruses (CoVs). Several regular/mild symptoms are cough and fever. Infection in the respiratory system that develops into pneumonia and exacerbates to ARD is the most severe case and results in death.[2,3]

The coronaviruses (CoVs) are critical pathogenic agents that cause respiratory, neurological, gastrointestinal, and systemic diseases in humans and animals. The name "coronavirus" is derived from "corona" which reflects the appearance of the spiky outer protein cover of the virus.[5] The coronavirus family consists of various genera, namely *Gamma*-, *Delta*-, *Beta*-, and *Alphacoronavirus*. The novel virus belongs to *Betacoronavirus*, a genus that has formerly caused epidemics, SARS-CoV-1 and MERS-CoV.[6] It has a genome of 29,890 bp (GenBank NC_045512.2), similar to another class of CoVs. The CoVs

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genome is a single-stranded RNA. E, M, N, and S, are the various types of structural proteins encoded by this viral genome.[7,8,9] The S protein has recently emerged as a prime prospective antigen within vaccine formulation to fight SARS-CoV-2. Its main objective is to interact with host cells through the ACE2 receptor and immediately be recognized by the host immune system.[10]

Indonesia is one of the Association of Southeast Asian Nations (ASEAN) that have reported the entire strain of SARS-CoV-2 genomes in their respective regions along with Brunei, Myanmar, Vietnam, Singapore, Malaysia, the Philippines, Thailand, Cambodia, and Laos.[11] Presently, Laos is the ASEAN nation with the fewest whole-genome sequences of the virus published to databases, such as GenBank or GISAID. The data is essential to support the epidemiology investigation which is the notable instrument in the observation of emerging and re-emerging viruses. New concepts and insights must be applied and adopted carefully as information emerges every day at a rapid pace. Furthermore, GISAD EpiCoV recently defined the various clades for isolates originated from Indonesia, such as G, GH, GR, and so on. Therefore, due to the recent data, we unlocked the phylogenetic analysis of SARS-CoV-2 isolates in Indonesia and compared these isolates to other Southeast Asian countries.

MATERIAL AND METHOD

a. *SARS-CoV-2 Isolates*

All SARS-CoV-2 isolates from Indonesia were regained from the database (GenBank and GISAID EpiCoV) until December 2020. All 105 virus isolates were collected from GISAID EpiCoV. Moreover, we used the isolate of Wuhan-Hu-1 (extracted from GenBank, NCBI) as a reference, according to Ansori *et al*. (2020).[11]

b. *Nucleotide Sequence Preparation*

We extracted S protein gene from all isolates. MSA of the sequences were completed using MUSCLE in MEGA X software (Pennsylvania State University, USA).[11]

c. *Phylogenetic Tree Analysis*

In the present study, we constructed the molecular phylogenetic design and visualization by employing MEGA X software on the S protein gene of all isolates with a maximum likelihood approach. In addition, the molecular phylogenetic construction was tested according to our previous study.[12,13]

RESULTS AND DISCUSSION

The *Alphacoronavirus* and *Betacoronavirus* infect animals and humans, whereas *Deltacoronavirus* and *Gammacoronavirus* only infect animals.[10,14,15,16] There were six CoVs that caused problems for humans by the end of 2019.[17,18] The SARS-CoV-2, the seventh CoVs, is the most recent identified CoV strain which was detected in Wuhan, China in December 2019. It was postulated to be transmitted to humans through animals in the live animal markets in Wuhan, China. Up until today, based on the CSSE, Johns Hopkins University, USA, more than 100 million people worldwide have been infected by this novel virus.[4]

The S protein mediates the access of SARS-CoV-2 through the membrane fusion of human cells and has become the main purpose for few researches of vaccines and antiviral drugs. Both S1 and S2 domains of the S protein are part of the novel virus that are essential for infection. In brief, the S1 domain is the most essential domain for binding into cellular receptors of the host. The efficacy of various remedies, including fusion blockers, disrupting protease inhibitors, neutralizing antibodies, S protein inhibitors, small RNAs, and ACE2 blockers show that in vitro researches are unacceptable. Various tools have been performed to generate vaccines by employing the S protein

(antigen).[12,19,20] Therefore, in this study we focus on the S protein of the novel virus isolates in Indonesia.

As new data on the novel virus is issued rapidly, updated theories and arrangements should be persistently adopted. Recently, the database established various clades of SARS-CoV-2, such as S, G, V, and so on.[2,3] In general, viruses have a much higher mutation rate than prokaryotes and eukaryotes. Viruses with a genome in the form of RNA have a high mutation rate, about one million times higher compared to their host, thus increasing their virulence. The mutation rate for CoVs is estimated at 4×10^{-4} nucleotide substitutions/site/year.[21,22] Therefore, it can be said that the novel virus has high mutation rate. Nonetheless, its mutation rate increases the potency of zoonotic viral pathogenicity for human-to-human transmission and it might be more virulent.[23]

Phylogenetic analysis is an analysis which is commonly used for targeting both the fundamental and applied issues of virology, including evolution, taxonomy, diagnostics, phylogeography, origin, and epidemiology. It might supply an overview of the virus evolution, which can be investigated to know the cluster of viruses.[24,25,26,27,28] In this study, we demonstrated the relation of 105 Indonesian SARS-CoV-2 isolates to another Southeast Asian SARS-CoV-2 isolates from humans, bats, mink, and pangolin (Figure 1). Interestingly, we found that based on the viral S protein gene isolated from Indonesia and various other countries, there was not much difference in them. Previously, an investigation of molecular phylogenetic analysis of Indonesian isolates was established by Ansori *et al*. (2020). However, it still has limitation regarding to the number of isolates.[12]

Our study demonstrated that the other CoVs isolated from humans, such as CoV-229E, -NL63, and -HKU1 are reflected as a pathogen lead to upper respiratory infection and conscientious for more than 15% of the common cold. The HCoV-229E replicates within the upper respiratory tract epithelial cells. Unlike SARS-CoV that spreads from the upper airway and causes a severe lower respiratory infection.[29,30,31] Moreover, we also used another CoVs sample originated from bats, namely CoV-HKU4-1, -ZC45, -YN2018D, -ZXC21, and so on. In addition, the results of a five-year study in twenty countries in three continents found that bats harbor a high number of probably zoonotic CoVs.[32,33,34] Since the emergence of SARS-CoV, it is known that various animal-borne CoVs have mutated and made the leap to humans, causing severe infections. All of SARS-CoV-2, HCoV-229E, HCoV-NL63, MERS-CoV, and SARS-CoV are believed to originate from bats, whereas HCoV-OC43 and HCoV-HKU1 are believed to derive from rodents. Additionally, all seven CoVs that cause human diseases have crossed the species barrier, as the progenitor viruses are found in different host animals.[35,36,37,38]

In recent findings, it is known that CoVs exists in several wild animals in Asia, especially mammals.[39] Thus, research related to this matter is very important in order to investigate the possible host roles of this new virus. Pangolin CoV derived from Malayan pangolin or Manis javanica, is 91.02% identic to the novel virus in the whole-genome level.[40] Hitherto, another study mentioned that the novel virus shares 96% of the whole genome with a BatCoV RaTG13 isolated from China.[41] Snakes are also considered as feasible virus reservoir to human infection.[42] Minks and bats are also prospective hosts of SARS-CoV-2.[43] Additionally, there is a possibility that domesticated animals could serve as an intermediate host and enable the transmission of the virus from their natural reservoirs to humans. Moreover, there are various intermediate hosts involved in virus transmission, such as camelids for human CoV-229E, dromedary camels for MERS-CoV, and civets for SARS-CoV.[44] Therefore, we suggest that supporting surveillance researches should be conducted on minks,

pangolins, bats, and other mammals in wild habitats, remarkably in East Asia, in order to understand the risk of forthcoming zoonotic diseases.

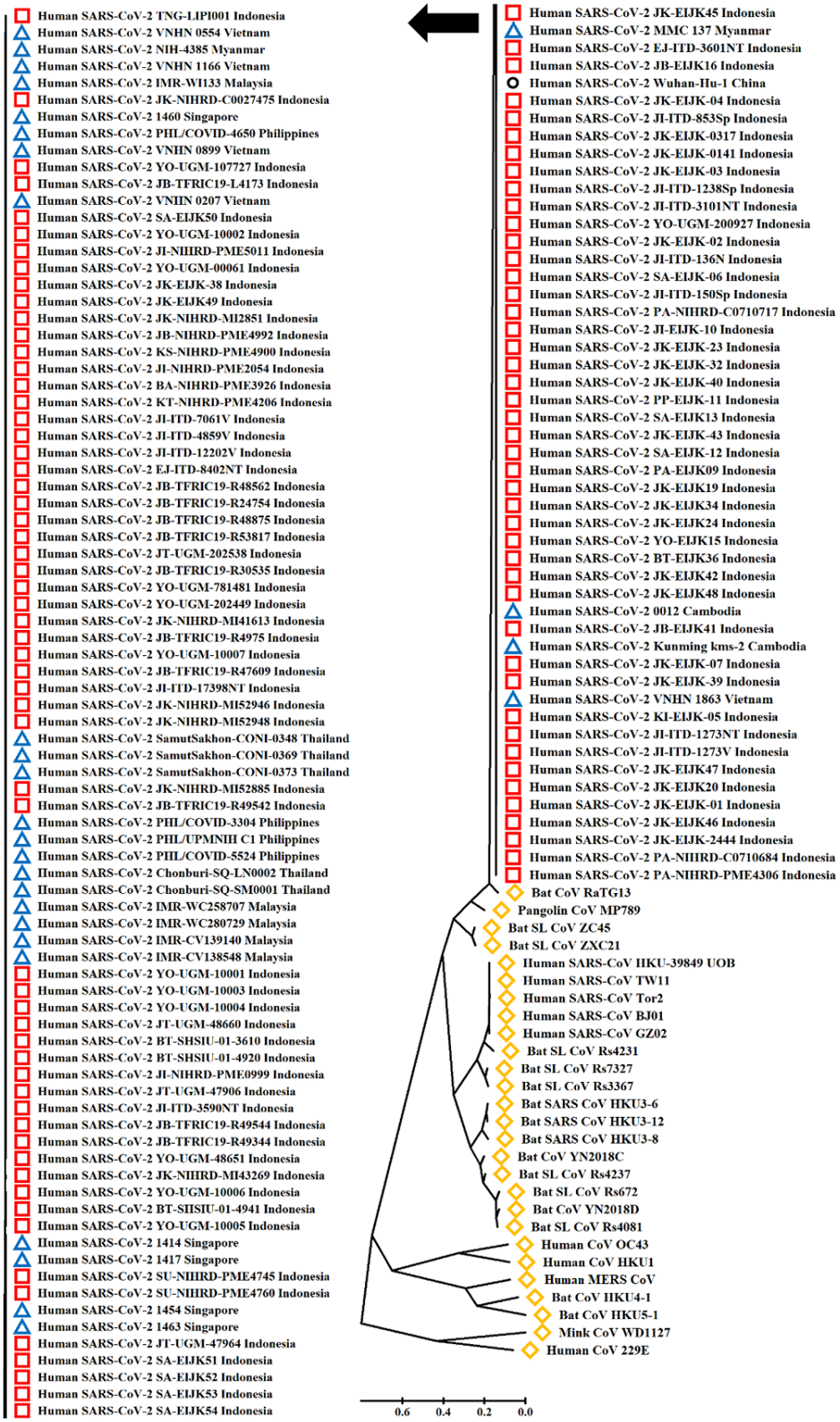

Figure 1. Phylogenetic tree showing the close relation of SARS-CoV-2 isolates in Indonesia to the Southeast Asian SARS-CoV-2 isolates and other CoVs (bat, human, mink, and pangolin).

Outbreak of the novel virus has led to economic and medical emergency worldwide.[45,46,47] Thus, unlocking the features of the novel virus genome and

establishing the procedures to observe the novel virus during the pandemic is a crucial move for monitoring the COVID-19 pandemic.[48,49] Genomic data should be used to monitor and track the spread of the novel virus. This is also related to the recognition of genotypes related to temporal infectious clusters and specific geographics.[10]

Furthermore, recent finding showed that another variant of SARS-CoV-2 had spread in Indonesia. The new variant which termed as B.1.617.2 variant or Delta variant has become the dominant variant to cause 78.8% cases. It was detected on April 2021 in Indonesia and transmitted widely in countrywide.[50] In brief, the lineage of Delta variant was consisted of various mutation in N-terminal domain (NTD). Therefore, those mutation positions were stated as B.1.617.1, B.1.617.2, and B.1.617.3. Result demonstrated that Delta mutation had ability to be resistant for neutralization by antibodies, including anti-NTD. It was caused by inability of antibody to bind into spike protein of SARS-CoV-2.[51] Based on the phylogenetic analysis in general, Delta variant or B.1617 variant was showed as derived from D614G lineage.[52]

Moreover, the SARS-CoV-2 virus seems keep evolving and the new variant is detected as MU variant. It is termed as B.1.621 based on the official Phylogenetic Assignment of Named Global Outbreak lineage designation. In detail, this variant is characterized by several substitution in spike protein, such as T95I, Y144T, Y145S, 146N, R346K, E484K, and N501Y. It known for having an ability for virus to escape from immune effect.[53] In Indonesia, this variant is estimated carefully in order to minimize the effect of infection. However, the specific number of infections remains unknown.

Hence, the quick finding of variation in genomic level within the investigation of the novel virus in Indonesia is urgently needed for a streamlined retort to the COVID-19 pandemic.[11,12,13] Furthermore, recognizing unique variants of the novel virus and linking them to employ a molecular epidemiology approach might enable scientists to establish the ancestry of a unique variant and observe the virus spreading. This data might be a crucial instrument in controlling the COVID-19 pandemic.

CONCLUSION

In summary, our work presents the phylogenetic analysis of 105 isolates in Indonesia. Our study assists in monitoring the deployment of the disease spreading. Furthermore, we suggest the genomics and epidemiological surveillance investigation to be enhanced on COVID-19, especially in Indonesia.

AUTHORS' CONTRIBUTIONS

Arif Nur Muhammad Ansori prepared the samples, designed the protocols, executed the protocols, and wrote the manuscript. Yulanda Antonius reviewed and supervised the manuscript. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The datasets used during the present work are available in the GISAID (https://www.gisaid.org/) and GenBank, NCBI

(https://www.ncbi.nlm.nih.gov/genbank/) database. We gratefully acknowledge the authors from originating and submitting laboratories of GISAID data in this study.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

REFERENCES

1. Kharisma V. D., A. N. M. Ansori (2020). Construction of Epitope-based Peptide Vaccine against SARS-CoV-2: Immunoinformatics Study. Journal of Pure and Applied Microbiology, 14, 999-1005.
2. Huang C., Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, L. Zhang, G. Fan, J. Xu, X. Gu, Z. Cheng, T. Yu, J. Xia, Y. Wei, W. Wu, X. Xie, W. Yin, H. Li, M. Liu, Y. Xiao, H. Gao, L. Guo, J. Xie, G. Wang, R. Jiang, Z. Gao, Q. Jin, J. Wang, B. Cao (2020). Clinical Features of Patients Infected with 2019 Novel Coronavirus in Wuhan, China. Lancet, 395(10223), 497-506.
3. Li X., E. G. Elena, M. H. Marichannegowda, B. Foley, C. Xiao, X. P. Kong, Y. Chen, S. Gnanakaran, B. Korber, F. Gao (2020). Emergence of SARS-CoV-2 through Recombination and Strong Purifying Selection. Science Advances, 6(27), eabb9153.
4. Dong E, H. Du, L. Gardner (2020). An Interactive Web-based Dashboard to Track COVID-19 in Real Time. Lancet Infectious Diseases, S1473-3099(20), 30120-30121.
5. Guo YR, Q. D. Cao, Z. S. Hong, Y. Y. Tan, S. D. Chen, H. J. Jin, K. S. Tan, D. Y. Wang, Y. Yan. (2020). The Origin, Transmission and Clinical Therapies on Coronavirus Disease 2019 (COVID-19) Outbreak - An Update on the Status. Military Medical Research, 7(1), 11.
6. LaTourrette K, Holste NM, Rodriguez-Peña R, Leme RA, Garcia-Ruiz H. Genome-Wide Variation in Betacoronaviruses. J Virol. 2021 Jul 12;95(15):e0049621. doi: 10.1128/JVI.00496-21. Epub 2021 Jul 12. PMID: 34037417; PMCID: PMC8274613.
7. Castillo A. E., B. Parra, P. Tapia, A. Acevedo, J. Lagos, W. Andrade, L. Arata, G. Leal, G. Barra, C. Tambley, J. Tognarelli, P. Bustos, S. Ulloa, R. Fasce, J. Fernández (2020). Phylogenetic Analysis of the First Four SARS-CoV-2 Cases in Chile. Journal of Medical Virology, 92(9), 1562-1566.
8. Ou X., Y. Liu, X. Lei, P. Li, D. Mi, L. Ren, L. Guo, R. Guo, T. Chen, J. Hu, Z. Xiang, Z. Mu, X. Chen, J. Chen, K. Hu, Q. Jin, J. Wang, Z. Qian (2020). Characterization of Spike Glycoprotein of SARS-CoV-2 on Virus Entry and Its Immune Cross-Reactivity with SARS-CoV. Nature Communications, 11, 1620.
9. Shereen M. A., S. Khan, A. Kazmi, N. Bashir, R. Siddique (2020). COVID-19 Infection: Origin, Transmission, and Characteristics of Human Coronaviruses. Journal of Advanced Research, 24, 91-98.
10. Phan T. (2020). Genetic Diversity and Evolution of SARS-CoV-2. Infection, Genetics and Evolution, 81, 104260.
11. Ansori A. N. M., V. D. Kharishma, S. S. Muttaqin, Y. Antonius, A. A. Parikesit (2020). Genetic Variant of SARS-CoV-2 Isolates in Indonesia: Spike Glycoprotein Gene, Journal of Pure and Applied Microbiology, 14, 971-978.
12. Ansori A. N. M., V. D. Kharisma, Y. Antonius, M. R. Tacharina, F. Rantam (2020). Immunobioinformatics Analysis and Phylogenetic Tree Construction of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): Spike Glycoprotein Gene. Jurnal Teknologi Laboratorium, 9(1), 13-20.

13. Turista D. D. R., A. Islamy, V. D. Kharisma, A. N. M. Ansori (2020). Distribution of COVID-19 and Phylogenetic Tree Construction of SARS-CoV-2 in Indonesia. Journal of Pure and Applied Microbiology, 14, 1035-1042.
14. Hasöksüz M, Kiliç S, Saraç F. Coronaviruses and SARS-COV-2. Turk J Med Sci. 2020; 50(SI-1): 549-556.
15. Malik YA. Properties of Coronavirus and SARS-CoV-2. Malays J Pathol. 2020; 42(1): 3-11.
16. Fahmi M, Kharisma VD, Ansori ANM, Ito M. Retrieval and Investigation of Data on SARS-CoV-2 and COVID-19 Using Bioinformatics Approach. Adv Exp Med Biol. 2021; 1318: 839-857.
17. Bchetnia M, Girard C, Duchaine C, Laprise C. The outbreak of the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): A review of the current global status. J Infect Public Health. 2020 Nov;13(11):1601-1610. doi: 10.1016/j.jiph.2020.07.011. Epub 2020 Aug 4. PMID: 32778421; PMCID: PMC7402212.
18. Chen B, Tian EK, He B, Tian L, Han R, Wang S, Xiang Q, Zhang S, El Arnaout T, Cheng W. Overview of lethal human coronaviruses. Signal Transduct Target Ther. 2020 Jun 10;5(1):89. doi: 10.1038/s41392-020-0190-2. PMID: 32533062; PMCID: PMC7289715.
19. Du L, Y. He, Y. Zhou, S. Liu, B. J. Zheng, S. Jiang (2009). The Spike Protein of SARS-CoV–A Target for Vaccine and Therapeutic Development. Nature Reviews Microbiology. 2009; 7: 226e36.
20. Yin C. (2020). Genotyping Coronavirus SARS-CoV-2: Methods and Implications. Genomics, S0888-7543(20), 30318-30319.
21. Pachetti M, Marini B, Benedetti F, Giudici F, Mauro E, Storici P, Masciovecchio C, Angeletti S, Ciccozzi M, Gallo RC, Zella D, Ippodrino R. Emerging SARS-CoV-2 mutation hot spots include a novel RNA-dependent-RNA polymerase variant. J Transl Med. 2020 Apr 22;18(1):179. doi: 10.1186/s12967-020-02344-6. PMID: 32321524; PMCID: PMC7174922.
22. Khan M. I., Z. A. Khan, M. H. Baig, I. Ahmad, A. E. Farouk, Y. G. Song, J. J. Dong (2020). Comparative Genome Analysis of Novel Coronavirus (SARS-CoV-2) from Different Geographical Locations and the Effect of Mutations on Major Target Proteins: An *In Silico* Insight, PLoS One, 15(9), e0238344.
23. Wang C., Z. Liu, Z. Chen, X. Huang, M. Xu, T. He, Z. Zhang (2020). The Establishment of Reference Sequence for SARS-CoV-2 and Variation Analysis. Journal of Medical Virology, 92(6), 667-674.
24. Cui J., F. Li, Z. L. Shi (2019). Origin and Evolution of Pathogenic Coronaviruses. Nature Reviews Microbiology, 17(3), 181-192.
25. Lu R., X. Zhao, J. Li, P. Niu, B. Yang, H. Wu, W. Wang, H. Song, B. Huang, N. Zhu, Y. Bi, X. Ma, F. Zhan, L. Wang, T. Hu, H. Zhou, Z. Hu, W. Zhou, L. Zhao, J. Chen, Y. Meng, J. Wang, Y. Lin, J. Yuan, Z. Xie, J. Ma, W. J. Liu, D. Wang, W. Xu, E. C. Holmes, G. F. Gao, G. Wu, W. Chen, W. Shi, W. Tan (2020). Genomic Characterisation and Epidemiology of 2019 Novel Coronavirus: Implications for Virus Origins and Receptor Binding. Lancet, 395(10224), 565-574.
26. Kumar SU, Priya NM, Nithya SR, Kannan P, Jain N, Kumar DT, Magesh R, Younes S, Zayed H, Doss CGP. A review of novel coronavirus disease (COVID-19): based on genomic structure, phylogeny, current shreds of evidence, candidate vaccines, and drug repurposing. 3 Biotech. 2021; 11(4): 198.
27. Tang X., C. Wu, X. Li, Y. Song, X. Yao, X. Wu, Y. Duan, H. Zhang, Y. Wang, Z. Qian, J. Cui, J. Lu (2020). On the Origin and Continuing Evolution of SARS-CoV-2. National Science Review, nwaa036.

28. Wu F., S. Zhao, B. Yu, Y. M. Chen, W. Wang, Z. G. Song, Y. Hu, Z. W. Tao, J. H. Tian, Y. Y. Pei, M. L. Yuan, Y. L. Zhang, F. H. Dai, Y. Liu, Q. M. Wang, J. J. Zheng, L. Xu, E. C. Holmes, Y. Z. Zhang (2020). A New Coronavirus Associated with Human Respiratory Disease in China. Nature, 579(7798), 265-269.
29. Greenhalgh T, Jimenez JL, Prather KA, Tufekci Z, Fisman D, Schooley R. Ten scientific reasons in support of airborne transmission of SARS-CoV-2. Lancet. 2021 May 1;397(10285):1603-1605.
30. Giwa A., A. Desai (2020). Novel coronavirus COVID-19: An Overview for Emergency Clinicians. Emergency Medicine Practice, 22, 1-21.
31. Liu D. X., J. Q. Liang, T. S. Fung (2020). Human coronavirus-229E, -OC43, -NL63, and -HKU1, In: Reference Module in Life Sciences. Elsevier.
32. Anthony S. J., C. K. Johnson, D. J. Greig, S. Kramer, X. Che, H. Wells, A. L. Hicks, D. O. Joly, N. D. Wolfe, P. Daszak, W. Karesh, W. I. Lipkin, S. S. Morse, PREDICT Consortium, J. Mazet, T. Goldstein (2017). Global Patterns in Coronavirus Diversity. Virus Evolution, 3(1), vex012.
33. Payne S (2017). Family Coronaviridae. Viruses, 2017, 149-158.
34. Su S., G. Wong, W. Shi, J. Liu, A. C. K. Lai, J. Zhou, W. Liu, Y. Bi, G. F. Gao (2016). Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. Trends in Microbiology, 24(6), 490-502.
35. Forni D, R. Cagliani, M. Clerici, M. Sironi (2017). Molecular Evolution of Human Coronavirus Genomes. Trends in Microbiology, 25, 35-48.
36. Müller M. A., V. M. Corman, J. Jores, B. Meyer, M. Younan, A. Liljander, B. J. Bosch, E. Lattwein, M. Hilali, B. E. Musa, S. Bornstein, C. Drosten (2014). MERS Coronavirus Neutralizing Antibodies in Camels, Eastern Africa, 1983–1997. Emerging Infectious Diseases, 20, 2093-2095.
37. Munir K., S. Ashraf, I. Munir, H. Khalid, M. A. Muneer, N. Mukhtar, S. Amin, S. Ashraf, M. A. Imran, U. Chaudhry, M. U. Zaheer, M. Arshad, R. Munir, A. Ahmad, X. Zhao (2020). Zoonotic and Reverse Zoonotic Events of SARS-CoV-2 and Their Impact on Global Health. Emerging Microbes & Infections, 9(1), 2222-2235.
38. Ye Z. W., S. Yuan, K. S. Yuen, S. Y. Fung, C. P. Chan, D. Y. Jin (2020). Zoonotic Origins of Human Coronaviruses. International Journal of Biological Sciences, 16(10), 1686-1697.
39. Zhang T., Q. Wu, Z. Zhang (2020) Probable Pangolin Origin of SARS-CoV-2 Associated with the COVID-19 Outbreak. Current Biology, 30(7), 1346-1351.e2.
40. Lam T. T., N. Jia, Y. W. Zhang, M. H. Shum, J. F. Jiang, H. C. Zhu, Y. G. Tong, Y. X. Shi, X. B. Ni, Y. S. Liao, W. J. Li, B. G. Jiang, W. Wei, T. T. Yuan, K. Zheng, X. M. Cui, J. Li, G. Q. Pei, X. Qiang, W. Y. Cheung, L. F. Li, F. F. Sun, S. Qin, J. C. Huang, G. M. Leung, E. C. Holmes, Y. L. Hu, Y. Guan, W. C. Cao (2020). Identifying SARS-CoV-2-Related Coronaviruses in Malayan Pangolins. Nature, 583(7815), 282-285.
41. Zhou P., X. L. Yang, X. G. Wang, B. Hu, L. Zhang, W. Zhang, H. R. Si, Y. Zhu, B. Li, C. L. Huang, H. D. Chen, J. Chen, Y. Luo, H. Guo, R. D. Jiang, M. Q. Liu, Y. Chen, X. R. Shen, X. Wang, X. S. Zheng, K. Zhao, Q. J. Chen, F. Deng, L. L. Liu, B. Yan, F. X. Zhan, Y. Y. Wang, G. F. Xiao, Z. L. Shi. Addendum: A Pneumonia Outbreak Associated with A New Coronavirus of Probable Bat Origin. Nature, 588(7836), E6.
42. Ji W., W. Wan, X. Zhao, J. Zai, X. Li (2020). Cross-Species Transmission of the Newly Identified Coronavirus 2019-nCoV. Journal of Medical Virology, 92(4), 433-440.
43. Cheng Z. J., J. Shan (2019). Novel Coronavirus: Where We Are and What We Know. Infection, 48(2), 155-163.
44. Wu D., T. Wu, Q. Liu, Z. Yang (2020). The SARS-CoV-2 Outbreak: What We Know. International Journal of Infectious Diseases, 94, 44-48.

45. Bitanihirwe B. K. Y., D. Ssewanyana (2021) The health and economic burden of the coronavirus in sub-Saharan Africa. Global Health Promotion, 28(1): 70-74.
46. Synhorst DC, Bettenhausen JL, Hall M, Thurm C, Shah SS, Auger KA, Williams DJ, Morse R, Berry JG. Healthcare Encounter and Financial Impact of COVID-19 on Children's Hospitals. J Hosp Med. 2021; 16(4): 223-226.
47. Pines JM, Zocchi MS, Black BS, Kornas R, Celedon P, Moghtaderi A, Venkat A; US Acute Care Solutions Research Group. The Effect of the COVID-19 Pandemic on the Economics of United States Emergency Care. Ann Emerg Med. 2021; S0196-0644(21)00333-4.
48. Jungreis I, Sealfon R, Kellis M. SARS-CoV-2 gene content and COVID-19 mutation impact by comparing 44 Sarbecovirus genomes. Nat Commun. 2021 May 11;12(1):2642. doi: 10.1038/s41467-021-22905-7. PMID: 33976134; PMCID: PMC8113528.
49. Harvey WT, Carabelli AM, Jackson B, Gupta RK, Thomson EC, Harrison EM, Ludden C, Reeve R, Rambaut A; COVID-19 Genomics UK (COG-UK) Consortium, Peacock SJ, Robertson DL. SARS-CoV-2 variants, spike mutations and immune escape. Nat Rev Microbiol. 2021; 19(7): 409-424.
50. Dyer O. Covid-19: Indonesia becomes Asia's new pandemic epicentre as delta variant spreads. BMJ. 2021; 374: n1815.
51. Planas D, Veyer D, Baidaliuk A, Staropoli I, Guivel-Benhassine F, Rajah MM, Planchais C, Porrot F, Robillard N, Puech J, Prot M, Gallais F, Gantner P, Velay A, Le Guen J, Kassis-Chikhani N, Edriss D, Belec L, Seve A, Courtellemont L, Péré H, Hocqueloux L, Fafi-Kremer S, Prazuck T, Mouquet H, Bruel T, Simon-Lorière E, Rey FA, Schwartz O. Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. Nature. 2021; 596(7871): 276-280.
52. Rambaut A, Holmes EC, O'Toole Á, Hill V, McCrone JT, Ruis C, du Plessis L, Pybus OG. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat Microbiol. 2020; 5(11): 1403-1407.
53. Laiton-Donato K, Franco-Muñoz C, Álvarez-Díaz DA, Ruiz-Moreno HA, Usme-Ciro JA, Prada DA, Reales-González J, Corchuelo S, Herrera-Sepúlveda MT, Naizaque J, Santamaría G, Rivera J, Rojas P, Ortiz JH, Cardona A, Malo D, Prieto-Alvarado F, Gómez FR, Wiesner M, Martínez MLO, Mercado-Reyes M. Characterization of the emerging B.1.621 variant of interest of SARS-CoV-2. Infect Genet Evol. 2021; 95: 105038.

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Original Research

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Prophylactic effects of Kaempferia Galanga against Plasmodium berghei: in vivo study

Check for updates

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Abstract: Recently, medicinal plants have been the main source in treating malaria. *Kaempferia galanga* was investigated as an antioxidant, and anti-inflammation. In vivo study of *K. galanga* had been verified as antimalarial for severe malaria. The objective of this study is to investigate the chemoprophylactic effect, body composition, and blood parameters of *K. galanga* against *Plasmodium berghei*. The prophylactic effects were determined by employing *K. galanga* (50; 100 and 200 mg/kg) in mice infected with *P. berghei*. Mice were subdivided into 4 groups (control negative and 3 treatment groups). The treatment groups received *K. galanga* daily for 3 days before the inoculation of *P. berghei.* Each mice were inoculated with the infected blood containing 10^6 of *P. berghei*. Parasitaemia and body weight were calculated every day until 5 days post-inoculation, and the blood parameters was monitored in day 5. The parasitaemia on the fifth days after inoculation revealed a significant suppresses effect compared to control ($p<0.05$). The suppressed effect by doses 50 mg/kg; 100 mg/kg; 200 mg/kg and negative control were 0.83%; 1.96%; 2.82%, and 17.8% respectively. The body weight on treatment groups was normal, but the control group decreased ($p<0.05$). The blood parameters on treatment groups were normal compared to control group. The *K. galanga* possess the prophylactic effect, normal weight and blood parameters against *Plasmodium berghei*.

Keywords: *Kaempferia galanga*; Malaria; In vivo; Prophylactic.

INTRODUCTION

Malaria is an essential parasitic disease with high morbidity and mortality in the world. The absence of an effective vaccine against malaria and the resistance of the antimalarial agent require a new agent to protect the human from this pathogen. In fact that malaria is a deadly disease, however, these diseases can be prevented.[1] Malaria causes organ dysfunction such as liver, lung, and brain.[2] One of the dangerous sign of malaria is anaemia, hypoglycaemia detected from the blood test.[3,4] These conditions impact cognitive impairment in children and productivity in adults.[5] People who are most at risk are those who live in the malaria-endemic area. This condition creates people in the endemic area to have a habit to consume herbs.[6] One of the wide herbs employed is from Zingiberaceae family.[7]

Kaempferia galanga is a family of *Zingiberaceae* widely distributed in tropical regions.[8] These plants are also identified as kencur, sand ginger, aromatic ginger, and resurrection lily. *Kaempferia galanga* is used not only as medicinal plants but also for cooking.[9,10,11] In Indonesia, these plants are employed as prophylactic from many diseases because these plants are believed has a prophylactic effect.[12] Several studies have revealed the active compounds of *K. galanga* containing alkaloids, saponin, tannin, flavonoids, terpenoids, phytosterols, phenols, and essential oils. [7,10] Based on the previous study, those compounds

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own beneficial effects as an antioxidant.[13] The antioxidant effect of kencur tubers is primarily obtained from flavonoid and phenolic compounds.[14,15] The phenolic compound of *K. galanga* possessed moderate antioxidant activity and less toxicity.[13] *Kaempferia galanga* tubers also own anti-inflammatory effects.[16] The anti-inflammatory effects of kencur tubers are by suppressing the progression of acute and chronic inflammation by inhibiting neutrophil cell infiltration.[17] *Kaempferia galanga* was acknowledged as an antimalarial activity, but there was no report of prophylactic effect. Based on the above background, the objective of this study is to investigate the prophylactic effect of *K. galanga* for malaria prevention.

MATERIAL AND METHOD

Plant collection and extract preparation

The *K. galanga* tubers were obtained from Gunung Kidul Yogyakarta. The tubers were air-dried at room temperature and powdered. One hundred grams of *K. galanga* was macerated in 1000 ml of 80% ethanol for 72 hours and then filtered. The filtrate was concentrated with a rotary evaporator to dryness. The residue was stored in a desiccator until it was used.

Ethical consideration

The protocol and experimental procedures employed in this study were confirmed with the Ethical Commission, Faculty of Medicine, Universitas Islam Indonesia with number 35/Ka.Kom.Et/70/KE/V/2018.

The parasite and treatment

Plasmodium berghei in this study was procured from the Faculty of Medicine, Universitas Islam Indonesia. Male Swiss mice weighing 20-30 g administered in this study were acclimatize for a week before treatment. The animal was kept in a standard laboratory with temperature 4±7^{o}C; humidity 70±5% with 12 hour light/dark cycles. Foods for mice were provided daily and water supplied ad libitum. Treatment mice were infected with 10^6 parasitized erythrocytes intra peritoneal. The day of infection was defined as days D1, D2, D3, D4, and D5.

Experimental design

Experimental groups were divided in accordance with wether they were control or treatment groups**.** Mice were divided into five groups of 4 mice for each group. The groups were as follows:

Group 1 (negative control): received solution solvent only
Groups 2 (treatment): ethanol extract of *K. galanga* 50 mg kg^{-1}
Groups 3(treatment): ethanol extract of *K. galanga* 100 mg kg^{-1}
Groups 4 (treatment): ethanol extract of *K. galanga* 200 mg kg^{-1}

Four animals per group were housed together in a cage with food, and water ad libitum. Food, water, and weight gain were monitored every day at 09.00 am. Treatment mice were treated orally by oral cannular to intra gastric (i.g). A ball-tipped, 18-gauge gavage needle was attached to a 1 cc syringe. Prophylactic treatment was performed once a day for 3 days. Four mice in each group were provided with an oral dose of 50, 100, and 200 mg kg^{-1} of *K. galanga* and observed for the mortality for 5 days. After the prophylactic treatment, mice were injected by 10^6 of *P. berghei* intra peritoneal.

Each mice was inoculated with 0.2 µl intra peritoneal which contain 10^6 *P. berghei* from donor mice which possess 30% parasitemia. Parasitemia was calculated by Giemsa stained thin blood smears from the mice's tail until day 5 (D5). The percentage of parasitemia was determined by calculating the number of parasitized red blood cells out of 1000 erythrocyte in random microscopic fields.

Full blood count was applied to identify the influence of treatment on malaria and its co-morbidities. The blood count was obtained from a cardiac puncture on day 6 after the re-passage of *P. berghei*. The blood count analysis found hemoglobin, hematocrit, and white cell count (WBC).

Statistical analysis

Data were presented as mean plus standard deviation of the mean. The analysis was performed by statistical analysis administering Kruskal's Wallis, followed by Mann Whitney. The $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

The prophylactic effect of *Kaempferia galanga*

Most of the people believe that traditional herbs could prevent infectious diseases. The result of this study revealed that local traditional herbs can indeed prevent people from the *Plasmodium* sp. infection. This study supports other research which local herbs own a potential effect as a prophylactic against *Plasmodium* infection.[18] The lack of local herbs consumption evidence in the community is the absence of standard dose. The herbal preparation is based on experience and drunk ad libitum. This practice induces adverse effect due to overdose or accumulation of herbal ingredient in several organs such as kidney.[18]

This study revealed that pretreatment of mice with *K. galanga* for 3 days, delayed the establishment of parasitemia compared to control (Figure 1). On day 3 after *P. berghei* injection, average parasitemia of mice pretreated with *K. galanga* doses 50 mg kg^{-1} was 0.03%, while the group 100 mg kg^{-1}, and 200 mg kg^{-1} were 0.06% and 0.65%, and the control group was 10.15%. The lowest decrease of parasitemia was discovered in 50 mg kg^{-1} group among groups and control groups. The similar result has been discovered until the fifth day after induction

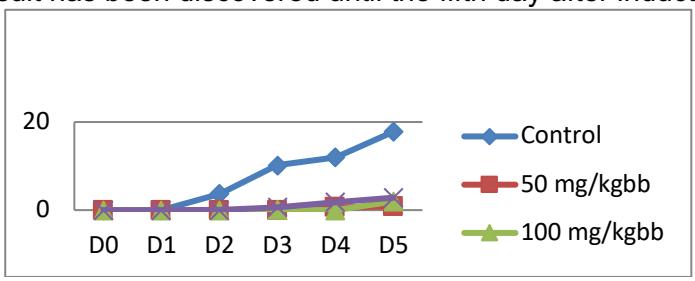

Figure 1. The prophylactic effect of *K. galanga* among groups and control

Based on Table 1 and Figure 1, five days after the *Plasmodium* injection, the average of parasitemia in the treatment groups 50 mg kg^{-1}, 100 mg kg^{-1}, and 200 mg kg^{-1} was 0.83%, 1.96%, and 2.82% respectively. The prophylactic effect was good in the treatment group with a dose of 50 mg kg^{-1} compared to other treatment doses. The parasitemia rate of treatments groups was lower and significantly different compared to the control groups (p=0.47). The prophylactic effect evidence is able to reduce parasitemia about 15% of the negative control group.

This study presents that *K. galanga* may delay the parasitemia growth until 5 days after the injection of *P. berghei* compared to control, indicating the chemoprophylactic effects. Based on the resulting study displayed, it is implied that ethanol extract of *K. galanga* had a good potential prophylactic effect. The result of this study is also supported by other studies which discovered that traditional plants have an effect as prophylactic against *Plasmodium*.[19,18] Several in vitro and in vivo studies had also investigated the antiplasmodial activity of the *K. galanga's* family.[20]

The best prophylactic effect was discovered in low concentration, whereas at the high dose, the parasitemia was not lower than low and middle concentration. Another study exhibited that the best prophylactic effect was identified at the highest concentration.[18] However, other studies had presented that the best prophylactic effect was noticed at low doses.[19] This study consisted of other studies that *K. galanga* had a chemo-prophylactic effect on human cells.[10]

Kaempferia galanga effect to hematological profile

The hematological parameter of hemoglobin in all treatment groups was normal compared to the control group. Table 1 presents that at all dosage of *K. galanga* extract, the hemoglobin, white blood cells were normal compared to the control group. All treatment groups show a normal value of those parameters than control groups. The 100 mg kg^{-1} group displays the highest value on the parameters of hemoglobin (13.1 g/dl), hematocrit (45.23 %), and the lowest white blood cell count (5.33 mmk), implying that this dose has better result than other doses (50 mg kg^{-1} and 200 mg kg^{-1}). The lowest dosage and highest dosage possess an almost similar parameter in blood count hemoglobin, hematocrit, and white blood cell. This result demonstrates that both in the low and high dosage groups, there was no different effect in a blood test.

Table 1. Parasitemia and hematological profile of *K. galanga* on infected mice

Information	Groups (mg kg^{-1})			
	Control	50	100	200
Parasitemia (%)	17.8±4.38	0.83±0.61	1.96±0.61	2.82±2.94
Hemoglobin (g/dl)	9.6±3.25	11.97±1.00	13.1±0.78	11.97±1.55
Hematocrit (%)	32.05±7.0	40.1±4.54	45.23±2.46	40.1±6.03
WBC (mmk)	18.205±3.57	13.09±7.04	5.33±2.19*	13.092±5.39

Kaempferia galanga contains ethyl-methoxycinnamate which is a derivative of cinnamic acid. Cinnamic acid is considered to have many benefits, such as an antimalarial. The antimalarial mechanism of cinnamic acid inhibits the ATP production in the parasite.[21] That information might explain the normal hemoglobin in the treatment group, compared to the control group. When the production of ATP Plasmodium decreased, it also reduced the Plasmodium ability to degrade the hemoglobin. Thus, the result of our study discovered that the ethanol extract of *K. galanga* revealed the ability to prevent anemia. In treatment groups, the Hb concentrations were normal than the control groups. In the control group, it was shown the Hb levels < 10 mg/dl indicating that anemia occurred. *Kaempferia galanga* contains phenolic compounds and flavonoids, understood as their antioxidant properties.[22,14,23] The phenolic compound of *K. galanga* possesses moderate and high antioxidant activity.[24,14] Typically, as antioxidants, flavonoids may reduce oxidative stress or increase the antioxidant capacity,[23], and radical scavenging.[24] Although the total phenolic level and radical scavenging activity were not as high as other Zingiberaceae family.[23] Phenolic compounds have also been widely acknowledged as an antimalarial.[25] Antimalarial activity of phenol compounds is accurately inhibiting heme polymerization.[26]

Our study revealed that the WBC parameter was normal in treatment groups compared to the control group. This result confirmed the previous study that *K. galanga* did not change the blood parameters.[27] The anti-inflammatory effects of *K. galanga* tubers were performed by suppressing the progression of acute and chronic inflammation which is by inhibiting neutrophil cell infiltration.[28] The anti-inflammatory effect of *K. galanga* caused by ethyl-methoxycinnamate was conducted by inhibiting the pro-inflammatory cytokines TNFα, IL-1, and also by inhibiting cyclooxygenase.[22,29,30] Research by [31] discovered that *K. galanga* tubers have anti-inflammatory benefits.

Kaempferia galanga effect to body weight

Bodyweight in all treatment groups increased every day (Figure 2). Control mice group owned significant decreased water and food intake as well as body weight until day 5 post-*Plasmodium* inoculation compared to treatment groups ($p<0.05$). *Plasmodium sp*. infection may cause weight loss both in human or animal models.[19,4] This study presented that weight loss appeared at 2 days after *Plasmodium* injection, but in the following day, the weight of treatment groups increased within the normal range. Bodyweight in the control group proved a

continuous decline until the end of the observation. The infected mice which are not treated indicated low food and water intake as well as negative weight gain.

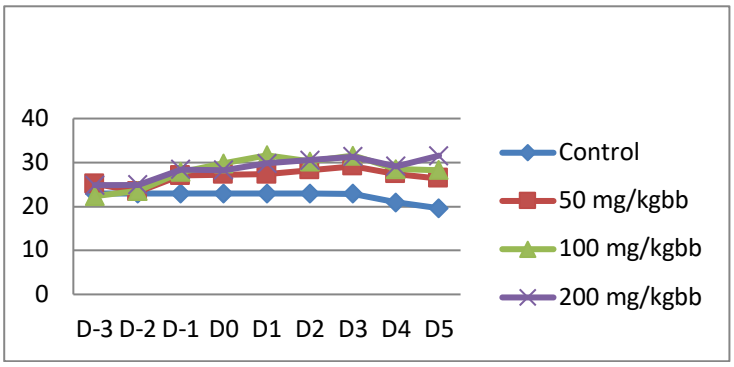

Figure 2. Bodyweight among groups

The result of this study is similar to the other study[19] and weight gain is better than other studies.[32] *Plasmodium* infection is contributing to the clinical manifestation of weight loss by producing IL 1 from the neutrophil.[30] This result occurred in the following control group but did not occur in the treatment group. *Kaempferia galanga* containing ethyl-methoxycinnamate suppresses the production of IL 1. This result also indicates that *K. galanga* is good received orally and possesses high tolerated dose.[33]

CONCLUSION

Kaempferia galanga demonstrates a positive effect on *Plasmodium* infection by suppressing the parasitaemia progression, maintaining body composition and normal blood parameters. It is indicated that *K. galanga* are save and good as a prophylactic candidate agent for malaria.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

REFERENCE

1. Tizifa TA, Kabaghe AN, Mccann RS, Berg H Van Den, Vugt M Van, Phiri KS. Prevention Efforts for Malaria. *Curr Trop Med Reports*. 2018;5:41-50. doi:https://doi.org/10.1007/s40475-018-0133-y
2. Akil SNH. The clinical and histopathological aspect of liver, lung, and kidney in malaria. *Qanun Med.* 2019;3:123–135.
3. White NJ. Anaemia and malaria. *Malar J.* 2018;17(371):1-17.

doi:10.1186/s12936-018-2509-9
4. Sakwe N, Bigoga J, Ngondi J, et al. Relationship between malaria, anaemia, nutritional and socio-economic status amongst under-ten children, in the North Region of Cameroon : A cross-sectional assessment. *PLoS One*. 2019;14(6):1-17. doi:https://doi.org/10.1371/journal.pone.0218442
5. Tapajós R, Castro D, Melo G, et al. Malaria impact on cognitive function of children in a peri-urban community in the Brazilian Amazon. *Malar J*. 2019;18(173):1-12. doi:10.1186/s12936-019-2802-2
6. Suswardany DL, Sibbritt DW, Supardi S, Pardosi JF, Chang S, Adams J. A cross-sectional analysis of traditional medicine use for malaria alongside free antimalarial drugs treatment amongst adults in high-risk malaria endemic provinces of Indonesia. *PLoS One*. 2017;12(3):1-15. doi:HTTPS://DOI.ORG/10.1371/journal.pone.0173522
7. Shetu HJ, Trisha KT, Sikta SA, *et al.* Pharmacological importance of Kaempferia galanga (Zingiberaceae): A mini review. *Int. J. Res. Pharm. Pharm. Sci.* 2018;3:32–39.
8. Subaryanti, Sulistyaningsih YC, Iswantini D, Triadiati T. The Growth and Production of Galanga (Kaempferia galanga L.) in Different Altitudes. *J. Ilmu Pertan. Indones.* 2020;25:167–177.
9. Jalil M. Pemanfaatan Curcuma longa dan Kaempferia galanga Sebagai Bahan Pembuatan Jamu " Beras Kencur " Bagi Ibu Pasca Persalinan. *Semin. Nas. Pendidik. Biol. dan Saintek*. 2019;167–173.
10. Srivastava N, Singh S, Chand A, Shanker K. Aromatic ginger (Kaempferia galanga L .) extracts with ameliorative and protective potential as a functional food , beyond its fl avor and nutritional bene fi ts. *Toxicol Reports*. 2019;6(May):521-528. doi:10.1016/j.toxrep.2019.05.014
11. Hermawan S, Aman IGM, Nyoman N, Dewi A. Comparison between Oral Administration of Kaempferia galanga Rhizome Extract and Simvastatin in Improving Lipid Profile of Dyslipidemic Male Wistar Rats. *Int. J. Sci. Res.* 2021;10:545–549.
12. Syahruddin AN, Dahlan CK, Taslim NA. The Effects of Kaempferia Galanga L . Extract on Pain , Stiffness and Functional Physic in Patient with Knee Osteoarthritis : Double Blind Randomized Clinical Trial. *Int. J. Sci. Healthc. Res.* 2017;2:37–43.
13. Rahman I, Kabir T, Islam N, *et al.* Investigation of antioxidant and cytotoxic activities of kaempferia galanga L. *Res. J. Pharm. Technol.* **12**, 2189–2194 (2019).
14. Ali, H., Yesmin, R., Satter, M. A., Habib, R. & Yeasmin, T. Journal of King Saud University – Science Antioxidant and antineoplastic activities of methanolic extract of Kaempferia galanga Linn . Rhizome against Ehrlich ascites carcinoma cells. *J. King Saud Univ. - Sci.* 2018;30:386–392.
15. Huyut Z, Beydemir F, Gülçin E. Antioxidant and Antiradical Properties of Selected Flavonoids and Phenolic Compounds. *Biochem Res Int*. 2017;2017:1-10. doi:10.1155/2017/7616791
16. Samodra G, Febrina D. Anti-Inflammatory Effects of Kaempferia galanga L. Rhizome Extract in Carrageenan-Induced Female Rats. *Adv. Heal. Sci. Res.* 2020;20:13–17.
17. Jagadish PC, Latha KP, Mudgal J, Nampurath GK. Extraction, characterization and evaluation of Kaempferia galanga L . (Zingiberaceae) rhizome extracts against acute and chronic in fl ammation in rats. *J Ethnopharmacol*. 2016;194(March):434-439. doi:10.1016/j.jep.2016.10.010
18. Olanlokun JO, David OM, Afolayan AJ. In vitro antiplasmodial activity and prophylactic potentials of extract and fractions of Trema orientalis (Linn.) stem bark. *BMC Complement Altern Med*. 2017;17(407):1-11. doi:10.1186/s12906-017-1914-x
19. Otegbade OO, Ojo JA, Adefokun DI, Abiodun OO, Thomas BN, Ojurongbe

O. Ethanol Extract of Blighia sapida Stem Bark Show Remarkable Prophylactic Activity in Experimental Plasmodium berghei–Infected Mice. *Drug Target Insight*. 2017;11:1-8. doi:10.1177/1177392817728725
20. Titanji VPK, Zofou D, Moses N. Ngemenya. The antimalarial potential of medicinal plants used for the treatment of malaria in Cameroonian folk medicine. *African J. Tradit. Complement. Altern. Med.* 2008;5:302–321.
21. Kanaani J, Ginsburg H. Effects of Cinnamic Acid Derivatives on In Vitro Growth of Plasmodium falciparum and on the Permeability of the Membrane of Malaria-Infected Erythrocytes. *Antimicrob. Agents Chemother.* 1992;36:1102–1108.
22. Sumazian Y, Syahida A, Hakiman M, Maziah M. Antioxidant activities, flavonoids, ascorbic acid and phenolic contents of Malaysian vegetables. *J Med Plants Res*. 2010;4(10):881-890. doi:10.5897/JMPR10.011
23. Da'i M, Setiawan D, Rosita Melannisa. Potency of Radical Scavenging Activity and Determination of Total Phenolic Content of Five Ethanolic Extract of Rhizome Zingiberaceae Family. *Indones J Cancer Chemoprevention*. 2013;4(1):457-462.
24. Yao F, Zhu X, Wang Y, He X. Phenolics from the Rhizomes of Kaempferia galanga L . and Their Antioxidant Activity. *J Complement Altern Med Res*. 2018;5(1):1-6. doi:10.9734/JOCAMR/2018/40630
25. Bekono BD, Kang FN, Onguéné PA, et al. The potential of anti - malarial compounds derived from African medicinal plants : a review of pharmacological evaluations from 2013 to 2019. *Malar J*. 2020:1-35. doi:10.1186/s12936-020-03231-7
26. Fitriastuti D, Julianto TS, Wahyu A, Iman N. Identification and Heme Polymerization Inhibition Activity (HPIA) Assay of Ethanolic Extract and Fraction of Temu Mangga (Curcuma mangga Val). Rhizome. *Eksakta*. 2020;1(1):64-72. doi:10.20885/EKSAKTA.vol1.iss1.art
27. Kanjanapothi D, Pathong A, Lertprasertsuke N, et al. Toxicity of crude rhizome extract of Kaempferia galanga L. (Proh Hom). *J Ethnopharmacol*. 2004;90(2-3):359-365. doi:10.1016/j.jep.2003.10.020
28. Jagadish PC, Latha KP, Mudgal J, Nampurath GK. Extraction, characterization and evaluation of Kaempferia galanga L. (Zingiberaceae) rhizome extracts against acute and chronic inflammation in rats. *J Ethnopharmacol*. 2016;December 2:434-439. doi:https:doi.org/10.1016/j.jep.2016.10.010
29. Umar MI, Asmawi MZ, Sadikun A, et al. Bioactivity-Guided Isolation of Ethyl-p-methoxycinnamate, an Anti-inflammatory Constituent, from Kaempferia galanga L. Extracts. *Molecules*. 2012;17:8720-8734. doi:10.3390/molecules17078720
30. Menezes MN De, Machado É, Vieira F, et al. IL-1 α promotes liver inflammation and necrosis during blood-stage Plasmodium chabaudi malaria. *Nature*. 2019;9(7575):1-12. doi:10.1038/s41598-019-44125-2
31. Ali H, Yesmin R, Satter MA, Habib R, Yeasmin T. Science Antioxidant and antineoplastic activities of methanolic extract of Kaempferia galanga Linn. Rhizome against Ehrlich ascites carcinoma cells. *J King Saud Univ Sci*. 2018;30(3):386-392. doi:10.1016/j.jksus.2017.05.009
32. Chaniad P, Techarang T, Phuwajaroanpong A, Punsawad C. Antimalarial Activity and Toxicological Assessment of Betula alnoides Extract against Plasmodium berghei Infections in Mice. *Evidence-Based Complement Altern Med*. 2019;November:1-8.
33. Amuamuta A, Plengsuriyakarn T, Na-bangchang K. Anticholangiocarcinoma activity and toxicity of the Kaempferia galanga Linn . Rhizome ethanolic extract. *BMC Complement Altern Med*. 2017;17(213):1-11. doi:10.1186/s12906-017-1713-4

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Short Communication

OPEN ACCESS

D18S51 A brief history of RT-PCR and our laboratory experience with SARS-CoV-2 analyses using RT-PCR

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Abstract: Polymerase Chain Reaction (PCR) is in vitro replication that allows accelerated amplification of certain sequences in small DNA fragments. A sensitive technique, only traces of DNA are needed for PCR to produce enough copies to be analyzed. In molecular diagnostic laboratories, rRT-PCR technique is applied to find target RNAs for the diagnosis of specific pathogens. Although the rRT-PCR method, which has high specificity and moderate sensitivity, is accepted by WHO as the gold standard test for the confirmation of COVID-19, there are many negative comments about this method that should be considered. While diagnosing SARS-CoV-2, it is possible to say that real-time PCR (RT-PCR) analysis is still valid but not sufficient to quickly distinguish similar infections. For this reason, there is a need for new analysis methods and new RT-PCR studies to be performed with newly developed unique rapid tests.

Keywords: Analytical error; COVID-19 virus; General protocol; Test kit; rRT-PCR.

INTRODUCTION

While studies on Deoxyribo Nucleic Acid (DNA) continue, PCR was first discovered by Kary Mullis in 1985. This technique, with its high sensitivity and specificity, has led to the evolution of diagnostic and research possibilities and has been awarded the Nobel Prize.[1] PCR is in vitro replication that allows accelerated amplification of specific sequences in small DNA fragments .[2] PCR; It has been applied in various fields such as biotechnology, cell biology, genetic engineering, forensic science, medical science, drug research. Methods for the efficient performance of PCR have been precisely optimized and have improved considerably over the past three decades.[3] High sensitivity and specificity of PCR; It allows the detection of rare microorganisms in diagnostic clinical applications, especially in body fluid infections. It is also a method that detects organisms in a sample faster, cheaper and more accurately compared to culturing. In recent years, it has been observed that multiple (multiplex) PCR technique, which identifies and distinguishes more bacteria than traditional urine culture, and enables direct urine analysis, has been applied in patients with urinary tract infection symptoms.[4] A sensitive technique, PCR only needs traces of DNA or RNA to produce enough copies to be analyzed. PCR can be performed after obtaining DNA from various tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes.[5]

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PCR Steps

In PCR; template DNA, four deoxyribonucleotides (dNTPs: dATP, dTTP, dGTP and dCTP), two primers or oligonucleotides, DNA polymerase enzyme, buffer solution and magnesium (Mg^{+2}) incorporated into nucleotides to be recognized by the polymerase enzyme, responsible for making the new strand from the template DNA; The template is subjected to a series of thermal cycles to reproduce millions of copies of DNA.[6] This cycle is basically the process that includes three steps: 1. Denaturation of the double-stranded DNA template, 2. Binding of target-specific primers, 3. Extension of bound primers by DNA polymerase.[4] The method is performed at a temperature between 94°C-96°C for 1 minute to 10 minutes, depending on the template DNA and polymerase type. This is followed by the denaturation step, typically carried out at a temperature between 93°C-98°C. Hydrogen bonds in double-stranded DNA (dsDNA) are broken, resulting in two single-stranded DNA (ssDNA) molecules from each dsDNA (denaturation step). In the binding step, the temperature is then lowered to the primer-specific binding temperature in the range of 55°C to 65°C, so that the primers bind to complementary sequences of single-stranded DNA molecules. The PCR mix is then heated to a temperature between 72°C-80°C, depending on the polymerase used. During the elongation step, the incomplete DNA sequence is extended by polymerase in the presence of free dNTPs that synthesize new double-stranded DNA, which is a copy of the original DNA template.[3]

PCR Optimization

Problems such as the presence of inhibitory substances in the samples, the risk of environmental contamination, incorrect use of the amount of components used, and the inability to adjust the temperature parameters are always the problems that can be encountered during PCR. In addition, the design of oligonucleotide primers is only possible with known strains of microorganisms and known sequences of these strains. Another factor that can cause problems in the functions of PCR is unexpected mutations in microbial genomes. One of the most important problems that may be encountered in routine PCR applications in diagnostic laboratories is false positives due to contaminations. This problem shows that the laboratories where PCR will be performed must be strictly controlled. Apart from the problems that we may encounter during the routine control and evaluation of PCR, there are standards and rules that should be known for the optimization of the reagents and materials to be used. Briefly, the procedures to be followed are the standards and parameters used in optimizing the amount of reagents used and the quality of the materials; DNA Extraction, Mg^{+2} Concentration, primers, dNTPs, temperature parameters used in the PCR reaction, PCR machines and tubes in which the PCR reaction is carried out.

Real-Time PCR

Real-time PCR is a highly preferred method today.[7] RT-PCR allows the target to be quantified relative to a calibrator and therefore the method is quantitative (qPCR).[8] qPCR represents an enhanced version of standard PCR. With this technique, products are continuously monitored throughout their reaction cycle using fluorescent dyes. starting amount of DNA sequence; It can be generated by comparing the fluorescence output

curve of the qPCR with the standard curve produced with known different starting numbers of DNA copies. The threshold cycle (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold and be detected. Ct levels are inversely proportional to the amount of target nucleic acid in the sample.[6] The sample output of real-time PCR is fast, and it is more sensitive and specific than conventional methods.[7] qPCR is widely applied in clinical settings and remains the gold standard for nucleic acid measurement.[8] Today, due to its high sensitivity level, qPCR technique is frequently used to detect malignant cells in different types of hematological malignancies.[6]

RT-PCR and SARS-CoV-2

PCR is accepted as a highly sensitive laboratory technique that can provide qualitative and quantitative results, and its reliability has been proven in the fields of medicine and biology (Figure 1). In molecular diagnostic laboratories, rRT-PCR technique is applied to find target RNAs in the diagnosis of specific pathogens.[9] To diagnose SARS-CoV-2, although CT scan and other biochemical findings seem helpful in the diagnosis of COVID-19, as revealed in previous studies, they may have similarities with some infections with similar symptoms.[10,11] The gold standard method that can distinguish SARS-CoV-2 from other beta-coronaviruses such as SARS and MERS for molecular diagnosis using specific primers and probes is the rRT-PCR method.[12]

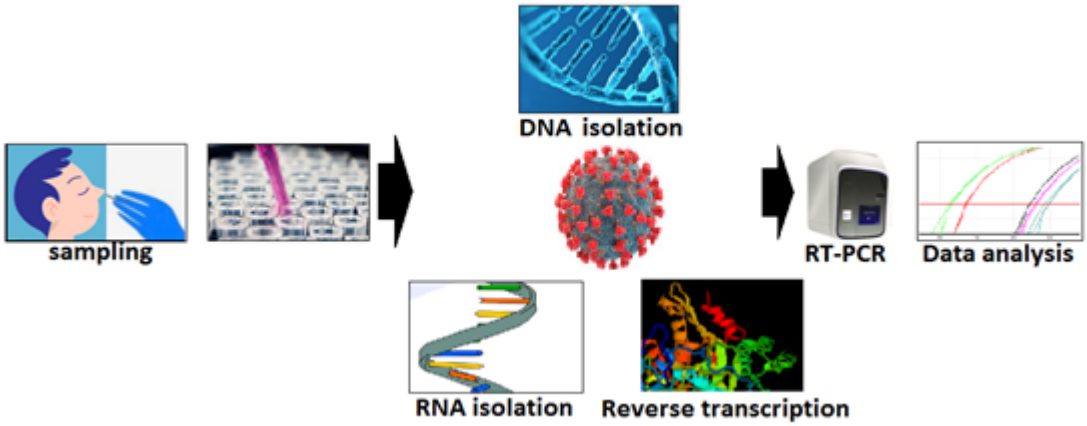

Figure 1. Working procedure of RT-PCR

Even though the rRT-PCR method, which has high specificity but moderate sensitivity, is accepted as the gold standard test for the confirmation of COVID-19 by WHO, there are many negative comments that should be considered on this method (Figure 1).[13] Considering the pre-analytical errors; Test results may be affected in the steps until the samples are taken and finalized in the laboratory.[14,15] Factors during analytical testing such as nucleic acid extraction, cDNA synthesis and PCR processing, and finally analytical errors such as interpretation and analysis of results and assay have also been reported.[13,15,16] In general, rRT-PCR troubleshooting pre-analysis, pre-analysis, and post-analysis phases, and by following the guidelines, it is possible to effectively increase the accuracy and precision of the results obtained.

RESULTS AND DISCUSSION

Based on the detection of SARS-CoV-2 virus RNA isolated from upper respiratory secretions, RNA copies per throat swab sample, virus RNA concentrations were readily isolated from throat or lung-derived samples.[17] Consistent diagnosis of COVID-19 is supported by viral tropism and high active replication rate in the pharyngeal region, but RNA isolation from blood, urine and stool samples is not preferred.[17] Diagnosis of SARS-CoV-2 infection is currently based on real-time reverse transcriptase-polymerase chain reaction (RT-PCR) performed on nasopharyngeal swabs (NPS) or oropharyngeal swabs (OPS).[18] Even though the diagnostic rates are not optimal with sample collection from the upper respiratory tract, it still represents the primary diagnostic method of COVID-19 patients with its NPS/OPS ratio.[13] Some studies mention that RT-PCR results for COVID-19 infection are false-negative and will be a non-negligible error, especially for symptomatic individuals suspected of being infected with COVID-19.[19,20] The use of CT to diagnose COVID-19 is known to be of great value in evaluating the course of the disease and treatment protocols. China uses CT instead of other research tools in the diagnosis of COVID-19, and the ability of CT to diagnose patients at an early stage may also be due to concerns about the specificity of other tests and the lack of virus test kits.[21] However, due to the low specificity of CT in distinguishing COVID-19 from other similar diseases, the American College of Radiology (ACR) opposes the use of CT for the diagnosis of COVID-19 disease in the first place.[22] In addition, the Royal College of Radiologists (RCR) state that CT has a very important role in the evaluation of patients with worsening clinical picture and severe respiratory distress, but that CT should not be used in the evaluation of coronavirus infection.[23] Ventilation, airflow and cleaning of scanner rooms, and other hygiene-related challenges in radiology areas are another reason not to view CT as the sole diagnostic tool for COVID-19 patients. Despite all this, it is noteworthy that RT-PCR has a low sensitivity (60-71%) compared to CT in the diagnosis of COVID-19 infection.[20,24,25] Studies supporting the high sensitivity of CT images (98%) compared to RT-PCR tests (71%) are frequently encountered.[25]

It has been reported that 3% of the patients from 167 people from whom nasopharyngeal and/or throat swabs were taken initially showed negative RT-PCR, but they were positively compatible with COVID-19 in simultaneous chest CT scans.[20] Multiple peripheral ground-glass opacities (GGO) can be observed in lingual segments known to be negative for RT-PCR laboratory test with a chest CT scan.[19] Such false negative results can be explained by the low viral load and/or laboratory errors in the samples.[20,25] The inadequacy of test kits may lead to the victimization of the patient and failure to detect similar errors again.[24] Therefore, more work falls on radiologists to diagnose COVID-19 (22). Wu et al. (2020) mentioned the role of chest CT scans in assessing the severity of COVID-19 infection, citing the fact that most patients had mild symptoms and a high fever, but the severity of lung findings on chest CT scans.[26]

The studies may suggest that RT-PCR testing alone is not sufficient to prove the diagnosis of COVID-19. Therefore, early chest CT scans may still be necessary, along with other research tools such as RT-PCR

testing. Considering the psychological status of COVID-19 patients and healthcare workers during diagnosis and treatment, it is clear that large-scale new studies are needed on the reliability of RT-PCR results.[27]

CONCLUSION

Although PCR-based methods, which are renewed with continuously developed tools, materials and ready-made kits, were initially developed for diagnostic purposes, they are currently used in many disciplines and fields. PCR-based methods, which require specialized molecular workers to be optimized, cause difficulties in laboratories until the optimization stages and can cause time and material loss. Even with repeated studies using information from optimized literature, the brand of materials and tools, their conditions of use, their misuse, and repeated reactions with inexperienced personnel may not yield the same results.

Even with the same instruments and brands, different results are obtained between different laboratories with experienced personnel. Even with all this in mind, the PCR technique continues to be an increasingly important and practical technique in diagnostic microbiology and other fields, despite its disadvantages and difficulties. This technique will continue to develop with increasing momentum in the coming years, with the PCR methods being renewed every day. In the current literature on COVID-19, although chest CT scans show high sensitivity in diagnosing COVID-19 compared to RT-PCR tests, chest CT scans alone are not sufficient to detect COVID-19.

The sensitivities of the RT-PCR tests in use are not sufficient to diagnose and guide the treatment of COVID-19. Judging from these research results, it is clear evidence that RT-PCR analysis is still not sufficient for the diagnosis of COVID-19 and that imaging methods and serum antibody tests should also be used. Considering the time, place and financial appropriations for the diagnosis of COVID-19; There is a need for RT-PCR studies to be conducted with newly developed unique tests.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The author report there are no competing interests to declare. The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

REFERENCE

1. Mullis K, Faloona F, Scharf S, et al. Spesific enzymatic amplification of DNA in vitro: polymerase chain reaction. Cold Spring Harbor Symposia on Quantitative Biology. 1986;51, 263-273.
2. Garcia LT, Cristancho LM, Vera EP, et al. A new multiplex-PCR for urinary tract pathogen detection using primer design based on an evolutionary computation method. J Microbiol Biotechnol. 2015;25(10):1714–27.
3. Sreejith KR, Ooi CH, Jin J, et al. Digital polymerase chain reaction tech - nology-recent advances and future per - spectives. Lab Chip. 2018;18(24):3717–32.
4. Dixon M, Sha S, Stefil M, et al. Is it Time to Say Goodbye to Culture and Sen - sitivity? The Case for Culture-independent Urology. Urology. 2019;136:112–8.
5. Garibyan L, Avashia N. Research Techniques Made Simple: Polymerase Chain Reac - tion(PCR). J Invest Dermatol. 2013;133(3).
6. Cilloni D, Petiti J, Rosso V, et al. Digital PCR in myeloid malignancies: Ready to replace quantitative PCR? Int J Mol Sci. 2019;20(9).
7. Kurkela S, Brown DWG. Molecular diag - nostic techniques. Medicine (Baltimore). 2009;37(10):535–40.
8. Quan PL, Sauzade M, Brouzes E. DPCR: A technology review. Sensors (Switzerland). 2018;18(4):1271.
9. Mayer G, Muller J, Lunse CE. RNA diagnostics: real-time RT-PCR strategies and promising novel target RNAs. *Wiley Interdiscip. Rev. RNA.* 2011;2(1):32–41.
10. Fu L, Wang B, Yuan T, et al. Clinical characteristics of coronavirus disease 2019 (COVID-19) in China: A systematic review and meta-analysis. *J. Infect.* 2020;80(6):656–665.
11. Alayunt NO, Ozudogru O, Yerlikaya E. First remarkable findings in comparison of patients in Siirt / Turkey in novel coronavirus (Covid-19) pandemic. Medicine Science 2020;9(4):1008-13
12. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro. Surveill.* 2020;25(3).
13. Lippi G, Simundic AM., Plebani M. Potential preanalytical and analytical vulnerabilities in the laboratory diagnosis of coronavirus disease 2019 (COVID-19) *Clin. Chem. Lab. Med.* 2020;58(7):1070–1076.
14. Espy MJ, Uhl JR, Sloan LM, et al. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev.* 2006;19(1):165–256.
15. Lippi G, Meyer A, Cadamuro J, et al. European Federation of Clinical, P. Laboratory Medicine Working Group for Preanalytical, PREDICT: a checklist for preventing preanalytical diagnostic errors in clinical trials, Clin. Chem. Lab Med. 58(4) (2020) 518–526.
16. Tang YW, Schmitz JE, Persing DH, et al. Laboratory Diagnosis of COVID-19: Current Issues and Challenges. *J. Clin. Microbiol.* 2020;58(6).
17. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature* 2020;581(7809):465–469.
18. Centers for Disease Control and Prevention. *Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons for Coronavirus Disease 2019 (COVID-19)*. Updated July 8, 2020. Accessed April 16, 2020.
19. Huang P, Liu T, Huang L, et al. Use of chest CT in combination with negative RT-PCR assay for the 2019 novel coronavirus but high clinical suspicion. *Radiology.* 2020 Apr;295(1):22–23.

20. Xie X, Zhong Z, Zhao W, et al. Chest CT for typical 2019-nCoV pneumonia: relationship to negative RT-PCR testing. *Radiology.* 2020 doi: 10.1148/radiol.2020200343.
21. Lee E, Ng M, Khong PL. COVID-19 pneumonia: what has CT taught us? *Lancet Infect Dis.* 2020;20(4):384–385.
22. American College of Radiology ACR Recommendations for the use of Chest Radiography and Computed Tomography (CT) for Suspected COVID-19 Infection. https://www.acr.org/Advocacy-and-Economics/ACR-Position-Statements/Recommendations-for-Chest-Radiography-and-CT-for-Suspected-COVID19-Infection
23. The Royal College of Radiologists The role of CT in patients suspected with COVID-19 infection. https://www.rcr.ac.uk/college/coronavirus-covid-19-what-rcr-doing/clinical-information/role-ct-chest/role-ct-patients
24. Kanne J, Little B, Chung J, et al. Essentials for radiologists on COVID-19: an update—radiology scientific expert panel. *Radiology.* 2020 doi: 10.1148/radiol.2020200527.
25. Fang Y, Zhang H, Xie J, et al. Sensitivity of chest CT for COVID-19: comparison to RT-PCR. *Radiology.* 2020 doi: 10.1148/radiol.2020200432.
26. Wu J., Wu X., Zeng W., Zeng W., Guo D., Fang Z. Chest CT findings in patients with coronavirus disease 2019 and its relationship with clinical features. *Invest Radiol.* 2020;55(5):257–261.
27. Alayunt NO, Ozudogru O, Cakan E. Psychological and biochemical Impact of Covid-19 pandemic on health workers in Siirt, Turkey. Medicine Science 2021;10(4):1486-91.

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Original Research

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Optimization of DCR1 and DCR2 epigenetic annealing temperatures for breast cancer biomarker using in-silico and in-vitro study

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Abstract: The epigenetics of methylated and unmethylated DCR1 and DCR2 (decoy receptors 1 and 2) are genes encoding membrane receptors that can bind to TRAIL causing TRAIL inhibition in the apoptotic pathway. Epigenetic detection of DCR1 and DCR2 was developed as a biomarker of breast cancer. One of the detection methods is using PCR. The most important step in the PCR process is the determination of the annealing temperature. This research performs Tm analysis using the insilico program from Neb, insilico, Thermofisher, and Promega and in vitro optimization. Methylated DCR1 can be amplified at annealing temperatures of 51.4°C, 52.4°C, 53.6°C, 54.7°C measuring about 600bp according to Tm analysis of insilico and promega. DCR1 could also be amplified at annealing temperatures of 50,1, 49, and 48.8 but the primers were also amplified at non-specific sites. Methylated DCR2 could be amplified at annealing temperatures of 48.8°C, 49°C and 50.1°C and a specific size of about 500 bp according to the Tm analysis of promega. Unmethylated DCR1 and DCR2 genes could not be amplified at the annealing temperature which were analyzed using Neb, insilico, promega, and thermofisher.

Keywords: Melting temperature; Annealing temperature; PCR.

INTRODUCTION

Breast cancer is the leading cause of woman death in America about 13%. The incidence of breast cancer in Indonesia ranks first, and the second highest cause of death after lung cancer [1]. Breast cancer develops from an early stage in the form of benign cancer that can divide continuously (hyperplasia and low grade dysplasia), pre-cancer (high grade dysplasia and carcinoma in situ), and cancer (invasive, and metastatic carcinoma). The incidence of breast cancer can be influenced by genetic and epigenetic factors. Epigenetic factors that can cause tumor development are DNA methylation and histone acetylation. DNA methylation aberrations are responsible for the silencing of tumor suppressor genes, leading to tumor development and cancer progression.

Research that has been carried out regarding DNA methylation modification in cancer cells occurs in the cytosine residue region at the CpG dinucleotide location on the gene promoter or the first exon where CpG islands are formed [2]. Changes in DNA methylation in the promoter gene cause the formation of suppressed chromatin structures so that it inhibits transcription factors from being able to bind to the active site, thereby causing uncontrolled DNA functions involved in tumor development, such as tumor suppressors and DNA repair, cell cycle regulators, and transcription factors [3]. One of the genes that control the process of apoptosis is DCR1 and DCR2. DNA methylation on the promoter site of DCR 1 and 2 genes causes inhibition of cell cytotoxic processes. DCR1 and 2

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are tumor necrosis factor receptor superfamily member 10C. DCR1 and DCR2 are receptors for TRAIL ligands that play a role in cytotoxic processes. DCR1 is able to prevent apoptosis by binding to TRAIL[4–6].

One of the detections is to determine the presence of DCR1 and DCR2 gene methylation by the PCR method. PCR is a method to amplify or duplicate specific genes in a short time [7,8]*. The PCR process produces specific data when using the right steps. One of the PCR steps that plays an important role in determining gene specificity is annealing* [9–12]*. Annealing is the stage of attaching the primers to the target gene. Annealing requires the right temperature by calculating the melting temperature (Tm). Melting temperature is highly dependent on the composition of adenine, guanine, cytosine, and thymine. The more guanine and cytosine content in the primers will determine the higher temperature, so the annealing temperature of each primer will be different* [13]*. Before carrying out the PCR process, the steps carried out were optimizing the annealing temperature* [11,12]*. The annealing temperature was determined manually or using the insilico program. The annealing temperature optimization process is an important step before PCR because the conditions of the samples, reagents, and tools to be used are different for each researcher* [14].

Insilico analysis simplifies trial and error annealing steps without clear references. Insilico analysis is the calculation of Tm using bioinformatics. There are several factors used to consider the annealing temperature calculation, namely the type of DNA polymerase, G and C content, and primers concentration. Insilico temperature melting analysis has many options including Neb, Thermofisher, Promega. Choosing the right insilico program is very important, especially if researchers don't have a thermocycler engine with a temperature gradient. This study aims to confirm the results of insilico annealing temperature analysis of several programs with in vitro PCR of methylated and unmethylated DCR1 and DCR2.

MATERIAL AND METHOD

The experimental study design used serum samples from breast cancer patients. The variables studied were the primers annealing temperature of methylated and unmethylated DCR1 and DCR2. Annealing temperature search analysis using the Insilico program. Promega Wizard®SV Genomic DNA Purification System kit, GoTaq® Green Master Mix 2x M7128(Promega), Primers methylated and unmethylated DCR1 and DCR2, DNA template, Nuclease free water, Agarose (GeneDireX), Nucleic Acid Gel Stain (Smobio NS 1000 FlouroVueTM), 5x DNA Loading Dye Smobio DL4000 Excel DyeTM, marker 1 kb vivantis.

Nanodrop MaestroGen, PCRmax 10570-1 version 2.41 thermocycler machine, UV transilluminator (MS Major Science), Mupid electrophoresis machine. DNA isolation using the promega Wizard®SV Genomic DNA Purification System kit. The isolated DNA was then calculated for concentration and purity using the MaestroGen nanodrop machine.

Insilico analysis of primary annealing temperature using https://tmcalculator.neb.com/, http://insilico.ehu.es/tm, https://worldwide.promega.com, https://www.thermofisher.com. The type of material used for the gene amplification process is a consideration for the annealing temperature data to be obtained. DNA polymerase using GoTaq® Green Master Mix 2x, M7128 Promega. Primers concentration was 100 pmol and template DNA was 3 ng/µl.

Specific gene amplification using the PCRmax 10570-1 version 2.41 thermocycler machine. 3 µl of 1 ng/µl template DNA was pipetted and put into a PCR microtube. Primers DCR 1 unmethylated F5'-GAATTTTTTTATGTGTATGAATTTAGTTAAT-3' and R5'-CCATCAAACAACCAAAACA-3' were added 2µl each reaction. GoTaq® Green

Master Mix 2x M7128 (Promega) added 12.5µl. Nuclease free water was added as much as 5.5µl. The reaction was homogenized using a vortex and spindown. The total volume of the reaction was 25µl. The PCR steps include predenaturation at 95°C for 15 minutes, denaturation at 95°C for 30 seconds, annealing at 48.8°C, 49°C, 50.1°C, 51.4°C, 52.4°C, 53.6°C, 54.7°C, 55.9°C, 57°C, 58.2°C, 59.1°C, 59.3°C for 1 minute, elongation temperature 72°C for 1 minute, extra extension at 72°C for 10 minutes, and cooling down at 4°C for 10 minutes. The same steps and PCR components were used to amplify the DCR1 methylated F5'-TTACGCGTACGAATTTAGTTAAC-3' and R5'-ATCAACGACCGACCGAAACG-3' genes, unmethylated DCR2 genes F5'-TTGGGGATAAAGTGTTTTGATT-3' and R5'-AAACCAACAACAAAACCA-3', and methylated DCR2 genes F5'-GGGATAAAGCGTTTCGATC-3' and R5'-CGACAACAAAACCGCG-3' (Tserga, 2011). Two (2)g of agarose (GeneDireX) was weighed, then the agarose was dissolved with 100 ml of TAE 1X. The solution is heated in a microwave until all the agarose powder is dissolved and the liquid is clear. Nucleic Acid Gel Stain (Smobio NS 1000 FlouroVueTM) as much as 4 µl was added to the agarose solution. The warm agarose solution is poured into the mold and the comb is installed. The hardened agarose gel is used for the electrophoresis process. 2 µl of loading dye (5x DNA Loading Dye Smobio DL4000 Excel DyeTM) was mixed with 8 µl of PCR product, then resuspended and put into agarose wells. 1 kb marker was used to determine the size of the PCR product. The sample running process was carried out using a voltage of 50V for 1 hour. The visualization process uses a UV transilluminator (MS Major Science).

The gene bands of DCR1 methylated at about 600 bp and DCR2 methylated at about 550 bp appeared at certain annealing temperatures and matched the suggested insilico data

RESULTS AND DISCUSSION

Insilico analysis using the programs https://tmcalculator.neb.com/, http://insilico.ehu.es/tm,https://worldwide.promega.com,https://www.thermofisher.com produces annealing temperatures that different (Table 1.). The Neb program produces one annealing temperature for a pair of primers. The information obtained from the NEB program is the annealing temperature, the number of nucleotides from a pair of primers, and the G+C content. The data entered in the Neb program are the type of polymerase kit, forward and reverse primers nucleotide sequences and the concentration of primers.

The insilico program from http://insilico.ehu.es/tm produces different annealing temperatures between the forward and reverse primers, so the annealing temperature of both primers must be confirmed simultaneously (Table 1). The information obtained was primers nucleotide length, G+C content, molecular weight, and annealing temperature. The data that is processed in the http://insilico.ehu.es/tm program is the primers nucleotide sequence, and the determination uses basic Tm or base-stacking Tm. In the base-stacking Tm data can be entered primary concentration, salt concentration, and concentration of Mg^{2+}.

The insilico program from https://worldwide.promega.com resulted in different Tm calculations between the forward and reverse primers (Table 1). The data needed in the Promega program are the primers nucleotide sequence, the concentration of the primers, and the type of buffer and polymerase used.

The insilico program from https://www.thermofisher.com generated Tm calculations for both primers (Table 1). In addition, the information provided is the annealing temperature of each primers, molecular weight, extinction coefficient, and annealing temperature.

Table 1. Annealing temperature based on insilico and in vitro analysis

Gen	Neb	Insilico	Promega	Thermo	Invitro
DCR1 unmet	31 °C	F-49.8 °C R-41.2 °C	F-53 °C R-47 °C	51,7 °C	
DCR1 met	39 °C	F-49.9 °C R-53.8 °C	F-52 °C R-57 °C	55,7 °C	52.4 °C 53.6 °C 54.7 °C 51.4 °C 50.1 °C 49 °C 48.8 °C
DCR2 unmet	38 °C	F-47.4 °C R-45.6 °C	F-51 °C R-50 °C	53,7 °C	
DCR2 met	37 °C	F-48.9 °C R-45.9 °C	F-50 °C R-49 °C	53,8 °C	48.8 °C 49 °C 50.1 °C

Based on the annealing temperature of several insilico programs, in vitro tests have been carried out using the PCR method. The annealing temperature range used is 50°C to 60°C with 12 different temperature gradients. The genes that were successfully amplified were methylated DCR1 and DCR2 (Table 1).

3.2 Invitro test optimization annealing temperature insilico program

The methylated DCR1 PCR product of about 600 bp was successfully amplified at an annealing temperature of 52.4°C (Figure 1). Thin amplified DNA bands indicate low amplicon. Low amplicon can be affected due to the low concentration of template DNA. The thick DNA band indicates a high concentration of template DNA [13,15]. The annealing temperature of DCR 1 methylated in vitro is almost the same as the insilico program from http://insilico.ehu.es/tm.

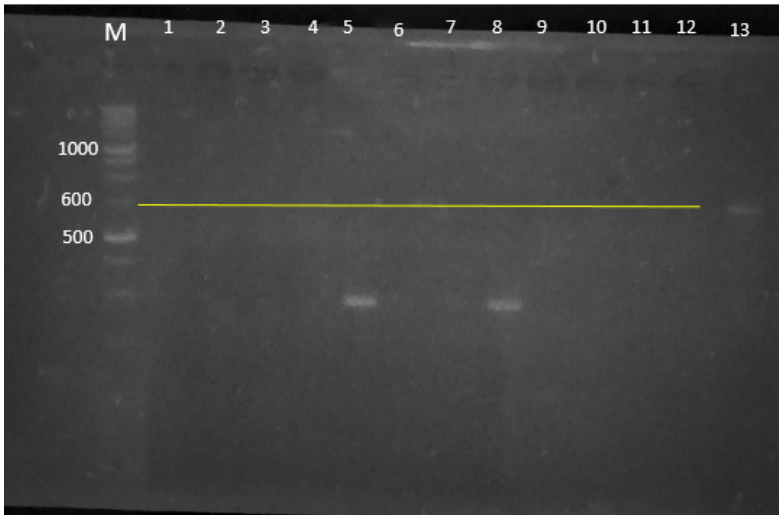

Figure 1. Optimization of the annealing temperature of the methylated DCR 2 gene (1) 50.1°C; (2) 49°C; (3) 48.8°C, LRP1 gene (5) 59.1°C ; (6) 58.2°C ; (7) 57°C ; (8) 55.9°C , unmethylated DCR2 gene (9) 57°C ; (10) 55.9°C , methylated DCR1 gene (11) 54.7°C ; (12) 53.6°C ; (13) 52.4°C, (M) marker

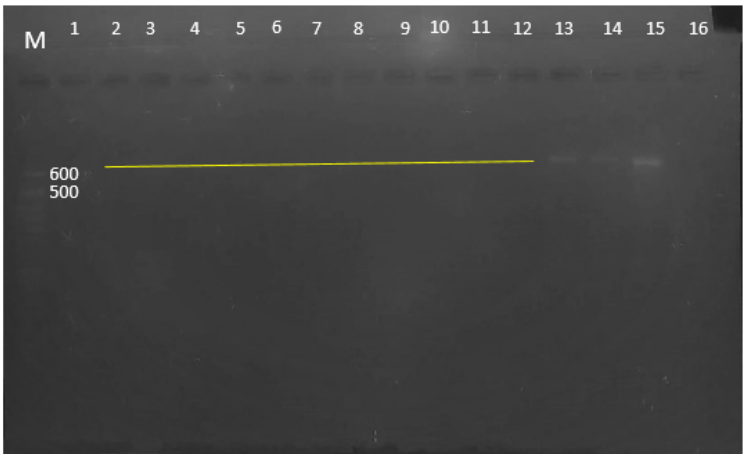

Figure 2. Optimization of annealing temperature of unmethylated DCR 2 gene (1) 53.6°C; (2) 52.4°C; (3) 51.4°C ; (5) 50.1°C ; (6) 49°C ; (7) 48.8°C ; methylated DCR 1 gene (8) 59.3°C ; (9) 59.1 °C ; (10) 58.2°C ; (11) 57 °C ; (12) 55.9°C ; (13) 54.7°C ; (14) 53.6°C ; (15) 52.4 °C , methylated DCR2 gene (16) 51.4 °C, (M) marker

Methylated DCR1 was also successfully amplified at annealing temperatures of 54.7°C, 53.6°C, 52.4°C (Figure 2). The methylated DCR1 gene produced the most amplicons at 52.4°C (Figure 2). The difference in the intensity and thickness of the thin or faint bands was due to the total DNA concentration being too small. However, if the DNA concentration is too high, dilution is also required [7]. The methylated DCR1 gene has almost the same annealing temperature as the insilico program http://insilico.ehu.es/tm.

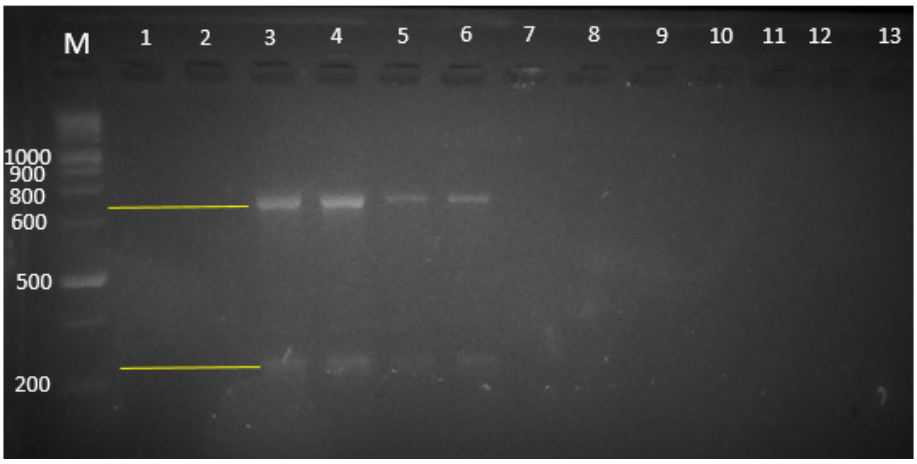

Figure 3. Optimization of the annealing temperature of the methylated DCR 2 gene (1) 53.6 °C; (2) 52.4 °C, methylated DCR 1 gene (3) 51.4 °C; (4) 50.1°C; (5) 49°C; (6) 48.8°C, unmethylated DCR 2 gene (7) 59.1°C; (8) 58.2°C ; (9) 57°C ; (10) 55.9°C ; (11) 54.7°C ; (12) 53.6°C ; (13) 52.4°C , (M) marker

The methylated DCR1 gene was amplified at 51.4°C, 50.1°C, 49°C, 48.8°C. The amplification temperature is too low, causing the PCR product to be non-specific. This is indicated by the appearance of two bands around 600 bp and 200 bp (Figure 3). The basis of the success of the PCR process lies in the suitability of the primers and the efficiency and optimization of the PCR process. The annealing stage requires a very specific and optimum temperature. Annealing temperature that is not specific can cause misspriming, namely primers amplify areas that are not the target or even do not amplify the target DNA [12]. Tm that is too high causes the release of the primer that has been attached to the DNA template so that the PCR product will not be formed, on the contrary if the tm is too low, the primer will stick to the non-specific side[14,16,17].

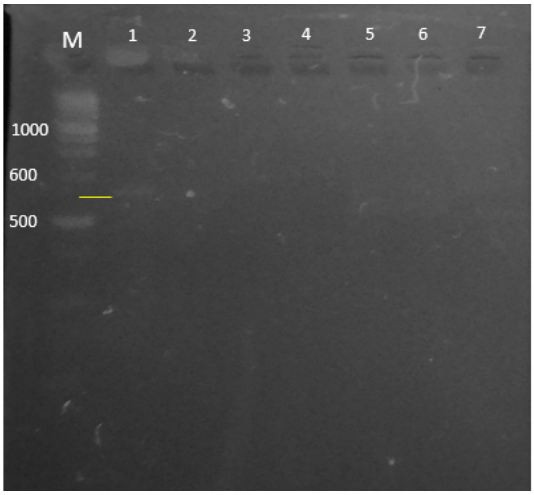

Figure 4. Optimization of annealing temperature of methylated DCR 2 gene (1) 48.8 °C ;(2) 59.3 °C ;(3)59.1 °C ; (4) 58.2 °C ;(5) 57 °C ;(6)55.9 °C ;(7) 54.7 °C, (M) marker

The methylated DCR2 gene was amplified at 48.8°C, 49°C, 50.1°C (Figures 4 and 5). The DNA band is about 500 bp in size and is single indicating a specific PCR product. The DNA band of the DCR2 gene is thin showing the slightly formed amplicons. The annealing temperature of the DCR2 gene in vitro is almost the same as the insilico annealing temperature of https://worldwide.promega.com.

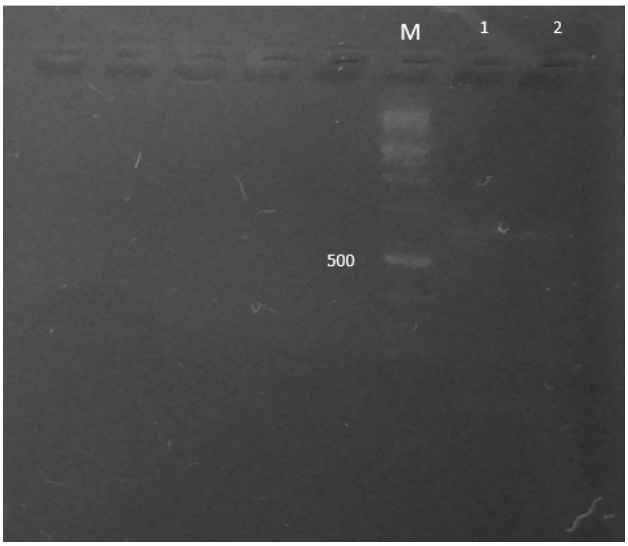

Figure 5. Optimization of the annealing temperature of the methylated DCR 2 gene (1) 50.1 °C ;(2) 49 °C (M) marker

The unmethylated DCR1 and DCR2 genes were not amplified in the annealing temperature range of 50-60 °C according to Tm analysis of various insilico programs. The basis of the success of the PCR process based on in the suitability of the temperature and time at each stage of the PCR, especially annealing. the annealing stage requires a very specific and optimum temperature[18]. The non-specific annealing temperature can cause the target DNA not to be amplified[19,20].

CONCLUSION

The methylated DCR1 gene had an annealing temperature of 51.4°C, 52.4°C, 53.6°C, 54.7°C almost the same as the Tm analysis from http://insilico.ehu.es/tm. The methylated DCR2 gene had an annealing temperature of 48.8°C, 49°C and 50.1°C almost the same as the Tm analysis program https://worldwide.promega.com. The unmethylated DCR1 and DCR2 genes could not be amplified at Tm as suggested by the insilico program.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

REFERENCE

1. A DL, Subtypes LB, Inic I, Pupic G, Jancic S. Clinical Medicine Insights : Oncology. 2014:107-111. doi:10.4137/CMO.S18006.Received
2. Lehmann U, La F, Feist H, Glo S, Hasemeier B. Quantitative Assessment of Promoter Hypermethylation during Breast Cancer Development. 2002;160(2):605-612. doi:10.1016/S0002-9440(10)64880-8
3. Tserga A, Michalopoulos N V, Levidou G, et al. Association of aberrant DNA methylation with clinicopathological features in breast cancer. 2012;(10):1630-1638. doi:10.3892/or.2011.1576
4. Fackler MJ, Malone K, Zhang Z, et al. Quantitative Multiplex Methylation-Specific PCR Analysis Doubles Detection of T umor Cells in Breast Ductal Fluid. 2006;12(11):3306-3311. doi:10.1158/1078-0432.CCR-05-2733
5. Liu JJ, Shen R, Chen L, et al. Piwil2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker. 2010;3(4):328-337.
6. Zhang HYAN, Liang F, Jia ZHIL, Song SANTAI, Jiang ZEFEI. PTEN mutation , methylation and expression in breast cancer patients. 2013:161-168. doi:10.3892/ol.2013.1331
7. Herman, Nainggolan M, Roslim DI. Optimizing Temperature Annealing for Four Primary RAPD in Mung bean (Vigna radiata L .). *J Din petanian*. 2018;34:41-46.
8. Amanda K, Sari R, Apridamayanti P. Optimasi suhu annealing proses PCR amplifikasi gen shv bakteri Escherichia coli pasien ulkus

diabetik. 2015;(10).
9. Kartika AI. Optimasi Annealing Temperature Primer mRNA RECK dengan Metode One Step qRT-PCR. *J labora medica*. 2018;2(1):22-31.
10. Triyaningsih, Nuringtyas TR, Purwestri YA, Sebastian A. Optimasi suhu annealing qRT-PCR gen WRKY45 sebagai deteksi gen ketahanan terhadap infeksi Xanthomonas oryzae pv . oryzae pada padi hitam cempo ireng. *J ilmu dasar*. 2022;23(1):23-28.
11. Kurniawati S, Hartati NS. Optimasi Suhu Annealing Primer Degenerate untuk Mengamplifikasi Fragmen Gen Arginine Decarboxylase (ADC) Genom Ubi Kayu Lokal Maluku Tenggara. *J ilmu dasar*. 2018;19(2):135-142.
12. Aulia SL, Suwignyo RA, Hasmeda M. Optimasi Suhu Annealing untuk Amplifikasi DNA Padi Hasil Persilangan Varietas Tahan Terendam dengan Metode Polymerase Chain Reaction. *J Ilm Mat dan Ilmu Pengetah Alam*. 2021;18(1):44-54. doi:10.31851/sainmatika.v18i1.5805
13. Sipos R, Szekely AJ, Palatinszky M, Revesz S, Marialigeti K, Nikolausz M. Effect of primer mismatch , annealing temperature and PCR cycle number on16S rRNA gene-targetting bacterial community analysis. *FEMS Microbiol Ecol*. 2007;60:341-350. doi:10.1111/j.1574-6941.2007.00283.x
14. Yuenleni. Langkah-langkah optimasi PCR. *Indones J Lab*. 2019;1(3):51-56.
15. Anggisti L, Roslim DI, Herman. Optimization of Annealing Temperature for Amplification of Actin Gene in Pandan (Pandanus sp). *J Din Pertan*. 2018;34:95-100.
16. Erjavec MS. Annealing temperature of 55C and specifity of primer binding in PCR reaction. In: *Synthetic Biology-New Interdisciplinary Science*. ; 2019:1-13.
17. Nurjayadi M, Efrianti UR, Azizah N, Julio E, Saamia V. Optimum temperature of the amplification of the fljB gene of Salmonella typhimurium. In: *Empowering Science and Mathematics for Global Competitiveness*. ; 2019:53-58.
18. Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. A practical approach to RT-qPCR — Publishing data that conform to the MIQE guidelines. *Methods*. 2010;50(4):S1-S5. doi:10.1016/j.ymeth.2010.01.005
19. Ludyasari A. Pengaruh suhu annealing pada program PCR terhadap keberhasilan amplifikasi DNA udang jari (metapenaeus elegans De Man, 1907) Laguna Segara Anakan, Cilacap, Jawa Tengah. 2005.
20. Deniariasih NW, Ratnayani K, Yowani SC. Optimasi PCR (Polymerase Chain Reaction) fragmen 724 pb gen katG multi drug resistance tuberculosis untuk meningkatkan produk amplifikasi. *J Farm Udayana*. 2013:110-115.

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Original Research

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Inhibitory potentials of ivermectin, nafamostat, and camostat on spike protein and some nonstructural proteins of SARS-CoV-2: Virtual screening approach

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Abstract: The search for potential oral drugs either through synthetic routes or by drug repurposing for combating the dreaded covid-19 virus is still ongoing. The coronavirus spike glycoprotein and several other non-structural proteins play crucial roles in the replication and transmission of this virus. Recent research have identified ivermectin, nafamostat, and camostat as promising drug inhibitors of SARS-CoV-2 target proteins. The broad-spectrum inhibitory action of ivermectin, nafamostat, and camostat on the spike glycoprotein and some non-structural proteins of this virus was studied in silico. The spike glycoprotein, nsp3, nsp5, nsp9, nsp10, nsp13, and nsp16 were selected for this study and were downloaded from the protein data bank. Flexible docking procedure implemented in Auto Dock Vina module was deployed for the docking procedure of the drugs with the protein receptors. Although ivermectin had the best inhibitory action on the viral spike protein and nsp10, nafamostat was identified as the compound with the best broad-spectrum activity on this virus, having the highest binding affinity values of – 9.4kcal/mol, – 7.9 Kcal/mol, – 6.1 Kcal/mol, – 8.0 Kcal/mol, and – 8.7 Kcal/mol for nsp3, nsp5, nsp9, nsp13, and nsp16 respectively. This drug, in combination with ivermectin could therefore be explored further as potential compounds that could be modified to curb the menace of the covid-19 pandemic.

Keywords: Camostat; Ivermectin; Nafamostat; Nonstructural protein; Spike protein; Virtual screening.

INTRODUCTION

Nearly a century after the Spanish flu, the coronavirus disease 2019 (COVID-19) is a pandemic currently being faced by the global community[1]. The current pandemic is because of a novel beta Covid, SARS-CoV-2, systematically having a place with the coronaviridae family, known to cause respiratory diseases in people[2]. SARS-CoV-2 is a wrapped, single-stranded positive-sense RNA infection. The viral RNA genome contains 29,903 nucleotide bases and has ten

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open reading frames (ORF). The ORF1ab encodes for the enormous replicase polyprotein PP1ab, which is separated by papain-like protease (PLpro) and 3-chymotrypsin-like protease (3CLpro) to generate nonstructural proteins (nsps) 1–16, required for the replication of the virus. The primary proteins S, N, E, M, and supplementary proteins are encoded by ORF2-10 [2]. The S protein, anchored on the virus envelope, serves to attach coronavirus receptors and internalization[3]. This protein plays a crucial role in receptor recognition as well as the cell membrane fusion process. As soon as the virus interacts with the host cell, an extensive structural reorganization of the S protein occurs. This activity allows the virus to fuse with the host cell membrane. Polysaccharide molecules coat the spikes to camouflage them, thereby dodging surveillance of the host immune system during entrance [4].

Several non-structural proteins contribute to the replication and transcription of coronaviruses. Nsp3 is a multi-domain protein produced by coronaviruses. It is the largest of the non-structural proteins. It plays many roles in the viral life cycle, acting as a framework of protein that interacts with itself and binds to other viral nsps or host proteins [5] .Generally, nsp3 is crucial in coronaviruses for the formation of replication transcription complexes (RTC) assembly on the host cell membrane, where replication and transcription of the viral genome take occur [6]. Nsp5, often referred to as 3C-like protease, plays a crucial role in synthesizing viral proteins and generates many nonstructural viral proteins through its protease activity. Nsp5 plays a vital role in the coronavirus life cycle, making it a desirable target for producing antiviral drugs [7,8]. Nsp9 is an essential non-structural protein that links coronavirus replication to RNA. Several ways of nsp9 dimerization improve their binding affinity to nucleic acid[9]. Nsp10 is also a significant replication regulator with 148 amino acids and two zinc finger domains for enzymatic interactions. It could interact with nsp14 and nsp16 [10,11]. Nsp13 is one of the most conserved ancestral proteins in nido-viruses, making it an essential drug discovery target [12]. This protein can unwind double-stranded DNA and RNA through hydrolysis of deoxyribonucleotide triphosphates (dNTPs) and ribonucleotide triphosphates. This activity can be facilitated by nsp12 [13]. All the non-structural proteins, nsp16 is crucial in the viral replication cycle because it is important for coronavirus immune evasion [14]. Nsp16 being a 2'-O-methyltransferase, forms part of the replication transcription complex [15]. This protein particularly promotes the transfer of a methyl group from its S-adenosylmethionine cofactor to the 2-hydroxyl of ribose sugar of viral Mrna [16]. This activity improves translation efficiency and camouflages the mRNA so that intracellular pathogen recognition receptors do not recognize it. Essentially, the inhibition or knocking out of 2'-O-mTase activity severely reduces viral replication and infectivity of coronaviruses [17]. Therefore, developing inhibitors of nsp16 is a potential therapeutic approach.

Numerous studies related to identifying effective therapeutics for SARS-CoV-2 have been reported [18-20]. In our previous study [21], using in silico techniques, we evaluated the efficiency of eleven drugs, including chloroquine, hydroxychloroquine, lopinavir, ritonavir, nafamostat, camostat, famotidine, umifenovir, nitazoxanide, ivermectin, and fluvoxamine, in blocking the interactions between human ACE2 and coronavirus spike glycoprotein. Lopinavir, ritonavir, and nafamostat showed good binding affinity on ACE2, while ivermectin, nafamostat, and camostat had the best binding affinity on the coronavirus spike glycoprotein. In this study, the binding affinities of ivermectin, nafamostat, and camostaton the spike and some non-structural proteins of theSARS-CoV-2 were investigated in silico to identify the compound with the largest broad-spectrum inhibitory activity on this virus.

MATERIAL AND METHOD

Protein selection and preparation

Three dimensional (3D) X-ray crystallographic structure of SARS-CoV-2 spike protein, non-structural proteins 3, 5, 9, 10, 13, and 16 weresourced from the protein data bank (PDB) through protein-plus webserver of Hamburg University, Germany. These selected proteins were then prepared for *in silico* docking and minimization implemented via the appropriate tools in Cresset Flare© software, version 4.0 (https://www.cresset-group.com/flare/). The minimization was implemented by choosing the General Amber Force Field (GAFF) option, with a gradient cutoff of 0.200 Kcal/mol/A, and iteration was set to 2000 iterations [22].

Selection and preparations of drugs

Three dimensional (3D) structures of camostat, nafamostat, and ivermectin were recovered from an online chemical curation server called PubChem in simple document format (SDF). Open babel in Python Prescription (version 0.8) was deployed for the optimization of our selected ligands. This process converts ligands into the most stable structures energetically by choosing Universal Force Field (UFF) option.

Computational docking procedure

Flexible docking procedure implemented in the Auto Dock Vina module in Python Prescription suite [23] was deployed for the docking procedure of the drugs with the protein receptors. Target site specific to each protein receptor was adjusted through the grid box with parameters provided in Table 1, containing the dimensions and the binding regions of each protein. The binding affinity with the protein-drug complex was retrieved at the end of the docking run.

RESULTS AND DISCUSSION

The binding affinity of ivermectin, nafamostat, and camostat on the spike glycoprotein and some other non-structural proteins of SARS-CoV-2 are shown in Table 2 and the interactions of the drugs with the amino acids at the binding site of the proteins are given in Table 3.

Table 2. Binding affinity of the selected drugs on some SARS-CoV-2 proteins

Drugs	ΔG (Kcal/mol)						
	Spike	Nsp3	Nsp5	Nsp9	Nsp10	Nsp13	Nsp16
Ivermectin	**-8.4**	**-**6.4	**-**6.9	**-**3.7	**-8.0**	**-**4.1	**-**5.7
Nafamostat	**-**7.8	**-9.4**	**-7.9**	**-6.1**	**-**7.7	**-8.0**	**-8.7**
Camostat	**-**7.2	**-**8.3	**-**6.7	**-**5.4	**-**7.0	**-**7.3	**-**7.6

The antiviral agent camostat is a serine protease inhibitor that attacks SARS-CoV and SARS-CoV-2. Clinically, it is used to treat pancreatitis and reflux oesophagitis. It fights and reduces viral infection by blocking virus-membrane fusion. Studies show thatSARS-CoV-2 utilizes the human transmembrane protease serine 2, TMPRSS2, to enter the human cell, cleave and activate the spike protein [31,32]. This shows that the drug attacks and prevents virus-cell membrane fusion, thereby inhibiting viral replication.

Nafamostat approved for the treatment of acute pancreatitis is being studied as a drug that can block the viral entry of the new coronavirus, SARS-CoV-2. According to recent studies on SARS-CoV-2 cell entry on ACE2 and TMPRSS2, nafamostat can very well inhibit the membrane fusion of the virus's envelope with host cell surface membranes [32]. Results show that it efficiently blocked SARS-CoV-2 infection of human lungs. It has also been reported to block the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection in vitro [33]

Ivermectin is widely used as a broad-spectrum antiparasitic drug with known efficacy of antiviral properties. It is commonly used to treat several tropical diseases that include onchocerciasis, helminthiases, and scabies. It is also used

to control malaria transmission as it is appreciably tolerated and used. Reports from studies suggest that ivermectin inhibits key intracellular transport proteins hijacked by viruses that infect by attacking the host's antiviral response. A study by Chaccour [34] reported that patients treated with a single 400 mcg/kg dose of ivermectin for mild COVID-19 showed a tendency to lower viral load and cough within 72h. They suggested that there could be a down-regulation of the ACE-2 receptor and viral entry into the cells of the respiratory epithelium and olfactory bulb. It could also result from inhibition of the activation of pro-inflammatory pathways in the olfactory epithelium. It has also been shown to inhibit the replication of SARS-CoV-2 in cell cultures [35].

The binding affinity of nafamostat was significantly greater than those of camostat and ivermectin on all the non-structural proteins apart from nsp10 that showed the best binding with ivermectin. This observation indicated that nafamostat would give the broadest spectrum of inhibitory action on these SARS-CoV-2 non-structural proteins but may however not be as efficient as ivermectin in preventing the replication of the virus. Ivermectin had the best inhibitory action on the spike glycoprotein and nsp10, which showed that it could prevent the penetration of the viral spike protein into the host and prevent the virus's replication.

A greater number of hydrogen bond interactions were found in the binding of nafamostat and camostat with the amino acid residues at the active sites of the proteins than what was observed with their interaction with ivermectin. This indicated that the drug-protein complexes formed by these two drugs would be more stable than those formed by ivermectin [36]. All the drugs interacted with Arg403 in the spike glycoprotein, showing that this amino acid is essential in inhibiting the action of this protein. Also, nafamostat and camostat interacted with Tyr495 and Thr500 in the spike glycoprotein, suggesting that their mechanism of action are similar.

All the drugs studied interacted with Pro248 and Tyr268 at the active site of nsp3. Nafamostat and camostat showed very similar mode of action at this site by their interaction with Gly163, Asp164, Arg166, Pro248, Tyr268, Gln269, and Glu167. Different amino acids interacted with the drugs at nsp5, indicating that their mechanisms of action at this protein site were not related.At nsp9 and nsp16, the mode of action of ivermectin was different from the other two drugs. Nafamostat and camostatinteracted withAla8, Leu9, Gln11, and Met101 at nsp9, and also with the amino acids Asp130, Gly71, Leu100, and Phe149 at nsp16,suggesting a similar mode of action by these two drugs at these sites. All the drugs interacted with ILE55 and VAL116 at nsp10, with nafamostat and camostat having a closer relationship in their mode of action by having additional interactions with Asp91 and Thr111. At nsp13, similarity in the interaction of all the drugs was observed at Lys288 and Asp374. The remaining interactions by all the drugs occurred at different amino acids in the protein.

CONCLUSION

The potentials of camostat, nafamostat, and ivermectin to inhibit the spike glycoprotein, nsp3, nsp5, nsp9, nsp10, nsp13, and nsp16 of SARS-CoV-2 were studied in silico. Nafamostat showed good binding affinity on all target proteins. However, ivermectin was better at binding with the spike glycoprotein and nsp10 than this drug. The mechanism of action of nafamostat and camostat on the studied proteins were very similar but varied markedly with ivermectin. The good binding affinity demonstrated by nafamostat at nsp3, nsp5, nsp9, nsp13, and nsp16 showed that it could influence multi-target interactions of the five proteins of the virus and hence curtail the infection. The potentials of nafamostat and ivermectin in SARS-CoV-2 prevention could therefore be explored for the possible production of a single compound that can inhibit spike glycoprotein and human ACE2 binding,

and interfere with the replication and transcription of coronaviruses in Homo sapiens when the infection has already occurred.

AUTHORS' CONTRIBUTIONS

CED: Conceptualization, Data curation, Supervision, Methodology, Software HIU: Conceptualization, Supervision, Methodology, Data curation, Software. IAD: Visualization, Investigation. UEE: Visualization, Investigation. LCN: Original draft preparation, Writing- Reviewing and Editing. CEE: Original draft preparation, Writing- Reviewing and Editing.

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All data generated or analyzed during this study are included in this published article.

DISCLOSURE STATEMENT

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REFERENCE

1. Shi Y., Y. Wang, C. Shao, J. Huang, J. Gan, X. Huang, E. Bucci, M. Piacentini, G. Ippolito, G. Melino, COVID-19 infection: the perspectives on immune responses, Cell Death Differ. (2020), https://doi.org/10.1038/s41418-020-0530-3
2. Faheem, Kumar BK, Sekhar KVGC, Kunjiappan S, Jamalis J, Balaña-Fouce R, Tekwani BL, Sankaranarayanan M. Druggable targets of SARS-CoV-2 and treatment opportunities for COVID-19. Bioorg Chem104:104269. https://doi.org/10.1016/j.bioorg.2020.104269
3. Yan R., ZhangY., Li Y., Xia, L.,Guo Y., Zhou Q. (2020). Structural basis for the recognition of SARS Cov-2 by full-lenght human ACE2. Science 367, 1444-1448.
4. Watanabe Y., Allen JD.,Wrapp O., Mclellan JS., Crispin M. (2020). Site-specific glycan analysis of the SARS-Cov-2 spike.Science. 2020.369;330-333.
5. Ma-Lauer Y, Carbajo -Lozoya J, Hein MY, Muller MA, Deng W, Lei J, Meyer B, Kusov Y, Von-Brun B, Bairad DR, Hunten S, Drosten C, Hermeking H, Leonhardt H, Mann M, Hilgenfield R (2016). Nsp3 down-regulates SARS corona virus replication and is targeted by the SARS-unique domain and PL^{pro} via E_3 ubiquitin ligase RCHY1. Proc. Natl. Acad. Sci. 113:E55192-E5201.
6. Wolff G., Melia CE., Snijder EJ., Barcena R. (2020).Double membrane vessicles as platforms for viral relication.Trends in Microbiology, https://doi.org/10.1016/j.tim.2020.05.009
7. Macchiagodena M., Pagliai M., Procacci P. (2020). Identification and potential binders of the main protease 3CL(pro) of the covid-19 via structure-based ligand design and molecular modeling. ChemPhysLett. 750:137489.
8. Durdagi S., Aksoydan B., Dogan B., Sahin K., Shahraki A. (2020). Sreening of clinically approved and investigation drugs as potential inhibitors of COVID-19 main protease: A virtual drug repurposing study. DOI:10.26434/chemrxiv.12032712.v1.

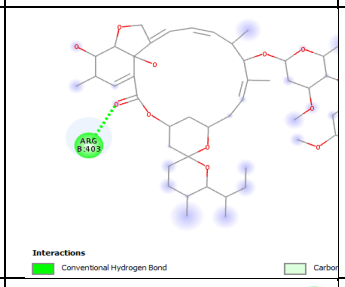
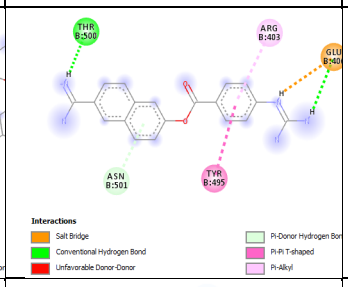
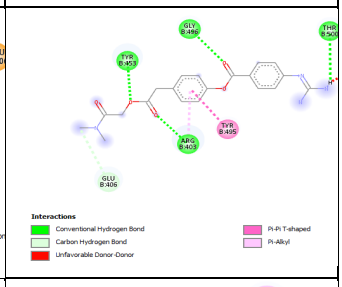
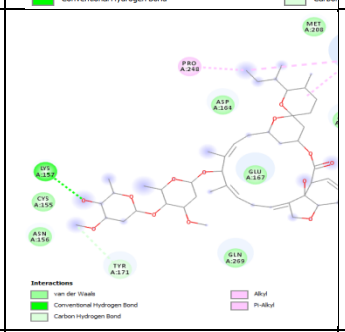
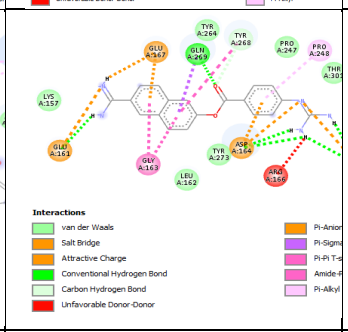
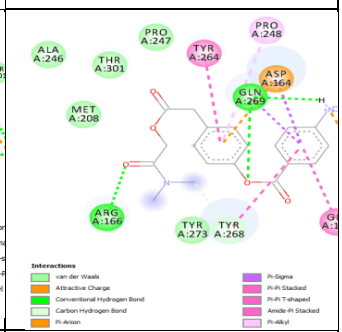
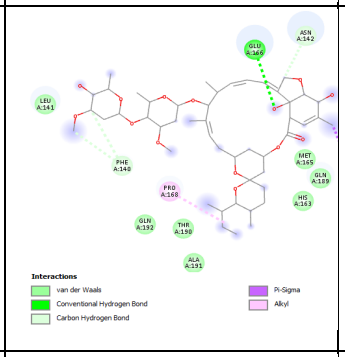
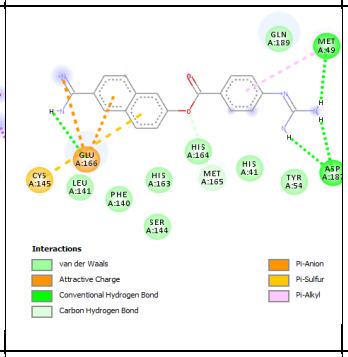
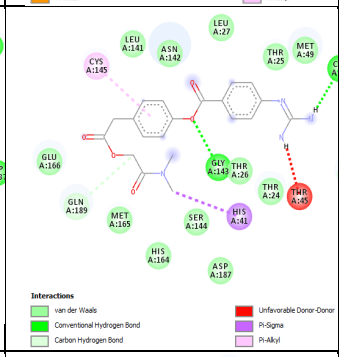
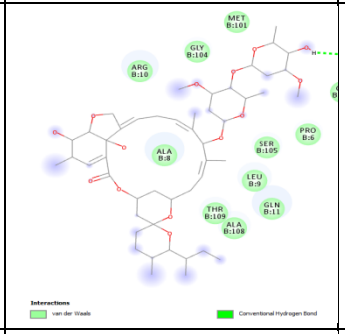
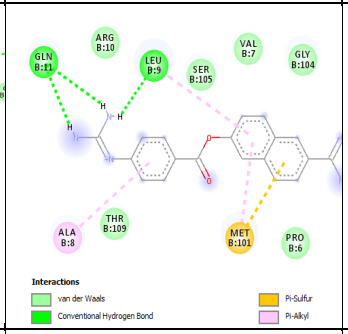
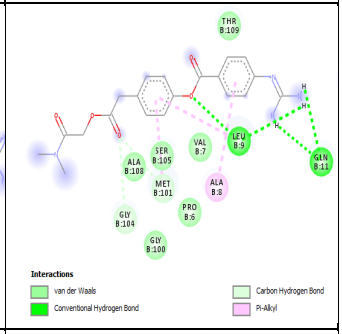
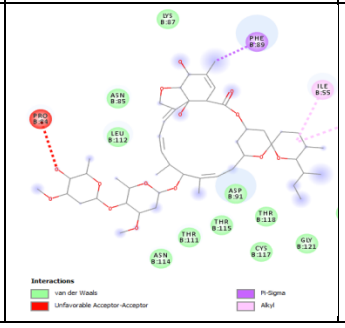
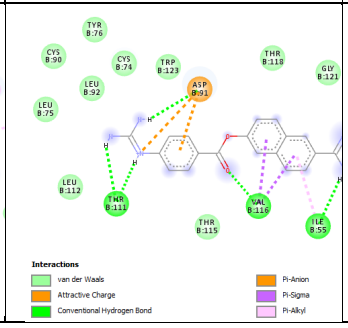
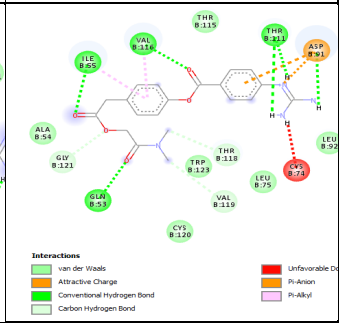
9. Zeng Z., Deng F., Shi K., Ye G., Wang G., Fang L. (2018). Dimerization of coronavirus nsp9 with diverse modes enhances its nucleic acid binding affinity. J Virol.92, e00692-18.
10. Ma T., Wu L., Shaw N., Gao Y., Wang J., Sun Y. (2015). Structural basis and functional analysis of the SARS-corona virus nsp14-nsp10 complex.ProcNatlAcad Sci, 112:9436-9441.
11. Rosas-Lemus M, G. Minasov, L. Shuvalova, N. L. Inniss, O. Kiryukhina, J. Brunzelle,nK. J. F. Satchell, High-resolution structures of the SARS-CoV-2 2′-*O*-methyltransferase reveal strategies for structure-based inhibitor design. Sci. Signal. 13, eabe1202 (2020).
12. Hao W., Wojdyla J.A., Zhao R., Han R., Das R., Zlatev I. (2017). Crystal structure of middle East respiratory syndrome coronavirus helicase. PLoSPathog 13, e1006474.
13. 13.Jia Z., Yan L., Ren Z., Wu L., Wang J., Guo J. (2019). Delicate structural coordination of the severe acute respiratory syndrome coronavirus nsp13 upon ATP hydrolysis.Nucleic acids Research, 47, 6538-6550.
14. Ramanathan A., Robb GB., Chan S.H. (2016). mRNA capping: Biological functions and applications. Nucleic acid Research. 44:7511-7526
15. Sawicki S.G., Sawicki D.L., Younker D., Meyer Y., Thiel V., Stokes H., Sidell S.G. (2005). Functional and genetic analysis of coronavirus replicase-transcriptase proteins.PLoSPathog. 1:e39.
16. Decroly E., Imbert I., Coutard B., Bouvet M., Selisko B., Alvarez K., Gorbalenya A.E., Snijder E.J., Canard B (2008). Corona virus nonstructural protein 16 is a Cap-0 binding enzyme possessing (Nucleoside-2'O)-methyltransferase. Activity J. Virol. 82: 8071-8084.
17. Snijder E.J., Decroly E.J., Zierbuhr J. (2016). The non structural proteins directing corona virus RNA synthesis and processing. Adv. Virus. Res. 96: 59-126.
18. Marcolino VA, Pimentel TC, Barão CE (2020). What to expect from different drugs used in the treatment of COVID-19: A study on applications and in vivo and in vitro results. European journal of pharmacology, 887, 173467.https://doi.org/10.1016/j.ejphar.2020.173467
19. Umar HI, Josiah SS, Saliu TP, Jimoh TO, Ajayi A, Danjuma JB.In-silico analysis of the inhibition of the SARS-CoV-2 main protease by some active compounds from select African plants. J TaibahUniv Medical Sci 2021; https://doi.org/10.1016/j.jtumed.2020.12.005
20. Duru CE, I.A. Duru, and A.E. Adegboyega (2021a). In Silico identification of compounds from *Nigella sativa* seed oil as potential inhibitors of SARS-CoV-2 targets. Bulletin of the National Research Centre, 45:57. https://doi.org/10.1186/s42269-021-00517-x
21. Duru C.E., Haruna Umar H.I., Duru I.A., Enenbeaku U.E., Ngozi-Olehi L.C., Enyoh C.E. (2021b). Blocking the interactions between human ACE2 and coronavirus spike glycoprotein by selected drugs: a computational perspective. Environmental Analysis Health and Toxicology, 36(2), e2021010. https://doi.org/10.5620/eaht.2021010
22. Stroganov OV, Novikov FN, Zeifman AA, Stroylov VS, Chilov GG. TSAR, a new graph-theoretical approach to computational modeling of protein side-chain flexibility: modeling of ionization properties of proteins. Proteins, 2011 79(9):2693-2710. https://doi.org/10.1002/prot.23099
23. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Computational Chemistry 2010; 31: 455–461.
24. Gao X, Qin B, Chen P, Zhu K, Hou P,Wojdyla JA, Wang M, Cui S.Crystal structure of SARS-CoV-2 papain-like protease. Acta PharmaceuticaSinica B 2021;11(1):237e245. https://doi.org/10.1016/j.apsb.2020.08.014

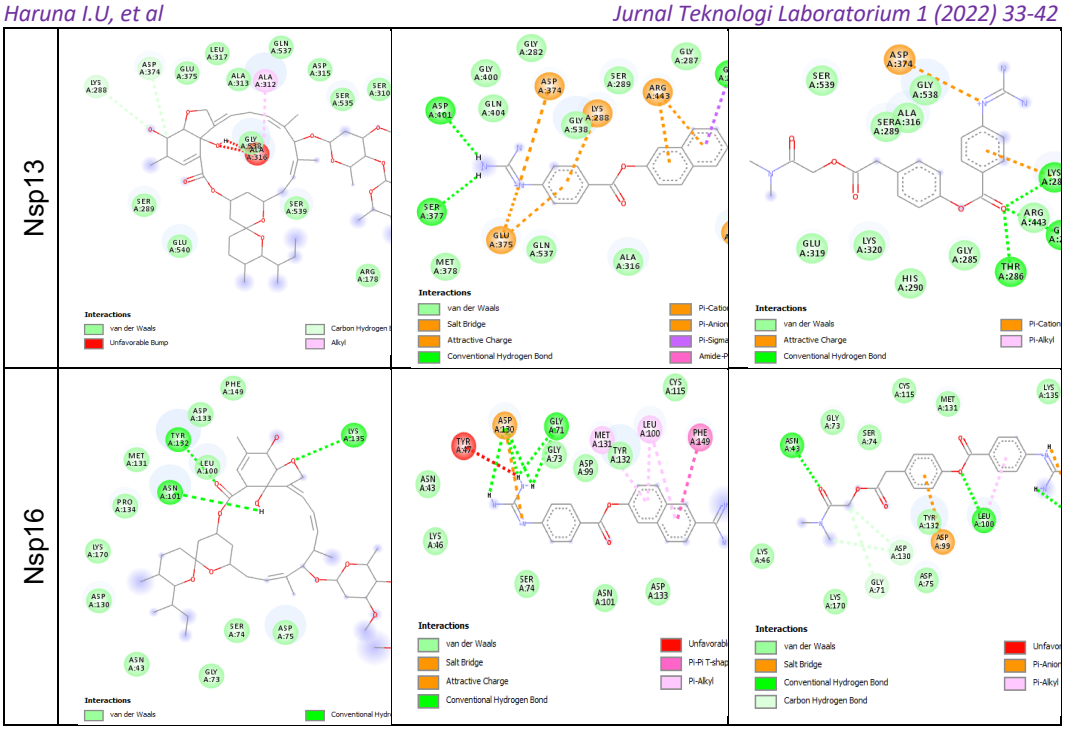
25. Jin Z, Du X, Xu Y, Deng Y, Liu M, Zhao Y (2020) Structure of M pro from SARS-CoV-2 and discovery of its inhibitors. Nature 582:1–24. http://dx.doi.org/10.1038/s41586-020-2223-y
26. Littler DR, Gully BS, Colson RN,Rossjohn J. Crystal structure of the SARS-CoV-2 non-structural protein 9, Nsp9. iScience 2020; 23:101258. https://doi.org/10.1016/j.isci.2020.101258
27. Rosas-Lemus M., Minasov G., Shuvalova L., Inniss N., Kiryukhina O., Wiersum G. (2020a). The crystal structure of nsp10-nsp16 heterodimer from SARS-Cov-2 in complex with S-adenosylmethionine. https://doi.org/10.1101/2020.04.17.047498
28. Chen J, Malone B, Llewellyn E, Grasso M, Shelton PMM, Olinares PDB, Maruthi K, Eng ET, Vatandaslar H, Chait BT, Kapoor TM, Darst SA, Campbell EA. Structural basis for helicase-polymerase coupling in the SARS-CoV-2 replication-transcription complex. Cell, 182(6):1560-1573. https://doi.org/10.1016/j.cell.2020.07.033
29. Krafcikova P, Silhan J, Nencka R, Boura E. Structural analysis of the SARS-CoV-2 methyltransferase complex involved in RNA cap creation bound to Sinefungin. Nature Communications 2020; 11:1-7. https://doi.org/10.1038/s41467-020-17495-9
30. Wang Q, Zhang Y, Wu L, Niu S, Song C, Zhang Z, Lu G, Qiao C, Hu Y, Yuen K, Wang Q, Zhou H, Yan J, Qi J. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. Cell, 2020; 181, 894–904. https://doi.org/10.1016/j.cell.2020.03.045
31. Breining P, Frølund AL, Højen JF, et al. Camostatmesylate against SARS-CoV-2 and COVID-19-Rationale, dosing and safety. Basic Clin. PharmacolToxicol. 2021, 128:204–212.
32. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell, 2020, 181(2):271-280.
33. Yamamoto M, Matsuyama S, Li X, Takeda M, Kawaguchi Y, Inoue J, Matsuda Z (2016). Identification of nafamostat as a potent inhibitor of Middle Eastrespiratory syndrome coronavirus S Protein-mediated membranefusion using the split-protein-based cell-cell fusion assay. Antimicrobial Agents and Chemotherapy, 60(11):6532-6537.
34. Chaccour C, Casellas A, Blanco-Di Matteo A, Pineda I, Fernandez-Montero A, Ruiz-Castillo P, Richardson MA, Rodríguez-Mateos M, Jordán-Iborra C, Brew J, Carmona-Torre F, Giráldez M, Laso E, Gabaldón-Figueira JC, Dobaño C, Moncunill G, Yuste JR, Del Pozo JL, Rabinovich NR, Schöning V, Hammann F, Reina G, Sadaba B, Fernández-Alonso M. The effect of early treatment with ivermectin on viral load, symptoms and humoral response in patients with non-severe COVID-19: A pilot, double-blind, placebo-controlled, randomized clinical trial. EClinicalMedicine, 2021, 32:100720.http://dx.doi.org/10.1016/j.eclinm.2020.100720
35. Caly L, Druce JD, Catton MG, Jans DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. Antiviral Res. 2020, 178:104787.
36. Duru CE, I.A. Duru, B.A.A. García, and U.E. Enenebeaku (2021c). Computational modeling of the activity of metronidazole against EhGα1 of *Entamoeba histolytica* enhanced by its copper and zinc complexes. Chemistry Africa. https://doi.org/10.1007/s42250-021-00245-9

Table 1: Grid box parameters and amino acids in the binding site of our selected protein receptors

S/N	Target Proteins	Center grid box (XYZ), Å	Dimension (XYZ), Å	Active site amino acid residues
1.	Nsp3 (PDB ID: 7CMD)	-22.808 × -16.359 × -19.925	33.668 × 29.582 × 31.718	Gly286, Trp106, Gly271, His73, Cys111, His272, Asp286,Arg140 and Asn109 (Gao et al, 2020)
2.	Nsp5 (PDB ID: 6LU7)	-14.85 × 14.923 × 69.59	25.02 × 27.98 × 30.87	Thr25, Cys44, Thr26, His41, Met49, Tyr54, Phe140, Leu141, Gly143, Cys145, Asn142, His163, His164, Met165, Ser144, Glu166, Pro168, His172, Val186, Asp187, Arg188, Gln189, Phe185, Thr190 and Gln192 (Jin et al., 2020).
3.	Nsp9 (PDB ID: 6M71)	38.221 × -15.213 × 14.62	16.848 × 25.00 × 19.496	Leu9, Ser105, Val7, Pro6, Tyr31, Leu106, Ala8, Met101, Leu103 and Ala107 (Littler et al., 2020)
4.	Nsp10 (PDB ID: 6YZ1)	67.062 × -19.238 × 9.243	28.835 × 37.509 × 35.771	Val42, Leu45, Lys93, Thr106, Ala107, asn40, Thr49, Cys120, Cys128, Cys130, Cys117, Cys74, Cys77, Cys90 and His83 (Rosas-Lemus *et al*.,2020b).
5.	Nsp13 (PDB ID: 6XEZ)	-13.877 × 14.581 × -74.112	20.448 × 20.567 × 25.581	Lys288, Ser289, Asp374, Glu375, Gln404 and Arg567 (Chen et al., 2020).
6.	Nsp 16 (PDB ID: 6YZ1)	83.813 × 16.651 × 25.451	20.977 × 26.335 × 20.158	Tyr47, Asn43, His69, Asp99, Asn101, Asp114, Asp130 and Lys170(Krafcikova et al., 2020)
7.	Spike protein (PDB ID: 6LZG)	-37.386 × 31.021 × 12.733	22.603 × 43.431 × 41.774	Trp353, Arg355, Lys417, Gly446, Tyr449, Tyr453, Ala475, Gln484, Phe486, Thr478, Tyr489, Gly496, Gln498, Thr500 and Gly502 (Wang et al., 2020)

Table 3. Drug interactions with amino acids at the active sites of the SARS-CoV-2 proteins

Protein	Protein-Ligand interactions		
	Ivermectin	Nafamostat	Camostat
Spike			
Nsp3			
Nsp5			
Nsp9			
Nsp10			


Nsp13
Nsp16
Interactions
van der Waals
Unfavorable Bump
Carbon Hydrogen
Alkyl
Salt Bridge
Attractive Charge
Conventional Hydrogen Bond
Pi-Cation
Pi-Alkyl
Unfavorable
Pi-Pi T-shap
Carbon Hydrogen Bond

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Original Research

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Antibacterial activities of bacteria asssociated with Marine sponges of *Axinella sp.* on Carbapenem-Resistant *Acinetobacter Baumannii* (CRAB)

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Abstract: *Carbapenem-resistant Acinetobacter baumannii* (CRAB) is a gram-negative bacilli that commonly causes nosocomial infection found in Indonesia. CRAB infection caused by *Acinetobacter baumannii* that is resistant to Carbapenem. Resistance occurred because bacteria that cause infections easily treated with antibiotics become difficult to treat due to the uncontrolled use of antibiotics. *A. baumannii* has been resistant against the carbapenem class of antibiotics; and therefore, antibiotics are required to be obtained from natural ingredients with optimal working power, such as from the marine sponges of *Axinella sp*. The purpose of this study was to determine the antibacterial activities of bacteria associated with marine sponges of *Axinella sp.* against CRAB. Methods: bacteria associated with *Axinella sp.* were isolated by differential dilution and cultures on Zobell 2216E media. Antibacterial activity test was performed using the overlay method. The antibacterial activity test was carried out to determine the presence of inhibition zones. The test results showed the bacteria associated with a marine sponge of *Axinella sp* strain 3 KD had antibacterial activity against CRAB growth with the formation of an inhibition zone of 16 mm. The results of the catalase test and oxidase test depicted that the isolates belong to the family of *Staphylococcaceae*. Conclusion: isolates bacteria associated with a marine sponge of *Axinella sp*. were potential antibacterial agents against CRAB growth.
Keywords: CRAB; Antibacterial activity; *Axinella sp*. isolate; overlay method; *Staphylococcaceae*

INTRODUCTION

Nosocomial infections are defined as infections found in hospitals that attack patients and appear within 48-72 hours after treatments[1]. Microorganisms can live and reproduce in the hospital environment, on either the floor or medical and non-medical equipment[2]. In Indonesia, nosocomial infections caused by *A. Baumannii* reach 25.8%, one of the causes of nosocomial infection worldwide and the highest mortality rate worldwide can reach 52%[3].

Manifestations of nosocomial infections attributed to *A. baumannii* include pneumonia, secondary meningitis, bloodstream site infection (BSI), and urinary tract infection (UTI)[4]. Some strains of *A. baumannii* are highly resistant to antibiotics[5]. Inappropriate use of antibiotics can cause antibiotic resistance because infectious bacteria become difficult to treat[6]. Antibacterial therapy regimens currently considered to be potent in treating *A. baumannii*-related infection are carbapenem, sulbactam (with a beta-lactamase-inhibitor), and

colistin[7]. *A. baumannii* is resistant to carbapenemase antibiotics (Carbapenem-resistant *Acinetobacter baumannii* (CRAB)). Carbapenem is the main antibiotic used for treating patients with *A. baumannii*-related infection, causing resistance to many carbapenem antibiotics[8]. Antibacterial agents derived from biological sources include lactic acid bacteria[9], mushrooms[10,11], latex , fruits[12,13], and seeds[14]. Bioactive compounds from marine sources have been widely studied in previous studies. Currently, there are many studies examining bioactive compounds from marine resources.

Indonesia is one of the countries with a lot of potential marine resources; therefore, it is necessary to develop research on marine life to look for bioactive compounds with the ability as an effective antibacterial and few side effects[15]. Many researchers were interested in conducting studies on antibacterial from marine sponges[16].

Several studies have been reported on the antibacterial activity of microbes associated with marine sponge *Axinella* sp. Fungi associated with the marine sponge *Axinella* sp have potential as an antibacterial against *Staphylococcus aureus* and *Escherichia coli*[17]. Another study reported that bacteria associated with the fungus *Axinella* sp from the Mediterranean Sea had antibacterial activity against several non-resistant pathogenic bacteria[18]. There have been no reports of bacteria associated with sponge *Axinella* sp to CRAB. To minimize the research gap, the aim of this study was to evaluate the anti-CRAB potential of bacteria associated with *Axinella* sp.

MATERIAL AND METHOD

Tools and Materials

The tools used in the research to test the antibacterial activity were autoclave (HMC Hirayama HICLAVE HVE-50), incubator (WTC Binder), laminar airflow cabinet (Labcono Purifier Class II Biosafety Cabinet), refrigerator (Sharp), petri dish, inoculation loop and needle, alcohol lamp, test tubes, and test tube racks.
The materials used in this study were Zobell 2216E media, MHB (Mueller Hinton Broth) antibacterial activity test media, MC media (Mac Conkey), BHI (Brain Heart Infusion), Standard Mc Farland, and physiological NaCl.

Tested microorganisms

CRAB was isolated from wound sample collected from patients suffering from diabetes in RS. Dr. Kariadi Semarang, Central Java, Indonesia. Isolates were identified using MacConkey Agar (MCA) media, as well as biochemical tests with Vitek®MS and bacterial sensitivity tests using the Clinical Laboratory Standard Institute M100-S25 for minimum inhibitory concentration(MIC) interpretation (CLSI2019).

Isolation of bacteria from *Axinella sp*.

Axinella sp. sponges were obtained from the waters of Tanjung Gelam Beach, Karimunjawa Islands, Jepara, Central Java, with a sampling technique at a depth of 0.5-1m. *Axinella* sp has been isolated from marine and fishery laboratory Universitas Diponegoro, Semarang. Then, isolation was performed by weighing ± 1 gram cleaned with sterile seawater, mashed, carried out a differential dilution of 10 to 10 4 then spread on Zobell 2216E media, incubated at a temperature of 35 ± 2 ° C for 48 hours, and then observed the incubation results. The Gram staining, catalase, and oxidase tests were used to know the bacterial family.

Antibacterial Activity Test

The marine sponge-associated isolates were screened, using agar overlay assays[19] for antibacterial activity against CRAB. Pure isolates bacteria associated with sponge *Axinella* sp were grown on Zobell 2216E media by spotting them on the media and forming small spheres with a diameter of ± 2 mm. The isolates were incubated at 35 ± 2°C for 48 hours and results were observed. After that, a suspension of the test bacteria was made. 1 ml physiological NaCl was taken and put into soft agar media at the temperature of ± 40°C. It was homogenized using a

vortex and once homogeneous, it was poured into Zobell media containing pure isolates of bacteria from *Axinella sp* sponges in the form of spheres with a diameter of ± 2 mm. Then, it was incubated at 35 ± 2°C for 48 hours and the formation of an inhibition zone was observed.

RESULTS AND DISCUSSION

CRAB isolates from wound infections

CRAB was isolated from wound samples. The result of the sensitivity test of bacteria to antibiotics showed that *A. baumanii* from wound patients was a CRAB stain because it was resistant to Carbapenem (Meropenem) besides resistant to Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Amikacin Gentamicin, Ciprofloxacine, Tigecycline, Nitrofurantoin, Sulfamethoxazole showed that *A. baumanii* not only included in the CRAB strain but also in the Multidrug-Resistant Bacteria (MDR).

Isolation of *Axinella sp.* Associated Bacteria

The isolation of bacteria from *Axinella sp* produced eight bacterial isolates, coded with 1 KA, 1 KB, 2 KA, 3 KA, 3 KB, 3 KC, 3 KD, and 4 KA.

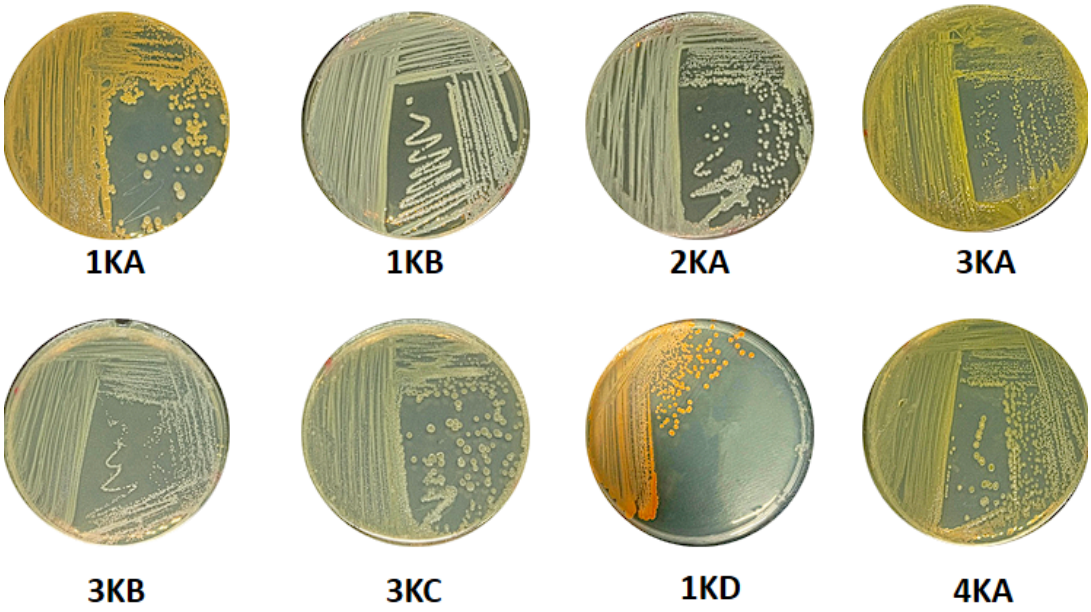

Figure 1. The results of pure bacterial isolation from *Axinella sp*

Table 1. Macroscopic Characteristics of Bacteria Isolates from *Axinella sp*.

No.	Code of Bacterial Isolate	Characteristic of Colony			
		Shape	Edge	Surface	Color
1.	1 KA	Round	Entire	Flat	Yellow
2.	1 KB	Round	Entire	Convex	White
3.	2 KA	Round	Entire	Pulvinate	White
4.	3 KA	Round	Entire	Convex	Yellow
5.	3 KB	Round	Entire	Raised	White
6.	3 KC	Round	Entire	Raised	White
7.	3 KD	Round	Entire	Convex	Orange
8.	4 KA	Round	Entire	Raised	Yellow

The isolation of symbiont bacteria from *Axinella sp*. produced eight bacterial isolates with various characteristics. According to Dwijoseputro[20], macroscopic observations of colony morphology are: from above, the shape of the colony is dominantly round; from the side, the most dominant types of colony surface are flat, convex, pulvinate, and raised; from above, the colony edge is

entire; and the most dominant colors are yellow, white, and orange. Gram staining was performed on the pure isolates and microscopic observation was carried out to identify the cell shapes. Gram-positive bacteria were purple while gram-negative bacteria were pink.

Table 2. Macroscopic Characteristics of Bacteria Isolates from *Axinella sp*. with Gram Staining

No.	Code of Bacterial Isolate	Gram	Cell Shape
1.	1 K A	-	Bacillus
2.	1 K B	-	Bacillus
3.	2 K A	+	Bacillus
4.	3 K A	-	Coccus
5.	3 K B	-	Bacillus
6.	3 K C	-	Coccus
7.	3 K D	+	Coccus
8.	4 K A	-	Bacillus

Table 2 presents that the bacteria in the eight isolates were grouped into two, including two gram-positive bacterial isolates (2 K A) and six gram-negative bacterial isolates (1 K A, 1 K B, 3 K A, 3 K B, 3 K C and 4 K A). The microscopic observation identified coccus bacteria in two isolates and bacillus bacteria in six isolates.

The Results of Inhibitory Test

The inhibitory test showed the formation of an inhibition zone, producing a clear zone around the colony. The activities on the inhibition zone are presented in Table 3.

Table 3. The Results of Antibacterial Activity Test of Bacterial Isolates from *Axinella sp* on CRAB

No.	Codes of Bacterial Isolates	Inhibition zone (mm)
1.	1 K A	0
2.	1 K B	0
3.	2 K A	0
4.	3 K A	0
5.	3 K B	0
6.	3 K C	0
7.	3 K D	16
8.	4 K A	0

Table 3 details that the isolates of symbiont bacteria from *Axinella sp* coded 3 K D could form a clear zone around the colony with an average diameter of 16 mm.

Table 4. The Results of Antibiotic Control Test on CRAB

Antibiotic Control	Inhibitor Zone Diameter (mm)
Gentamicin	23
Ampicillin	25
Meropenem	0

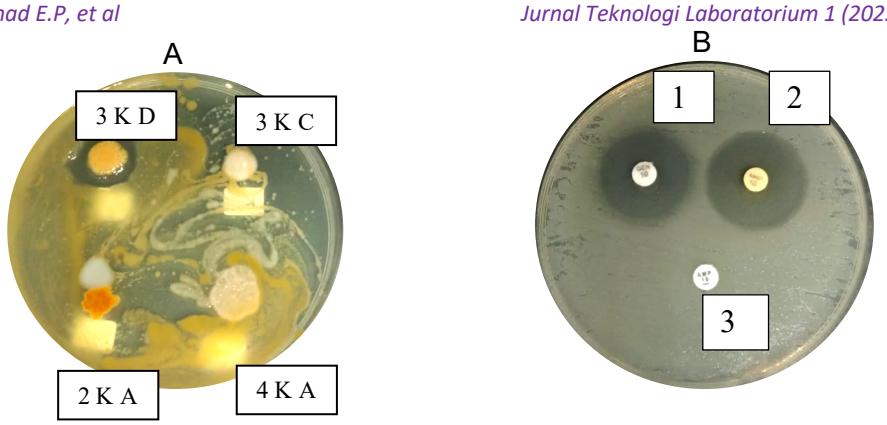

Figure 2. Inhibitory zone of (A) Isolates from *Axinella sp* Code P3-K9, (B) Antibiotics: 1. Gentamicin., 2. Ampicilin., 3. Meropenem

The results of the inhibitory test on isolates from *Axinella sp* (code: 3 K D) on CRAB, as presented in Table 4 and Figure 2, showed a clear zone around the colony with a diameter of 16 mm. The catalase test was carried out on the 3 K D isolate to determine its ability to produce catalase enzyme and its tolerance to oxygen. The catalase test was used to identify groups of catalase-producing bacteria. This test was performed to distinguish catalase-positive members of the *Micrococcaceae* or *Staphylococcaceae* from catalase-negative *Streptococccaceae*[21]. Meanwhile, the oxidase test was conducted to identify bacteria containing cytochrome c oxidase enzyme that could oxidize the reducing agents in the form of tetramethyl p-phenylenediamine (Kovac's reagent) or dimethyl p-phenylenediamine (Gordon and McLeod's reagent)[21]. The catalase test showed a positive result, indicated by the presence of foam or bubbles. Meanwhile, the oxidase test yielded a negative result with no blue-purple color change in the oxidase paper.

In this study, yellow marine sponges of *Axinella sp*. obtained from the waters of Tanjung Gelam Beach, Karimunjawa Islands, Jepara, Central Java. CRAB pathogenic bacteria causing nosocomial infections that were resistant to carbapenem antibiotics were also utilized. This study showed that one of eight isolates of *Axinella sp*. associated bacteria (code: 3 K D) have an antibacterial activity to inhibit CRAB bacteria by forming a clear zone around the colony. The previous studies explained that the *Axinella sp* contained bioactive compounds, including alkaloids and steroids[22]. Although identifications of compounds in *Axinella sp*. and the antibacterial activity of alkaloids and steroids were not performed in this study, another studies concluded that alkaloids could interfere with the peptidoglycan constituent components of bacterial cells so that the cell wall layer was not naturally formed, destroying the cytoplasmic membrane and bacterial cell wall[23]. The cell membrane turned into a fragile and lysed cell[24]. Bacterial cell walls were damaged, causing the disruption of bacterial cell metabolism and resulting in death[25]. Osmotic pressure happened and the cell walls became lysis, causing bacteria to die[26]. The results of the study showed that the *Axinella sp*. isolate (code: 3 K D) produced an inhibition zone of 16 mm.

The association process of marine sponges with their symbiotic bacteria began with the presence of bacteria in the waters of the surrounding environment[27]. The sponge will absorb specific bacteria through the sponge during filter-feeding and by vertical transmission of symbionts through the gametes of the sponge by the inclusion of the bacteria in the oocytes or larvae[28,29]. Sponges of the *Axinella* genus are known as producers of achemically diverse metabolites with excellent bioactive activity[30]. The metabolite compounds contained in the *Axinella sp*. sponge could kill or inhibit the growth of pathogenic bacteria[31]. According to Gunathilaka research, the bioactive compound from marine associated bacteria

has the potential to provide future drugs against sicknesses such as cancer, malaria, and inflammation[32]. Thus, bacteria with metabolites and bioactive compounds can perform as antibacterial agents.

The metabolites contained in the marine sponges of *Axinella sp*. are closely related to the metabolite compounds synthesized by the symbiotic microorganisms[33]. One of the isolates of the symbiotic bacteria of *Axinella sp*. is the potential to produce antibacterial compounds[34]. According to Konig research, several studies have reported that many bioactive compounds from marine biota are similar to the bioactive compounds from microorganism associated with these marine biotas[35]. They are true producers of bioactive compounds. Study by Gultom explained that isolates of bacteria associated with marine sponge *Axinella sp* contained alkaloid against *Staphylococcus aureus* and *Escherichia coli*[36]. According to Januar research that has found that the three compounds were hymeniadisine, 3-bromohymenialdiside, and dibromophakelin[37]. Zhang has been identified by detailed spectroscopic analysis reported 14-0-sulfate massadine has enhanced chemical stability that potential as antibacterial properties[38]. According to the research by Rastina, the ability of an antimicrobial to inhibit microbes depends on the concentration of antimicrobial material and the type of antimicrobial material produced[39]. The greater the concentration of an antimicrobial, the higher the concentration of bioactive substances, and thus, the inhibition will be higher, as indicated by an increase in the formed clear zone.

The antibacterial levels are divided into four categories: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20 mm). The identification of marine microbial isolates associated with *Axinella sp*. included morphological observations, gram staining, catalase test, and oxidase test[40]. According to Henstsel, various types of microorganisms have been found in sponges, such as symbiotic bacteria, which are members of *Bacillus sp*., *Staphylococcus sp.,* and *Vibrio sp*[41]*.* The most common *Axinella sp*. symbiotic bacteria found were *Pseudomonas sp*., *Bacillus sp.*, *Vibrio sp*., and *Staphylococcus sp.* groups[33].

The antibacterial activity test confirmed the formation of a 16 mm inhibitory zone, signifying that symbiont bacterial isolates have a strong inhibitory power. Further catalase test to identify the isolates of *Axinella sp.* symbiotic bacteria showed a positive result, while oxidase test yielded a negative result, indicating that the *Axinella sp*. 3 K D symbiotic bacterial isolates belonged to the catalase-positive members of *Staphylococcaceae*.

CONCLUSION

This research concluded that 3 K D bacterial isolates associated with *Axinella* sp. Are potential to be developed as antibacterial agents against the growth of CRAB, indicated by the formation of a clear inhibition zone around the colony with an average diameter of 16 mm. The bacteria are members of *Staphylococcaceae*. Further studies are recommended to be performed by incorporating different strains of pathogenic bacteria using *Axinella sp*. bacteria isolates and the overlay antibacterial test method. During the present research process, some obstacles occurred, such as uneven distribution of pathogenic test bacteria on the media. Therefore, proper homogenization with a vortex is required in further study. Moreover, media contamination also happened; and thus, it is recommended to use a biosafety cabinet to reduce the risk of contamination to obtain accurate and optimal results.

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All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

REFERENCE

1. Rezai MS, MD BN, M., Nikkhah A. Catheter-related urinary nosocomial infections in intensive care units: An epidemiologic study in North of Iran. *Casp J Intern Med Spring*. 8(2):76-82. doi:10.22088/cjim.8.2.76
2. Tombokan C, Waworuntu O, Buntuan V. Potensi penyebaran infeksi nosokomial di ruangan instalasi rawat inap khusus tuberkulosis (irina c5) BLU Rsup Prof. *Dr R D*. https://doi.org. 10.35790/ebm.v4i1.11247
3. Bulens SN, Yi SH, Walters MS. Carbapenems Nonsusceptible Acinetobacter baumannii, 8 US Metropolitan Areas, 2012-2015. *Emerg Infect Dis Wwwcdcgoveid*. 24(4).
4. Baran G, Erbay A, Bodur H, et al. Risk factors for nosocomial imipenem-resistant Acinetobacter baumannii infections. *Int J Infect Dis*. 2008;12(1):16-21. doi:10.1016/j.ijid.2007.03.005
5. Lin MF. Antimicrobial resistance in Acinetobacter baumannii : From bench to bedside. *World J Clin Cases*. 2014;2(12):787. doi:10.12998/wjcc.v2.i12.787
6. W.H.O. Combat Antimicrobial Resistance. *Available*. http://www.who.int/world-healthday/2011/en/
7. Luna CM, Aruj PK. Nosocomial Acinobacter pneumonia. *Respirology*. 12:787-791.
8. Soekiman S. *Infeksi Nosokomial Di Rumah Sakit*. CV sagung seto
9. Lestari SD, Sadiq ALO, Safitri WA, Dewi SS, Prastiyanto ME. The antibacterial activities of bacteriocin Pediococcus acidilactici of breast milk isolate to against methicillin-resistant Staphylococcus aureus. *J Phys Conf Ser*. 2019;1374(1):012021. doi:10.1088/1742-6596/1374/1/012021
10. Prastiyanto ME, Darmwati S, Iswara A, Setyaningtyas A, Trisnawati L, Syafira A. Antimicrobial Activity and Identification The Compounds of Methanol Extract from The Pleurotus Ostreatus Fruiting Body. *El-Hayah*. 2017;6(1):29. doi:10.18860/elha.v6i1.4082
11. Prastiyanto ME, Rukmana RM, Saraswati DK, S. D, Maharani ETW, Tursinawati Y. Anticancer potential of methanolic.
12. Prastiyanto ME, Wardoyo FA, Wilson W, Darmawati S. Antibacterial Activity of Various Extracts of Averrhoa bilimbi against Multidrug Resistant Bacteria. *Biosaintifika J Biol Biol Educ*. 2020;12(2):163-168. doi:10.15294/biosaintifika.v12i2.23600
13. Prastiyanto ME, Tama PD, Ananda N, Wilson W, Mukaromah AH. Antibacterial Potential of Jatropha sp. Latex against Multidrug-Resistant Bacteria. *Int J Microbiol*. 2020;2020:1-6. doi:10.1155/2020/8509650
14. Prastiyanto ME, Azizah IH, Haqi HD, et al. In-vitro antibacterial activity of the seed extract of three-member Artocarpus towards Methicillin-Resistant Staphylococcus aureus (MRSA). *J Teknol Lab*. 2020;9(2):128-135. doi:10.29238/teknolabjournal.v9i2.237

15. Rachmaniar R. Spons Indonesia Kawasan Timur Keragaman, Distribusi, Kelimpahan, dan Kandungan Metabolit Sekundernya. *Oseanologi Dan Limnol Indones*. 33(1):123-138.
16. Anjum K, Abbas SQ, Shah SAA, Akhter N, Batool S, Ul Hassan SS. Marine Sponges as a Drug Treasure. *Biomol Ther*. 2016;24(4):347-362. doi:10.4062/biomolther.2016.067
17. Trianto A, Widyaningsih S, Radjasa O, Pribadi R. Symbiotic Fungus of Marine Sponge Axinella sp. Producing Antibacterial Agent. *IOP Conf Ser Earth Environ Sci*. 2017;55(1):012005. doi:10.1088/1755-1315/55/1/012005
18. Haber M, Ilan M. Diversity and antibacterial activity of bacteria cultured from Mediterranean Axinella spp. *Sponges J Appl Microbiol*. 116(ue 3):519-532.
19. Matobole R, van Zyl L, Parker-Nance S, Davies-Coleman M, Trindade M. Antibacterial Activities of Bacteria Isolated from the Marine Sponges Isodictya compressa and Higginsia bidentifera Collected from Algoa Bay, South Africa. *Mar Drugs*. 2017;15(2):47. doi:10.3390/md15020047
20. Dwijoseputro. *Dasar-Dasar Mikrobiologi*. Djambatan
21. Leboffe MJ, Perce BE. *A Photographic Atlas for The Microbiology Laboratory*. 4Th ed. Morton Publishing Company. The USA
22. Heydari H, Gozcelioglu B, Konuklugil B. Biodiversity and Secondary Metabolites of Marine Sponges from Turkey. *ACG Publ Rec Nat Prod*. 13(5):367-4378.
23. Yan Y, Li X, Zhang C, Lv L, Gao B, Li M. Research Progress on Antibacterial Activities and Mechanisms of Natural Alkaloids. *Rev Antibiot*. 10:318. doi:10.3390/antibiotics
24. Ahmed B. *Chemistry of Natural Products*. Department of Pharmaceutical Chemistry of Science. Jamia Hamdard
25. Nuria MC, Faizatun. A, Sumantri. Uji Antibakteri Ekstrak Etanol Daun Jarak Pagar (Jatropha cuircas L) terhadap Bakteri Staphylococcus aureus ATCC 25923, Escherichiacoli ATCC 25922, dan Salmonella typhi ATCC 1408. *J Ilmu – Ilmu Pertan*. 5:26-37.
26. Sari FP, Sari SM. *Ekstraksi Zat Aktif Antimikroba Dari Tanaman Yodium (Jatropha Multifida Linn) Sebagai Bahan Baku Alternatif Antibiotik Alami*. Universitas Diponegoro
27. Brinkmann C, Marker A, Kurtböke D. An Overview on Marine Sponge-Symbiotic Bacteria as Unexhausted Sources for Natural Product Discovery. *Diversity*. 2017;9(4):40. doi:10.3390/d9040040
28. Radjasa OK, Martens T, Grossart H, Brinkhoff T, Sabdono A, Simmon M. Antagonistic activity of a marine bacterium Pseudoalteromonas luteoviolacea TAB4.2 associated with coral Acropora sp.
29. Thomas TRA, Kavlekar DP, LokaBharathi PA. Marine Drugs from Sponge-Microbe Association—A Review. *Mar Drugs*. 2010;8(4):1417-1468. doi:10.3390/md8041417
30. Mander L, Liu HW, LK TE, A K, I N, Y R. Comprehensive Natural Products II: Chemistry and Biology. *Elsevier Amst Neth*. 2009;3(11):838-43:345-350.
31. Manivasagan P, Venkatesan J, Sivakumar K, Kim SK. Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiol Res*. 2014;169(4):262-278. doi:10.1016/j.micres.2013.07.014
32. Gunathilake V. Arine bacteria and fungi as sources for bioactive compounds: present Status and future trends. *Int J Adv Res IJAR Int J Adv Res*. 5(9):610-614.
33. Lee KY, Lee HJ, Lee HK. Microbial Symbiosis in Marine Sponges. *J Microbiol*. 29(4. P):254-264.
34. Menna M, Aiello A, D'Aniello F, et al. Further Investigation of the Mediterranean Sponge Axinella polypoides: Isolation of a New Cyclonucleoside and a New Betaine. *Mar Drugs*. 2012;10(12):2509-2518. doi:10.3390/md10112509

35. König GM, Kehraus S, Seibert SF, Abdel-Lateff A, Müller D. Natural Products from Marine Organisms and Their Associated Microbes. *ChemBioChem*. 2006;7(2):229-238. doi:10.1002/cbic.200500087
36. Gultom ES, Suryanto D, Munir E, Diningrat DS. Bacteria extraxt activity associated with sponges Haliclona sp.2 and Axinellid sp as antibacterial. *Int J Adv Res IJAR Int J Adv Res*. 5(9):610-614. doi:10.21474/IJAR/2810
37. Januar HI, Motti C, Tapiolas D, Wright AD. Analisis Dereplikasi Untuk Identifikasi Senyawa Antibakteri Sponge Axinella Sp. Dari Perairan Kepulauan Karimunjawa. *J Pascapanen Dan Bioteknol Kelaut Dan Perikan*. 2009;4(1):79. doi:10.15578/jpbkp.v4i1.439
38. Zhang H, Khalil Z, Conte MM, Plisson F, Capon RJ. A search for kinase inhibitors and antibacterial agents: bromopyrrolo-2-aminoimidazoles from a deep-water Great Australian Bight sponge, Axinella sp. *Tetrahedron Lett*. 2012;53(29):3784-3787. doi:10.1016/j.tetlet.2012.05.051
39. R R, Sudarwanto M, Wientarsih I. Aktivitas Antibakteri Ekstrak Etanol Daun Kari (Murraya Koenigii) Terhadap Staphylococcus aureus, Escherichia coli, dan Pseudomonas sp. *J Kedokt Hewan - Indones J Vet Sci*. 2015;9(2). doi:10.21157/j.ked.hewan.v9i2.2842
40. Jackson EN, Lily Y. 2001. Ecology and industrial microbiology learning and earning from diversity. *J Curr Opin Microbiol Editor Overv*. 4:281-284.
41. Henstchel U, Michel S, Michael W, Lars F, Christine G, Jorg H. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges Aplysina aerophobia and Aplysina cavernicola. *FEMS Microbiol Ecol*. 35:305-3