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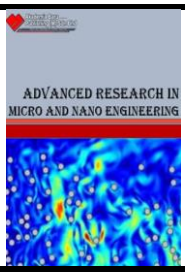

Prone-to-Infectivity of Omicron BA.2 Subvariant from Indonesia on ACE-2 Expressing Cell Lines

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ABSTRACT

SARS-CoV-2 virus undergoes mutation, leading to the virus's evolution and modifications in the characteristics of the virus. Omicron, including BA.2 subvariant, is currently the predominant variant in SARS-CoV-2 infection. There are no reports regarding its properties or the utilization of BA.2 Indonesian isolate for therapy and vaccine development. Therefore, this study evaluated appropriate host cells for Omicron BA.2 Indonesian isolate via susceptibility tests. The Omicron BA.2 from Indonesia was exposed to three mammalian-ACE2-expressing cell lines. Sharing amino acids between BA.2 from Indonesia and previous VOC Omicron subvariants was performed using a simple *in silico* comparison method. The results showed that the virus could not infect HepG2 and Huh-7D12 due to no foci forming on those cell lines. Moreover, we also found that BA.2 Indonesian isolate has a unique amino acid alteration on spike protein. According to the findings, the Omicron BA.2 from Indonesia could propagate on Vero E6 cell lines, and the mutations could play a role in the virus's changing infection mechanism. A deeper *in vitro* and *in silico* experiment could enhance the findings by comparing all BA.2 sequences from Indonesia and analyzing the infection mechanism by each single mutation using pseudovirus.

1. Introduction

A global health emergency has arisen as a result of SARS-CoV-2 virus infection. From 2020 to 2023, the world witnessed a high mortality rate, prompting the implementation of various strategies to combat the outbreak. These global initiatives, including social distancing, contact tracing, and a vaccine campaign, have been crucial in combating the COVID-19 pandemic. However, the disease's

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global impact underscores the need for further research and understanding of the virus [1,2]. Similar strategies were also implemented in Indonesia, a country that experienced a significant surge in SARS-CoV-2 infections and mortality rates. However, the effectiveness of these measures was hampered by the spread of false information and hoaxes, leading to a significant number of people not adhering to the policies. This non-compliance has played a role in the high prevalence and fatality rate of COVID-19 patients in Indonesia, underscoring the urgent need for more effective strategies and interventions.

Currently, there has been a global decline in the number of COVID-19 cases, and the pandemic has been officially proclaimed to be over. SARS-CoV-2 infection rates persist, particularly in Indonesia. By the end of 2023, Indonesia ranks second in terms of the highest number of COVID-19 cases in Southeast Asia [3]. COVID-19 incidence is closely associated with the SARS-CoV-2 viral mutation.

SARS-CoV-2 will evolve by occurring mutation as their adaptive mechanism [4]. SARS-CoV-2 has a lower mutation rate than HIV, Influenza virus, and Hepatitis C virus [5]. Though more than seven variations of SARS-CoV-2 have been identified since 2020, only roughly five of these are classified as variants of concern: Alpha (B.1.17), Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1), and Omicron. Because of their ability to elude neutralizing antibodies and relatively high infectivity, these variations are classified as such by the WHO [6].

November 2021 marked the discovery of Omicron (B.1.1.529) in South Africa which has become a dominant variant for SARS-CoV-2 infection globally [7,8]. Since then, several subvariants have been identified, including BA.1, BA.2, BA.3, BA.4, and BA.5. It indicates SARS-CoV-2 is rapidly mutating [9,10]. Comparing the mutations on Wuhan-Hu1, 50 amino acid alteration was experienced by Omicron, and most mutations occurred on spike protein. The mutations on most amino acids causing SARS-CoV-2 can evade neutralizing antibodies produced by vaccination, natural infection by previous variants, and antibody-based therapies [10]. Interestingly, Omicron is less pathogenic than previous variants, especially Delta [11]. Omicron BA.2 has been identified as a highly pathogenic Omicron subvariant because BA.2 was more resilient to neutralizing antibodies [12,13]. Additionally, it was stated that BA.2 is more pathogenic than BA.1 [14].

Previously, Indonesia was reported to have the most significant number of COVID-19 cases in South East Asia. Unfortunately, there are not many studies have been reported that use live virus to develop COVID-19 therapies and vaccines *in vitro* or *in vivo*. Multiple SARS-CoV-2 variants, specifically B.1.470, B.1.466.2, and AY.23, were shown to be the most prevalent during the initial and subsequent waves of COVID-19 in Indonesia. Of the three variants, only B.1.470 has been used to produce COVID-19 therapy.

At present, Omicron also dominates the COVID-19 infection in Indonesia. Omicron BA.1 was reported in Indonesia in December 2021. After identifying BA.1, another subvariant, BA.2, was discovered in Yogyakarta in January 2022. The incidence of Omicron BA.2 infection is 50% more than that of BA.1 infection. Our study is the first study about BA.2 Indonesian isolate. This study aims to determine the cell lines of ACE-2 expression to propagate the SARS-CoV-2 virus variant Omicron BA.2 from Indonesia. We compared three types of epithelial cell lines, namely Vero E6, Huh-7D12, and HepG2, well-known cells for propagating SARS-CoV-2 *in vitro* experiments. Moreover, we also performed a simple amino acids analysis for a deep insight of BA.2 Indonesian isolate.

2. Methodology

2.1 Ethical Clearance

The Health Research Ethics Committee-Faculty of Medicine Universitas Indonesia and Dr. Cipto Mangunkusumo National Hospital (HREC FMUI-CMH) has approved this research with ethics number

KET-42/UN2.F1/ETIK/PPM.00.02/2022. The participant has provided informed consent. Not a single medication administration procedure was used in this investigation. We employed Biosafety Laboratory Level 3 (BSL-3) environment to conduct the experiments on viral infection.

2.2 Virus

SARS-CoV-2 virus variant Omicron BA.2 was a collection of the Microbiology Department, Faculty of Medicine, University of Indonesia, isolated from the naso-oropharyngeal specimen of infected patients in June 2022.

2.3 Cell Lines

Our study utilized three cell lines: Vero E6, Huh-7D12, and HepG2. The Vero E6 cell lines were obtained from the Microbiology Department, Faculty of Medicine, University of Indonesia. The HepG2 cell lines were acquired from the National Research and Innovation Agency, Indonesia. On the other hand, we purchased the Huh-7D12 (ECACC 01042712) from the European Collection of Authenticated Cultures (ECACC), United Kingdom.

For the susceptibility test of the SARS-CoV-2 virus, we used Vero E6, Huh-7D12, and HepG2 cell lines. We cultured HepG2 and Huh-7D12 using Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 10% Fetal Bovine Serum (FBS standard, South America Origin, PAN-Biotech, Germany). The Vero E6 cell lines were co-cultured with Minimum Essential Medium (MEM, Gibco, USA) containing 10% FBS for cell maintenance. Every cell line was cultured in an incubator with 5% CO_2 and 37°C.

2.4 Susceptibility Test for SARS-CoV-2 Subvariant BA.2 on Vero Cell Line

We followed the protocol outlined by Yamasoba *et al.,* [12] with minor adjustments. Vero cell lines were cultured in 96-well plates using MEM supplemented with 10% FBS. The cells were maintained at 37°C with 5% CO_2 until they reached 90% confluency. Before infecting the Vero E6 cell lines, we performed a 10-fold serial dilution of the virus. Each dilution involved taking 400 µL of virus from the stock ($1{,}32 \times 10^5$ ffu/mL) and combining it with 400 µL of MEM medium, repeating this process until the sixth dilution was achieved. The virus was then inoculated onto the monolayer cell lines in MEM containing 2% FBS, with 30 µL per well. For two hours, the cells were incubated at 37°C and 5% CO_2, with agitation every 30 minutes. Subsequently, 100 µL of 1% methylcellulose (Sigma, USA) was added to each well, followed by further incubation at 37°C with 5% CO_2 for 48 hours. The number of BA.2 variant-infected cells was determined through immunostaining analysis.

2.5 Susceptibility Test for SARS-CoV-2 Subvariant BA.2 on HepG2 and Huh-7D12 Cell Line

For the susceptibility test, we followed the protocol outlined by Yamasoba *et al.,* [12]. HepG2 and Huh-7D12 cell lines were seeded on 96-well plates using Dulbelco's Minimum Essential Medium (DMEM, Gibco, USA) with 10% FBS. The plates were then incubated at 37°C and 5% CO_2. As mentioned in section 2.4, prior to infection, we prepared a 10-fold serial dilution of the virus stock. This involved taking 400 µL of the virus stock ($1{,}32 \times 10^5$ ffu/mL) and adding it to 400 µL of DMEM medium for the first dilution, and continuing this process until the sixth dilution. To infect the monolayer cell lines, we inoculated the virus into DMEM containing 2% FBS at a volume of 30 µL per well. The plates were then kept at 37°C with 5% CO_2 for a duration of 2 hours, with agitation every 30 minutes. After that, we added 100 µL of 1% methylcellulose (Sigma, USA) to each well and

incubated the plates at 37°C with 5% CO_2 for 48 hours. Finally, we calculated the number of BA.2 variant-infected cells by immunostaining.

2.6 Immunostaining

Following the administration of methylcellulose during the viral cell infection phase, the subsequent step was initiated. We conducted the immunostaining following the procedure described by Angelina *et al.,* [15] and Dewi *et al.,* [16] with slight modifications. After 48 hours, the infected cells in each well were treated with 100 µL of 10% formaldehyde (AppliChem, Germany) in Phosphate Buffer Salin (PBS, Sigma, USA). The cells were then allowed to incubate for an hour at room temperature before being repeatedly washed with PBS. Later, 1/1000 Human polyclonal IgG-anti BA.2 subvariant was added after blocking in PBS with 5% skim milk (Sigma, USA) and kept at room temperature for two hours. Then, 100 µL substrates of 3,3'-Diaminobenzidine (DAB, Thermo Scientific, USA) with H_2O_2 were applied to each well and incubated for 15 minutes upon washing. Manually, the foci forming on each well, including negative control, were observed and enumerated under a microscope. The number of foci forms was analyzed using GraphPad Prism version 10.0.0.

2.7 Alignment Amino Acids of BA.2 Indonesian Isolate

The Indonesian Omicron BA.2 isolate DNA sequence was translated and compared to the Omicron BA.1 (EPI_ISL_10633761), BA.2 (EPI_ISL_9092427), BA.3 (EPI_ISL_7605591), BA.4 (EPI_ISL_12268495.2), BA.5 (EPI_ISL_12268493) sequence using SeqScape™ 3.0 software. The Global Initiative on Sharing Avian Influenza Data (GISAID) obtained Omicron subvariant sequence data. The percentage of shared amino acids mutation between BA.2 Indonesian isolate and other subvariants Indonesian isolate and other subvariants (BA.1, BA.2, BA.3, BA.4, BA.5) was analyzed using Venny 2.1 [49], a bioinformatics web tool.

3. Results

3.1 Infectivity Ability of SARS-CoV-2 Subvariant BA.2 on ACE-2 Expressing Cell Lines

The ability of the Indonesian BA.2 virus isolate to infect cells was demonstrated by the formation of a focus on cells infected with the SARS-CoV-2 virus using the Focus Reduction Neutralization Test. This study used primary antibodies from recovered VTM donors to observe the foci forms.

After administration of DAB substrate, observations revealed that Huh-7D12 and HepG2 cells, whether exposed to the virus stock or a 10-fold serial dilution, exhibit similar conditions to unexposed cells (negative control) (Figures 1(a) and 1(b)). These findings indicate that the SARS-CoV-2 subvariant BA.2 did not infect these cell types. In contrast, Vero E6 cells developed brown-colored foci formation in all virus-exposed cells, with the number of foci correlating with the dilution level (Figure 1(c) and 2). Similar findings were found in other variants (data not shown).

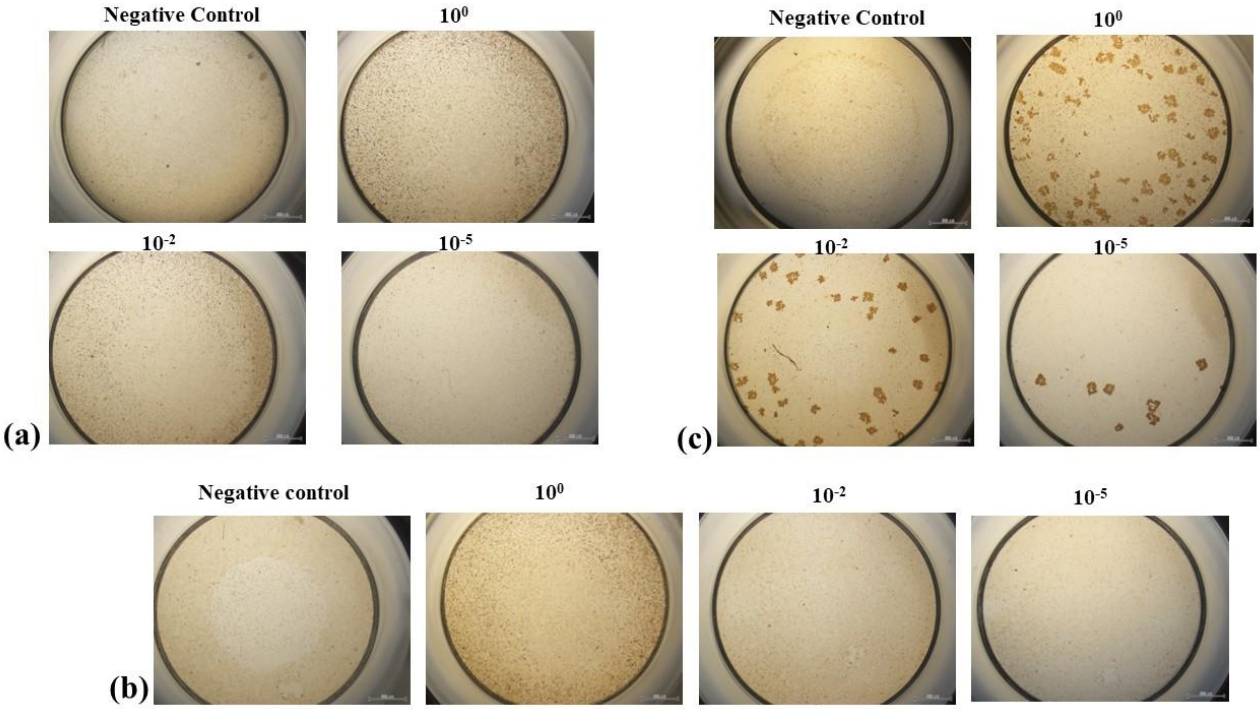

Fig. 1. Representatives of foci formation on ACE-2 expressing cells following infection with the Omicron BA.2 virus infection at a 10-fold serial virus dilution. Infected cells appear brown, indicating the presence of foci on the cell. The scale bar measured 200 µm. (a) HepG2 cell lines, (b) Huh-7D12 cell lines, (c). Vero E6 cell lines

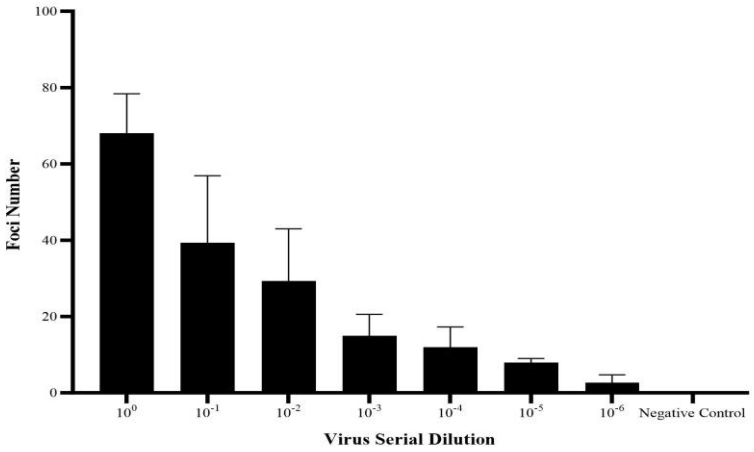

Fig. 2. The number of foci forming after SARS-CoV-2 infection on Vero E6, Huh-7D12, and HepG2 cells

3.2 A Comparison of Omicron BA.2 Amino Acid Sequences in Indonesian Isolates to Omicron Subvariants

The translated amino acids sequence of BA.2 Indonesian isolate was compared to other Omicron subvariants. We utilized VENNY 2.1 software to determine the proportion of amino acid alteration similarities between Omicron BA.2 Indonesian isolate and the previous VOC Omicron subvariant.

Our findings show that BA.2 in Indonesian isolate contains 82.9% amino acid changes comparable to those found in BA.2, but only 42.5% of mutations are similar between BA.2 Indonesian isolate as shown in Figure 3(b). Moreover, 20 amino acid mutations in BA.2 Indonesian isolate are similar to

BA.4 and BA.5. Interestingly, S371L, N856K, and L981F of BA.2 Indonesian isolate were found in BA.1 (Figure 3(b) and (c)).

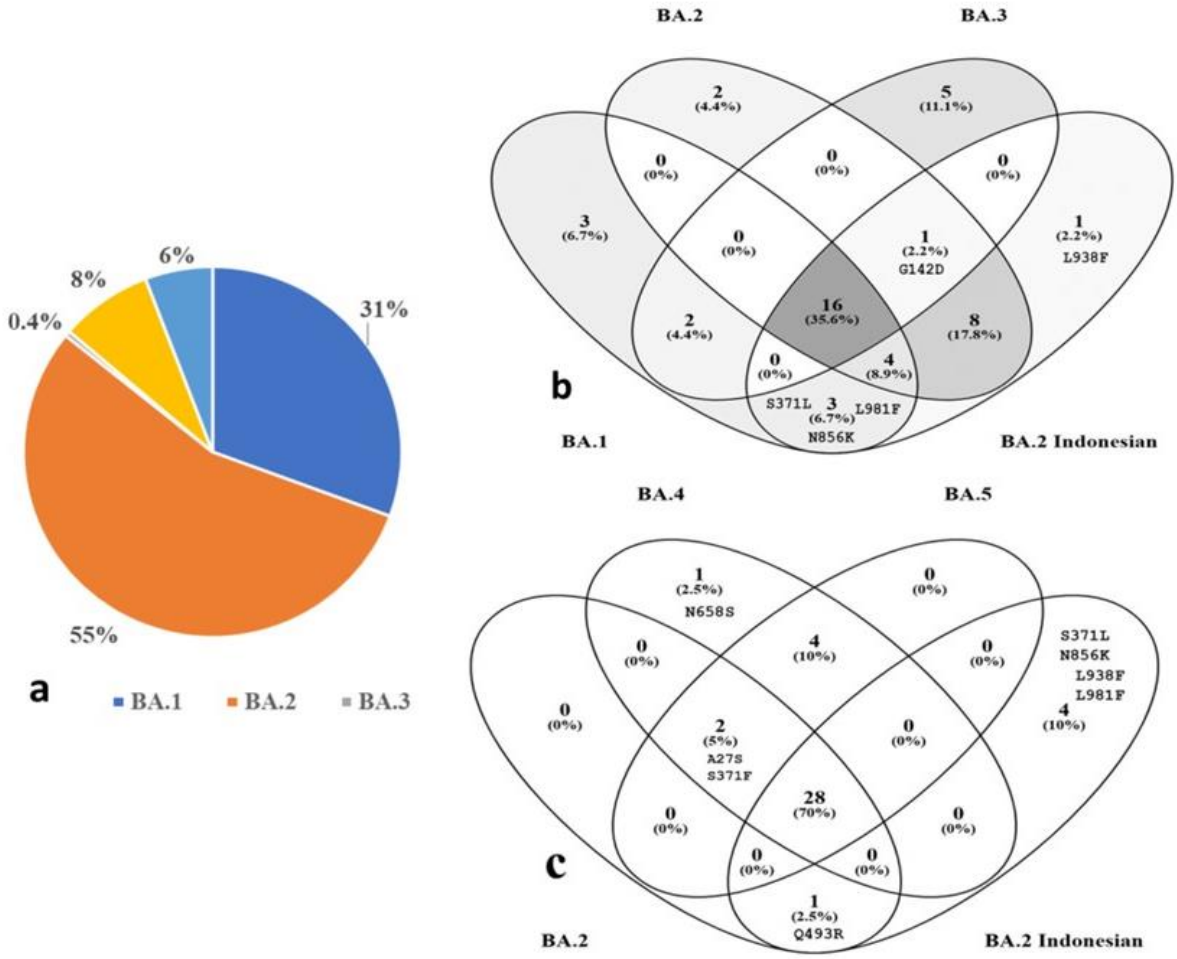

Fig. 3. Comparison of Omicron in Indonesia and Omicron protein amino acids. (a) Pie chart of the total number of Omicron BA.1, BA.1, BA.3, BA.4, and BA.5 genomes from Indonesia available on GISAID. The entire genome was collected from November 2021 to December 2023, (b) Ven diagram illustrating the differences in mutations in the Spike protein of BA.2 in Indonesian isolates compared to BA.1, BA.2, and BA.3. The analysis was conducted using a three-way Venn diagram, (c) Comparison of amino acid alterations in spike proteins between BA.2 isolated from Indonesia, BA.2, BA.4, and BA.5. Ven diagrams are created using a web-based application, VENNY 2.1 [49]

4. Discussion

A significant number of mutations in SARS-CoV-2 affected their development and infection mechanism. Since late 2021, the Omicron has become the dominant SARS-CoV-2 variant. Although the pathogenicity is lower than the latest variants, Omicron is easily mutated and more transmissible. BA.2 is one of the Omicron subvariants previously classified as a Variant of Concern. Here, we employed Omicron BA.2, which had previously been isolated from an Indonesian patient and identified as Omicron BA.2 by a previous study. No reports related to Omicron BA.2 from Indonesia utilization; therefore, we employed the virus infection in three mammalian cells: Vero E6, Huh-7D12, and HepG2. Our findings reveal that the Omicron BA.2 Indonesian isolate exclusively infects Vero E6 cells.

Indonesia reported the first Omicron BA.2 infection in January 2022, and more than 50% of Omicron genome deposits on GISAID, a genomic data platform, were BA.2 (Figure 3(a)). Similar events occurred in Denmark, the Philippines, and Hong Kong during the fourth wave of COVID-19 [17–19]. SARS-CoV-2 transmission is comparable with the number of deposited genomes in GISAID. SARS-CoV-2 genomic data and nucleic acid sequences in GISAID demonstrate infection rates and genetic variants. Due to the role of GISAID as an open-access genomic data platform, it becomes a reference for people to track the growth of infection and a new variant in an area [20].

Since 2006, GISAID has been used to monitor viruses circulating globally. This monitoring is crucial for biannual vaccine recommendations and risk assessment of animal influenza that frequently causes zoonotic diseases [21]. Furthermore, GISAID permits worldwide information sharing.

Due to some mutations and indels, changes in spike protein structure led to Omicron's dominance in SARS-CoV-2 infections [22]. Alterations in the RBD area cause an increased transmissibility of SARS-CoV-2. The ACE-2 receptors were critical in the viral internalization process in SARS-CoV infections between 2002 and 2003 [23].

Omicron RBD has up to 15 mutations compared to Wuhan-Hu, the ancestral variant. Mutations affected the affinity of RBD-ACE2, which became stronger and caused the virus penetration to be more effective [22,24,25]. Our findings indicate that Omicron BA.2 Indonesian isolate, BA.1, and BA.2 share 20 amino acid alterations in the spike protein. Moreover, twelve of the twenty mutations are in RBD. In agreement with a previous study, RBD changes in as much as 12 amino acids between BA.1 and BA.2 [25,26].

During the 2002–2003 SARS–CoV epidemics, the ACE-2 receptor is crucial to the viral internalization process [23]. The presence of a mutation in spike protein changes how the virus infects cells. Interaction between spike with ACE-2 receptors and cellular protease enzymes such as TMPRSS2 and Cathepsin are the criteria for SARS-CoV-2 infection. The protease enzyme determines whether SARS-CoV-2 infection occurs endosomally or fusogenically. The fusogenic process begins when TMPRSS2 enhances virus uptake after a spike-ACE-2 receptor interaction is created. This process is also known as the TMPRSS2-dependent route. On the contrary, endosomal pathway (TMPRSS2-independent pathway) is employed when cathepsin is involved in internalization [12,23].

The infection mechanism of Omicron is distinct from other variants due to the increased affinity of spike binding to ACE-2 receptors [27]. The previous VOC variants utilize both penetration mechanisms, while Omicron employs a TMPRSS2-independent pathway, allowing the virus to be internalized endosomally [2,28]. In agreement with our findings, an *in vitro* investigation comparing pseudoviruses of four Omicron subvariants and Delta variants utilizing TMPRSS2- and ACE-2 expressing cells. They found that compared to Vero E6-TMPRSS2 cells, Calu-3, and Caco-2, Omicron was more efficient in infecting ACE-2-expressing cell lines [29].

We found that BA.2 Indonesia isolate also uses the TMPRSS2-independent pathway, similar to their variants. Since Omicron BA2 Indonesian isolate is a new virus and there are no reports about the virus, determining the suitable host cells becomes essential. As a result, we will understand the biology of viral infections, including growth dynamics and tropism of the virus [30].

There are numerous ways to select suitable cells for propagating a novel virus, such as selecting cells based on their shared host, cells expressing central receptors for virus infection, and the infection process [30]. Investigating phylogenetically related viruses is important to determine the criteria of suitable cells. Vero E6, Huh-7D12, and HepG2 cells are frequently employed in SARS-CoV [31] and previous variants of SARS-CoV-2 [11,32,33]. Moreover, those cells originated from organs that were affected by SARS-CoV-2 infection and could express TMPRSS2 and ACE-2 receptors (table 1).

The level expression of protease enzyme produced by cells influences the infection mechanism. The virus will use the TMPRSS2-dependent route if the target cell significantly expresses TMPRSS2 [34]. Since the viral infection was detected in Vero E6 cells that do not express TMPRSS2, our investigation demonstrates that the Indonesian BA.2 isolate infects target cells without requiring the expression of a TMPRSS2, as evidenced by the fact that the virus infected Vero E6 cells lacking TMPRSS2. Our findings are consistent with a previous study; Omicron effectively infects cells expressing only ACE-2 [29,35,36].

Table 1
The ACE2 receptor and TMPRSS2 protease expression level by Vero E6, Huh-7D12, and HepG2 cell lines

Cell Line	Organ originated	Organism	ACE-2 expression	TMPRSS2[37]	Ref
Vero E6	Kidney	African Green Monkey	+++	-	[30,37]
Huh-7D12	Liver	Human	+	+++	[30,37]
HepG2	Liver	Human	+	+++	[30,37]

-: no expression, +: low, +++: very high

The ability of Omicron BA2 from Indonesia to infect cells through the TMPRSS2-independent pathway is inseparable from the changes in amino acids possessed by the virus. We found that there are four amino acid alteration differences between BA.2 Indonesia isolate and BA.2, S371L, N856K, L938F, and L981F. Interestingly, S371L, N856K, and L981F were similar to BA.1. Moreover, the amino acids have significant roles in immune evasion and infection mechanisms [25,38–41].

The discrepancy of amino acids among BA.2 Indonesian isolate and BA.2 (EPI_ISL_9092427) influence the utilization infection route by BA.2 Indonesian isolate. Omicron BA.2 Indonesian isolates prefer to infect cells endosomally. Meanwhile, BA.2 tends to use the fusogenicity pathway. An *in vitro* study using 293HKT-hACE2-TMPRSS2 cells reported that infection by BA.2 pseudovirus was more efficient than BA.1 pseudovirus [39]. Even though both BA.2 and BA.1 have S375F mutation, BA.1 has N856K residue, which reduces TMPRSS2 dependency [39]. An *in vivo* study also shows that BA.1 infection was only found in TMPRSS2 knockout mice [42]. Moreover, Omicron exclusively infects the upper respiratory tract of TMPRRS knockout mice, which makes Omicron less pathogenic than previous variants [42]. N856K also reported lead fusogenicity reduction of Omicron on Huh-7 [43].

S371L, N856K, and L981F residues are unique to Omicron and influence SARS-CoV-2 immune evasion. The residues cause Omicron to hinder the neutralizing antibodies [38,39]. The immune escaping was influenced by RBD conformation changing from open conformation on previous variants into closed conformation in Omicron. The S371L has been reported as important for RBD conformation. With S373P, S375S, and D614G, S371L creates non-polar residues that affect the down conformation, potentiating Omicron to evade antibodies [38,39,44].

Omicron BA.2 isolate Indonesia has a single unique mutation, L938F. This mutation has been found in the Wuhan Hu variant. However, its mutation rate is extremely low, ranging from 0.0025% to 0.055% [45]. The amino acid leucine 938 is found in the S2 area of the Spike protein, precisely in heptad region 1 (HR1). The subunit 2 region, especially H1HR2 formation, is believed to be more stable than the RBD region [45]. Huan Sun findings show that 100% similarity among SARS-CoV-2 was found at HR1HR2 formation. However, L938 has a bigger chance to mutate, but the mutation has no significant impact to change the HR1HR2 structure [46].

Protein S2 contains HR1 and HR2 regions involved in membrane fusion following proteolytic cleavage and forming the HR1HR2 structure. This stage is critical in SARS-CoV-2 infection because it allows the virus to release genetic material and create new virions [47]. The formation of the HR1HR2 structure is critical in the infection process of SARS-CoV-2, SAR-CoV and Mers-CoV. Therefore, anti-viral therapies target HR1HR2 to inhibit infections [46,47].

The formation of the HR1HR2 structure is crucial for MERS-CoV, SAR-CoV, and SARS-CoV-2. This was demonstrated by decreased SARS-CoV-2 infection in ACE-2/293T cell lines and Huh-7 after HR1 inhibitor addition [47,48]. The replication process will begin after the fusion membrane, which causes the viral genome release [38].

Our findings indicate that alteration in amino acids of BA.2 Indonesian isolate generates a distinct infection mechanism from BA.2, as evidenced by infection in three ACE-2 expressing cells. However, further research to determine the possibility of the same mutation in BA.2 originating from Indonesia and globally needs to be carried out to be used as a reference in developing drugs and vaccines. The *in silico* and *in vitro* research about the role of each mutation on spike structure and infection mechanism will enhance our findings. Furthermore, *in vitro* and *in vivo* experiments on comparing infection between our virus and other Omicron subvariants will also improve the study.

5. Conclusions

Due to spike protein mutations, the Omicron variant has dominated SARS-CoV-2 infections since late 2021. Because of the high mutation rate, it is critical to identify the appropriate host cell for hCOV-19 virus propagation. Cells producing SARS-CoV-2 receptors and the origin organs are considered when selecting host cells for hCoV-19 propagation. Omicron BA.2 Indonesian isolate cannot effectively infect cells with low ACE-2 expression due to amino acid alteration in BA.2 from Indonesia. The Indonesian Omicron BA.2 isolate, isolated in 2022, has 29 amino acid alterations identical to BA.2; 10% have distinct mutations from BA.2, BA.4, and BA.5, and one unique mutation, L938F. Further research could improve our findings and be advantageous for the COVID-19 vaccine and therapeutic development, such as analyzing the influence of each mutation on the structure of spike protein and mechanism of infection by *in silico*, *in vitro*, and *in vivo* experiments.

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