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Detection of ZIKA virus antibody in pregnant women in Makassar City, Indonesia: The first proof of the distribution of Zika virus in the area

Detección de anticuerpos del virus del ZIKA en mujeres embarazadas en la ciudad de Makassar, Indonesia: La primera evidencia de la distribución del virus del Zika en la zona

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SUMMARY

Objective*: The Zika virus (ZIKV) is a type of arbovirus belonging to the Flaviviridae family. It is primarily transmitted to humans through mosquito bites, with the Aedes aegypti mosquito being a common carrier of ZIKV and other viruses like dengue, chikungunya, and yellow fever. In Indonesia, with a population of 254.9 million people, there were no reports of Zika outbreaks until 2018. Although there have been no documented cases of Zika in pregnant women in Indonesia, there is evidence suggesting that ZIKV may already be present in the country, putting pregnant women at risk of infection. This study aimed to investigate the seroprevalence of Zika in pregnant women living in Makassar, Indonesia, using the ZIKV IgG ELISA method.* **Methodology and result:** *139 stored serum samples used in this study were tested for anti-ZIKV IgG antibodies. Additionally, the study sought to determine the potential for cross-reactivity by conducting dengue IgG ELISA (indirect and capture ELISA) on 46 samples that were seropositive for ZIKV*

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and nine randomly chosen negative samples to rule out the possibility of confusion with dengue antibodies. Employing anti-Zika virus IgG ELISA Kits (ABCAM, US lot # GR34470066-2), the study revealed that 46 (33 %) samples tested positive for ZIKV-specific IgG antibodies, and 93 samples (67 %) tested negative. Of the 46 positive ZIKV samples, 22 % (12) were also seroconverted for DENV IgG indirect ELISA and none for the capture ELISA. While the trends of antibodies checked with indirect and captured ELISA for DENV were comparable, antibodies of ZIKV showed a distinct trend that distinguished them from DENV antibodies. **Conclusion, significance, and impact of research:** *This study shows preliminary proof of Zika virus distribution among pregnant women in Makassar despite a possible immune cross-reaction between the two viruses.*

Keywords*: Zika, dengue, pregnant women, Makassar, ELISA.*

RESUMEN

Objetivo: *El virus del Zika (ZIKV) es un tipo de arbovirus perteneciente a la familia Flaviviridae. Se transmite principalmente a los humanos a través de picaduras de mosquitos, siendo el mosquito Aedes aegypti un portador común no solo del ZIKV, sino también de otros virus como el dengue, la chikunguña y la fiebre amarilla. En Indonesia, con una población de 254,9 millones de personas, no hubo informes de brotes de Zika hasta 2018. Aunque no hay casos documentados de Zika en mujeres embarazadas en Indonesia, hay evidencia que sugiere que el ZIKV podría estar presente en el país, poniendo a las mujeres embarazadas en riesgo de infección. El objetivo de este estudio es investigar la seroprevalencia del Zika en mujeres embarazadas que viven en Makassar, Indonesia, utilizando el método ZIKV IgG ELISA.* **Metodología y resultados:** *Se analizaron un total de 139 muestras de suero almacenadas en busca de anticuerpos IgG anti-ZIKV. Además, el estudio buscó determinar el potencial de reactividad cruzada realizando ELISA de IgG contra el dengue (ELISA indirecto y de captura) en 46 muestras que fueron seropositivas para ZIKV y 9 muestras negativas seleccionadas al azar, con el fin de descartar la posibilidad de confusión con anticuerpos del dengue. Utilizando kits de ELISA de IgG anti-Zika virus (ABCAM, lote estadounidense # GR34470066-2), el estudio reveló que 46 muestras (33 %) dieron positivo para anticuerpos IgG específicos de ZIKV y 93 muestras (67 %) dieron negativo. De las 46 muestras positivas para ZIKV, el 22 % (12 muestras) también presentaron seroconversión para el ELISA indirecto de IgG contra el DENV y ninguna para el ELISA de captura. Mientras que las tendencias de los anticuerpos analizados con ELISA indirecto y de captura para el DENV fueron comparables, los anticuerpos del ZIKV mostraron una tendencia distintiva que los diferenció de los anticuerpos del DENV.* **Conclusiones, importancia e impacto de la investigación***: Este estudio muestra una prueba preliminar de la distribución del virus del Zika entre las mujeres embarazadas en Makassar, a pesar de una posible reacción cruzada inmunológica entre ambos.*

Palabras clave*: Zika, Dengue, mujeres embarazadas, Makassar, ELISA.*

INTRODUCTION

The Zika virus (ZIKV) is a disease transmitted by mosquitoes, mainly the *Aedes genus*, with the *Aedes aegypti* mosquito being the most common vector. The ZIKV, Dengue (DENV), Yellow Fever (YFV), Japanese Encephalitis (JEV), and West Nile Virus (WNV) are the same group of arboviruses from the genus Flavivirus (1,2). Most people who are infected with ZIKV either have no symptoms or experience mild ones, including fever, skin rash, headache, joint and muscle pain, and eye irritation. These symptoms typically last for about two to seven days (3).

The ZIKV was initially discovered in Uganda in 1947, and human infections were first reported in the United Republic of Tanzania five years later in 1952. The outbreak of Zika virus disease in Brazil starting in early 2015 prompted the World Health Organization (WHO) to declare it a public health emergency in February 2016. This decision was made by the emergency committee, which assessed that Zika virus infection posed a significant global threat and required international cooperation (4-6).

Southeast Asia has been where the Zika virus has circulated for more than 60 years, but significant outbreaks have only recently been recognized. The virus was initially identified in mosquitoes in Malaysia in 1966, but it was not until 1977 that the first human cases were reported (7). In recent years, there have been confirmed cases of Zika in several Southeast Asian countries, including Singapore, Thailand, Malaysia, and Myanmar. In Singapore, a total of 455 cases were confirmed in 2016. Meanwhile, there were 386 reported cases in Thailand between 2015 and 2017, affecting 29 out of 76 provinces (7).

Indonesia, whith a massive population of 254.9 million people, did not report any Zika outbreaks until 2018 (8). However, there are signs of Zika presence in Indonesia based on the WHO Report 2016: one case from Central Java (1981), six from Lombok, West Nusa Tenggara (1983), two Australian tourists traveled from Jakarta (2013) and Bali (2015), and one case during a dengue surveillance (2015-2016) in Jambi Province. These instances suggest the presence of Zika in Indonesia despite no significant outbreaks being officially reported until 2018.

The global risk of ZIKV infection depends on two key factors: the suitability of *Ae. aegypti* mosquitoes as vectors for ZIKV in real-world conditions and the potential for ZIKV to spread to countries where *Ae. aegypti* and *Ae. albopictus* mosquitoes are present. ZIKV can likely co-circulate with other viruses like dengue and chikungunya. Notably, Indonesia, one of the largest dengue-endemic countries that has experienced multiple outbreaks of chikungunya fever, is the home to both Aedes species, which may also act as vectors for ZIKV (8,9). The mosquitoes are also commonly found in South Sulawesi, one of the fifteen provinces in Indonesia with the highest number of dengue cases recorded between 2008 and 2017. However, there have been no reported cases of ZIKV infection in the province. This study aims to investigate the presence of Zika virus antibodies in the community of Makassar City, particularly the pregnant women, the group that has the highest risk of being affected with the ZIKV infection, by testing the serum samples using the ELISA technique to shed light on the ZIKV in this population.

MATERIALS AND METHODS

Study design and samples

A sero-surveillance has been performed on 139 archive serum samples that were initially collected in a previous study on pregnant women living in residential slums of Makassar City within the working areas of Primary Health Centers (*Puskesmas*) of Jumpandang Baru, Kaluku Bodoa, and Rappokalling of Makassar City, Indonesia.

Ethical Approval and Demographic Data

The use of the archive samples in this study was granted by the Institutional Review Board of the Faculty of Medicine Universitas Hasanuddin (FWA00018968) with the approval reference number #182/UN4.6.4.5.3.1/PP36/2023. The serum samples were anonymously analyzed, with the investigator having no link to identify the sensitive data of the subjects. Confidentially, the data manager of the previous study at the Faculty of Public Health kept all data. Demographic data provided to investigators were limited to basic information, such as age, educational level, and occupational categories, to describe the samples' attribute profile as a group rather than as individuals. GPS coordinates were barely used to generate the map image to show the distribution of seroconverted ZIKV and DENV without any site visits or further exploration of privacy information.

ELISA Tests

The ELISA assay was performed at Hasanuddin University Medical Research Center (HUMRC) in Hasanuddin University Hospital using the ELISA Reader MULTISKAN FC (Thermo Scientific, USA, 2013) at the 450/620 nm wavelength.

ZIKV-specific IgG detection. According to the manufacturer's instructions, all 139 serum samples were tested for anti-ZIKV IgG antibodies using the ab221844 anti-Zika IgG capture ELISA kit (ABCAM, USA. Lot GR34470066-2). Briefly, the serum and kit's reagents were incubated at room temperature, and a 1:100 diluted serum was added to precoated anti-human IgG-class antibodies after washing, then ZIKV-HRP conjugate was added. The binding of the serum Ab with ZIKV-HRP conjugate-activated TMB substrate that was added later resulted in color changes of the solution from blue to yellow. The reaction was immediately frozen using a stop solution. The readings were performed within 30 minutes of the addition of stop solution.

***Cross-reactive confirmation with Dengue antibodies*:** Since DENV infection is the most common flavivirus infection in Makassar City with no case report of YFV, WNV and JEV infection, all the seroconverted samples for ZIKV

antibodies and some randomly chosen negative seronegative samples were re-assayed for the presence of DENV specific IgG antibodies using Panbio (Australia) ELISA kits, both Dengue IgG Capture ELISA (Lot 10321) and Dengue IgG Indirect ELISA (Lot 11080) to assess whether the positive result was due to cross-reaction with dengue antibodies. The examination of dengue IgG antibodies was conducted following the manufacturer's instructions. Briefly, serum and kit's reagents were incubated at room temperature, a 1:100 diluted serum was added to precoated anti-human IgG-class antibodies and then added by human anti-ZIKV specific antibodies, the bound Ag-Ab activated HRP substrate that was added later to result in color changes of the solution from blue to yellow that was immediately freeze using stop solution. The readings were performed within 30 minutes of the addition of stop solution.

Interpretation

ELISA results: The reading for ZIKV IgG capture ELISA results were interpreted as follows: Kit's index value < 9 was considered negative and > 11 a positive result, while index values between 9 to 11 were considered equivocal. The same was true for the DENV IgG indirect ELISA, while DENV IgG Capture ELISA has debatable values between 19-21, with >19 as negatives and >21 as positives. The seronegative samples indicated no previous infection, while seropositive or seroconverted samples indicated the presence of a previous infection. Samples with equivocal index values were in a gray area and needed to be re-tested. However, due to the limited kit's reagent and to simplify the analysis, the equivocal samples were considered negative in this analysis.

Confirmation for cross-reactivities: Although the plaque reduction neutralization test (PRNT) is currently recommended as the gold standard test for detecting human antibodies against certain viruses by showing their protective effect against the virus-induced cythopatic effect (CPE), we could not perform it due to facilities and fund limitations. Instead, we performed indirect methods to assess the cross-reactivities between ZIKV and DENV antibodies by comparing the ELISA values pattern (using ZIKV ELISA values as the standard order *vs*. DENV IgG Capture and Indirect ELISA) and using spatial distribution pattern of the patient addresses. Even though there were different cut-off points (COP) applied by other kits to determine the interpretation of the results, the typical absorbance pattern should show a similar trend in the case of cross-reactivity presence. The lack of a similar pattern of antibodies of ZIKV and DENV in the same individuals is less likely because of cross-reactivity.

RESULTS

Of the 139 serum samples of the pregnant women tested, the age group (years old) were: 15-25 (39.6 %), 25-34 (43.2 %) and 35-41 (17.3 %), while their highest educational levels were: elementary school (7.9 %), high school (69.1 %) and university degree (8.6 %). Their daily jobs were as housewives (85.6 %), government employees (2.9 %), and other jobs on their own (11.5 %).

Based on the ZIKV IgG capture ELISA results from 139 pregnant women samples, seropositive results were obtained in 46 samples (33 %), while seronegative results were found in 93 samples (67 %). DENV IgG indirect ELISA and DENV IgG Capture ELISA were also performed on all seropositive ZIKV samples to evaluate for cross-reactivities. The results showed that among 46 seropositive ZIKV samples, 26.1 % were also seropositive for DENV (12 samples by Indirect ELISA and 0 by capture ELISA). Additionally, randomly selected nine seronegative ZIKV samples were also re-tested. There were 6 out of 9 samples were seropositive by Dengue IgG indirect ELISA, while 0 for the captured ELISA (Figure 1). Most of the sero-positives serums were from pregnant women under 35 years old (89.1 %), with the highest educational level only until high school (97.8 %) and having no formal jobs but as housewives 84.4 % (Figure 2).

To check whether the positivity of ZIKV IgG serums was due to a cross-reaction to DENV, the most prevalent arboviruses in the area, we analyzed the pattern of ELISA results of ZIKV IgG and DENV IgG (both indirect and capture ELISA). It shows that while the pattern of ELISA results for DENV IgG for both indirect

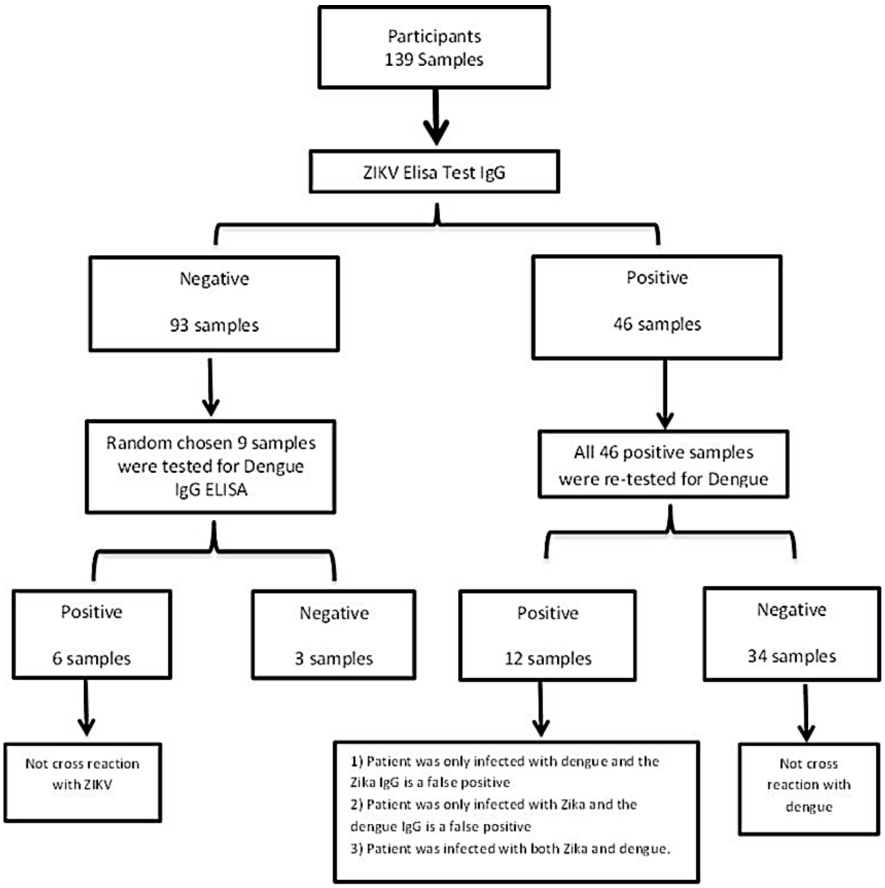

Figure 1. Flow chart of the samples and the results of ELISA testing.

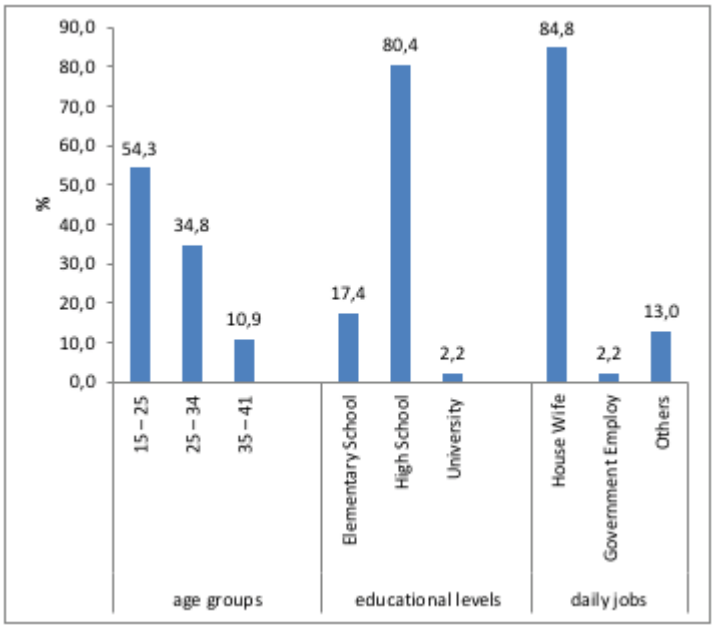

Figure 2: Sero-positive samples with their demographic attributes.

and capture methods were comparable, the results of ZIKV indirect ELISA show a distinct pattern that distinguishes it from the DENV antibody patterns (Figure 3).

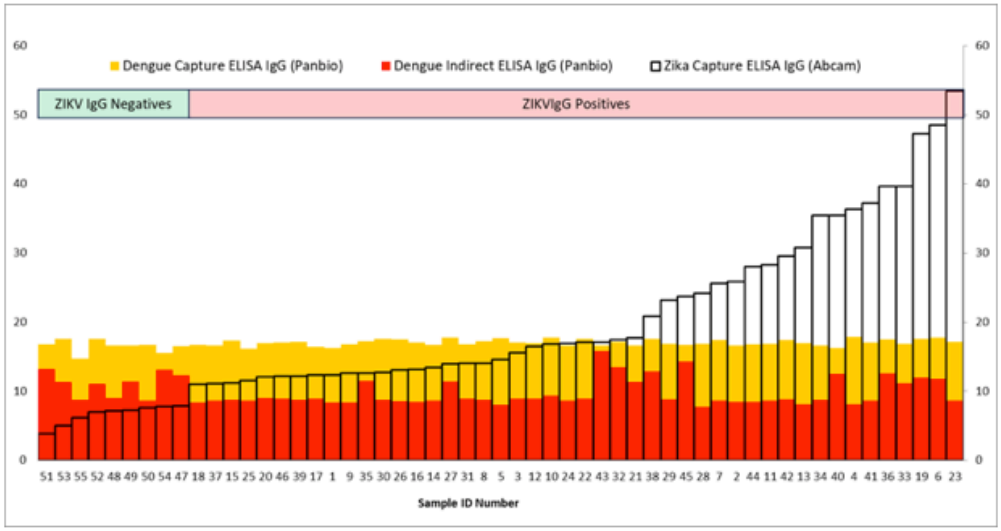

Figure 3. Overlay pattern of ELISA results for ZIKV IgG Capture ELISA (transparent, cut of point cop=11 Abcam unit value) vs. DENV IgG Capture ELISA (Yellow, cop=21 Panbio unit value) vs. DENV IgG Indirect ELISA (red, cop=11 Panbio unit value).

Further, the analysis of the spatial distribution of the ZIKV seropositive and DENV seropositive are clearly different, where most of the samples were spatially distributed as ZIKV sero-positives and show only about one-fifth of its overlap with DENV sero-positives (26.1 %). In contrast, out of 9 randomly chosen ZIKV seronegative serums, 6 (66.7 %) were DENV seropositive (Figure 4).

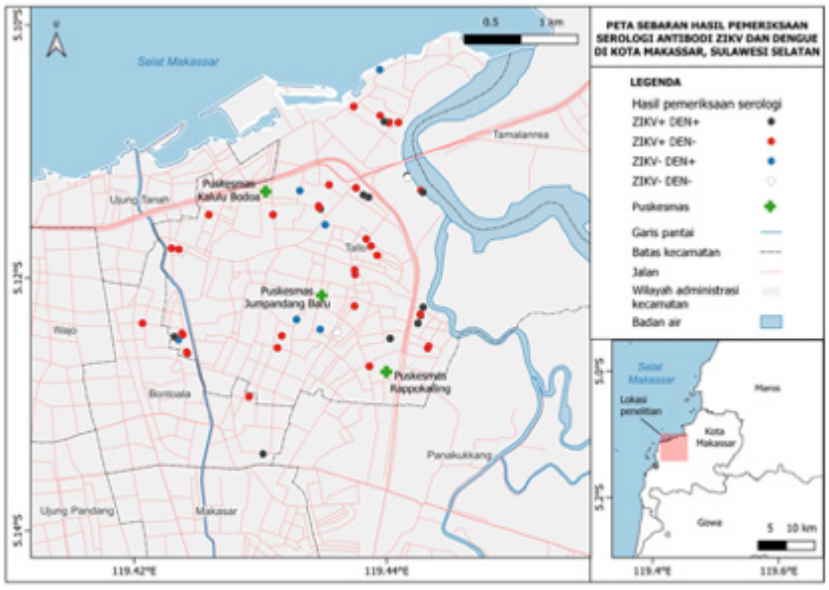

Figure 4. Spatial distribution of 55 serum samples with ZIKV seropositive and reconfirmed with DENV antibodies.

DISCUSSION

Pregnant women are susceptible to heightened risks associated with viral infections, with the potential for more severe illnesses that can culminate in fetal abnormalities. In light of these vulnerabilities, it is recommended that pregnant women proactively receive vaccinations against severe infectious diseases as a precautionary measure. Regrettably, no authorized vaccine or specific treatment for Zika virus (ZIKV) infection exists. However, in cases where a woman has previously encountered ZIKV, her immune system may possess the capacity to recognize and provide protection against subsequent infections. This immune safeguard results from generating ZIKV-specific or neutralizing antibodies, a vital component of the adaptive immune response. These antibodies are pivotal in conferring immunity against ZIKV in individuals previously exposed to the virus (10).

In our research, 46 samples from pregnant women were found positive for IgG antibodies specific to Zika virus (ZIKV), accounting for approximately 33 % of the samples. These findings align with a prior study conducted by Chayawat et al., wherein approximately 30.77 % of 650 pregnant women tested positive for ZIKV-specific IgG antibodies. Consequently, our results suggest that nearly 70 % of the participants in our study had yet to encounter the Zika virus, emphasizing a substantial portion of the population with no prior exposure.

Cross-reactivity among antibodies targeting different members of the Flavivirus family is a known concern in research studies, as it can introduce bias and complicate the interpretation of results. Flaviviruses share structural and genetic similarities, making developing antibodies that exclusively target a single species challenging. Efforts to minimize bias from cross-reactivity involve rigorous validation of antibodies, systematic testing for cross-reactivity, thoughtful experimental design, and transparent reporting. Collectively, these measures contribute to the reliability and accuracy of flavivirus studies.

In our study, 26.1 % of the serum gave positive results for both ZIKV and DENV IgGs. This may indicate two possibilities: the first is that subjects were exposed only with DENV but not ZIKV, resulting in false positives due to cross-reactivity between DENV and ZIKV antibodies; and the second is that the subjects were infected with both ZIKV and DENV either in sequential infection separated with enough time or mix infection at the same time. However, in most cases, those were only seropositive ZIKV and not DENV IgG, and some seronegative ZIKV IgG with seropositive DENV IgG may indicate that the cross-reactivity was less likely in this case. This was strengthened by the fact that the pattern of ELISA results of ZIKV antibody tests was clearly distinct from those of DENV antibody tests for both indirect and capture ELISA. The spatial distribution of the ZIKV seropositive, with only one-fifth overlapping with DENV sero-positives, further emphasizes the interpretation. However, we still do not close the space of cross-reactivity with other members of Flaviviruses even though no cases of dengue infection were reported.

Females face an elevated susceptibility to Zika virus infection, primarily through the bite of *Aedes spp*. Mosquitoes can occur during nighttime hours, both indoors and outdoors, including while commuting or at work. This heightened risk may influence their healthcare-seeking behavior in response to Zika symptoms. Additionally, findings from Lozier et al. have shed light on a noteworthy pattern. Specifically, the substantial prevalence of IgG antibodies against the Zika virus observed in individuals aged 15-24 appears to contrast with the antibody levels in individuals over 35. This underscores a notable disparity in Zika virus exposure among different age groups (11).

The incidence of arboviruses demonstrates a strong correlation with socioeconomic factors. Specifically, the presence of Chikungunya virus (CHIKV) and Dengue virus (DENV) is notably associated with individuals of lower socioeconomic status. At the same time, distinct research has indicated that higher socioeconomic status is linked with Zika virus (ZIKV) prevalence. Individuals of lower economic status often contend with substandard living conditions, creating an environment conducive to the proliferation of vectors. These individuals tend to reside in densely populated areas, including informal settlements and slums, where access to essential healthcare and sanitation is inadequate. The prevalence of ZIKV is mainly facilitated

in densely populated locales, where the risk of exposure to disease vectors and potential infection with ZIKV, DENV, and CHIKV is heightened. High population mobility in such areas further amplifies this risk and contributes to the transmission and dissemination of arboviruses. Notably, *Aedes aegypti*, an anthropophilic mosquito species known to transmit these viruses, thrives in close proximity to human habitation, exacerbating the risk of transmission in densely inhabited regions (12).

Residing in tropical and subtropical regions elevates the susceptibility to contracting Zika Virus (ZIKV) and Chikungunya Virus (CHIKV), whereas semi-arid territories foster the dissemination of Dengue Virus (DENV). The escalation in temperature and arid climatic conditions is correlated with the propagation of three arthropod-borne viruses (arboviruses). At the same time, an augmentation in rainfall and population density is associated with the prevalence of ZIKV, CHIKV, and DENV infections (12,13).

Scientifically, it has been noted that densely populated regions can represent a significant threat to the transmission of Zika Virus (ZIKV) (14). Furthermore, the utilization of public transportation has been linked to an elevated risk of contracting Dengue Virus (DENV) (15). Additionally, residing in areas characterized by substantial population mobility is recognized as a risk factor for heightened exposure to disease vectors, amplifying the potential for acquiring both ZIKV and DENV infections (14). Considering these findings, it is imperative to implement preventive measures in such locales to mitigate the dissemination of these diseases.

According to Santana and Braga's, a thorough analysis of kernel and choropleth maps has robustly demonstrated the expansion of arboviruses from their initial reported cases into neighboring regions, where they have successfully established themselves. These findings shed light on the distinctive spatial diffusion pattern and diffusion expansion characterizing the Zika and Dengue epidemics in Salvador-Bahia. This pattern is consistent with the transmission dynamics typical of infectious diseases, which tend to spread more easily among individuals in close spatial and temporal proximity (15).

The urban-dwelling mosquito species *Ae. aegypti* assumes a pivotal role in the transmission of Dengue Virus (DENV), Zika Virus (ZIKV), and Chikungunya Virus (CHIKV) worldwide. These mosquitoes thrive in densely populated urban areas and their environs, mainly due to their preference for human blood meals and the presence of human-made breeding sites, such as water containers and discarded tires. The transmission patterns of these viruses are influenced by multiple factors, encompassing the virus itself, the mosquito and human hosts, and environmental conditions. Furthermore, the coexistence of multiple arboviruses can foster novel interactions among them, rendering transmission dynamics even more intricate (16). In line with Santana and Braga's research and Kazazian et al., it becomes evident that when multiple arboviruses are concurrently present, the risk levels for each virus are not uniformly distributed throughout the city. Specifically, in instances where DENV and ZIKV co-occur, distinct clustering patterns emerge in both time and space, underscoring the complexity of arboviral transmission dynamics (15,16).

Despite the high prevalence of dengue and abundant mosquito vectors, the incidence of Zika Virus (ZIKV) infections in Indonesia remains significantly lower than that of dengue. Indonesia, situated within the tropical zone where the *Aedes aegypti* mosquito thrives, raises intriguing questions. One hypothesis posits that the intense endemicity of dengue may induce cross-reactive immunity to ZIKV, potentially constraining the scale of ZIKV outbreaks in Southeast Asia. This cross-reactivity between ZIKV and Dengue Virus (DENV) antibodies has sometimes complicated the interpretation of research findings. Indonesia ranks second among the 30 endemic countries regarding the highest number of reported dengue cases, with a staggering 1,213,324 cases documented over the past decade. Within Indonesia, the South Sulawesi Province emerges as one of the fifteen provinces with the highest dengue incidence between 2008 and 2017. Recent investigations have illuminated that while cross-reactivity exists between DENV and ZIKV, the extent of cross-

neutralization, and consequently, the level of protection against disease, remains limited (7,17).

Studies focused on ZIKV prevalence in Southeast Asia suggest varying immunity levels among different countries. Nations such as Malaysia, Laos, and Vietnam exhibit lower immunity rates, typically below 10 %, rendering them susceptible to severe ZIKV outbreaks. Conversely, countries like the Philippines, Cambodia, Thailand, and Indonesia demonstrate high population immunity, which could mitigate transmission to smaller groups (18).

Based on initial phylogenetic investigations, it is evident that specimens originating from Indonesia share a close genetic relationship with those from Thailand, Myanmar, Cambodia, and the Philippines. This intriguing genetic association implies that the Zika Virus (ZIKV) may have been circulating in Indonesia before its formal discovery in 1977. This is particularly noteworthy given that ZIKV was originally characterized in Uganda, and its origin and early presence in Southeast Asia remain enigmatic (19).

The outcomes of this research bear significant implications for healthcare strategies, particularly concerning monitoring pregnant women's health. This knowledge can aid in assessing the risk of ZIKV transmission through mosquito bites, contribute to the advancement of flavivirus vaccines, and inform the development of proactive public health policies. While the potential for cross-reactivity between ZIKV and subsequent Dengue Virus (DENV) infections necessitates further investigation and scrutiny, there is a pressing need to establish a swift diagnostic test capable of identifying ZIKV infections and potentially enhancing the capacity to distinguish between DENV and ZIKV infections (20).

Although this study might be affected by the possible cross-reactions from other members of flaviviruses, including DENV infection, which is the most prevalent arbovirus infection reported, the PRNT assays might be needed in future studies to confirm whether the result shows a true prevalence. The data presented here, however, were important enough to increase public awareness about the possible distribution of ZIKV in the area and can be used by the health authority to invest more in specific diagnostic kits to plan a strategy to manage emerging diseases.

CONCLUSION

While there was a possibility of cross-reaction of the ZIKV IgG kit with the dengue IgG as the most frequent flavivirus infection in the area, the distinct patterns observed from the ZIKV and DENV seroconverted serum and its spatial distribution showed the preliminary proof of Zika virus distribution that exposed pregnant women in Makassar. It may need a follow-up confirmation with PRNT assays in future studies.

Conflict of interests

The authors declare that there are no conflicts of interest.

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