Zika virus antibody-positivity among symptomatic/asymptomatic pregnant women in the Aseer region displays pre-exposure to dengue viruses

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ABSTRACT
Antibody cross-reactivity among flaviviruses is a major limitation in understanding the prevalence without vector control measures. In this study, we investigated the presence of Zika virus (ZIKV)-specific antibodies and the significance of their cross-reactivity with other flaviviruses, which could affect the serological specificity in both symptomatic and asymptomatic pregnant women. Among the results obtained from 217 serum samples tested for ZIKV-specific IgM and IgG, no specific predictions regarding seropositivity or exposure due to extensive cross-reactivity with dengue virus (DENV) serology could be made. Clear-cut positivity was observed in 1.8% (n = 4) and 1.0% (n = 2) for ZIKV IgM and IgG, respectively. The same samples assessed for DENV showed 1.3% (n = 3) seropositivity each for IgM and IgG levels. None of the samples were positive for ZIKV and DENV IgM or IgG. However, one sample (0.4%) tested positive for ZIKV and DENV IgM. No significant correlation was observed between DENV IgM and IgG when comparing the overlapped serotiters. On the other hand, the ZIKV IgG-positive sample showed higher serotiters for DENV IgG, indicating cross-reactivity with ZIKV but without statistical significance. Therefore, screening for the incidence of ZIKV becomes particularly challenging in a population where the presence or pre-exposure to DENV is observed. Our observations further suggest that unless flavivirus prevalence is properly addressed, determining the prevalence of ZIKV antibodies, which may be confounded with other uninvestigated flaviviruses, will be complicated.

Keywords: Zika; intrauterine virus transmission; flavivirus cross-reactivity; dengue; cross-reactive viral antibodies.

INTRODUCTION
In an advanced medical setting, the consequences and clinical presentation of the Zika virus (ZIKV) during pregnancy remain undisclosed. However, the frequency of such cases has not been adequately measured in the past due to the lack of passive surveillance (European Centre for Disease Prevention and Control, 2015; Antoniou et al., 2020). In 2007, a survey conducted on Yap Island, Micronesia, reported that 80% of the general population had asymptomatic ZIKV infection (Duffy et al., 2009). A study in the Dominican Republic during 2016-2017 found the prevalence of ZIKV among pregnant women to be 37%, with approximately 10% of reported pregnancies resulting in fetal loss (Pena et al., 2019).

Conversely, a systematic review from French Guiana revealed that out of the pregnant women studied, 573 (19%) tested positive for the ZIKV, of which only 133 (23.2%) experienced symptoms. Women over 30 were more likely to have symptomatic infections (p = 0.03, 28%) (Flamand et al., 2017). However, no explanation was provided for the high number of asymptomatic Zika-infected pregnant women (Flamand et al., 2017). Variations in the severity of symptoms and similar entry criteria, such as rash and fever, as reported in the previous research literature on affected pregnant women, can hinder the accurate evaluation of the exact ratio of adverse fetal outcomes. One cohort study in the U.S. reported a 1:1 ratio of adverse fetal outcomes when comparing asymptomatic and symptomatic Zika-affected women (Shapiro-Mendoza et al., 2017; Paixao et al., 2018).

ZIKV, along with other flaviviruses such as dengue virus (DENV) and West Nile virus (WNV), is known to be transmitted congenitally, perinatally, through breast milk, and postnatally (Colt et al., 2017; Blohm et al., 2018). These infectious viral diseases have recently been identified as a major cause of mortality in neonates and long-term morbidity in other age groups (Langerak et al., 2019). Mosquito vectors have been implicated in the postnatal transmission of the virus, leading to a wide range of clinical presentations from mild to severe asymptomatic cases (Montecillo-Aguado et al., 2019). Initial antibody screening and determining the prevalence of these viruses pose challenges due to the need to include most of the Flavivirus...
group, given the frequent cross-reactivity reported between them (Muller & Young, 2013; Gunawardana & Shaw, 2018; Tan et al., 2019). The cross-reactivity among flaviviruses can be attributed to numerous structural, envelope, and nonstructural proteins encoded within their polyproteins (Rathore & St John, 2020). While flaviviruses, including ZIKV, share antigenic and molecular determinants, which has led to the search for a common strategy for vaccine development (Hadiniggoro et al., 2015), common targets for antiviral treatment, such as flavivirus protease, have recently been demonstrated (Tokunaga et al., 2020). Despite the benefits of cross-reactivity, no common vaccine is available for the entire flavivirus group or among serotypes of the same type (Lai et al., 2020). Several studies have explored potential small molecules and compounds with broad-spectrum anti-flavivirus activity (Chuang et al., 2019; Hasan et al., 2019; Wilson, 2019).

The global prevalence and incidence of arthropod-borne viruses (ARBO) have been well characterized (Mackenzie et al., 2004; Daep et al., 2014; Burdmann, 2019; Guarner & Hale, 2019; Tamura et al., 2019). In the past, pockets of ARBO viruses, including flaviviruses such as DENV, Japanese Encephalitis, and Yellow Fever viruses, were identified, and yearly preventive measures were implemented. These measures included entomological screening for vectors, vector control measures, and the preparation of tertiary care hospitals to handle an upsurge of cases (Al-Saeed et al., 2017; Stewart-Ibarra et al., 2017).

However, the outbreaks of ZIKV and the increasing incidence of DENV have raised concerns in other parts of developing and developed countries, prompting them to investigate the presence of ZIKV (AlSalloum et al., 2015; Alayed et al., 2018). Numerous reports from Saudi Arabia and other parts of the Middle East region have documented the prevalence of ARBO viruses such as DENV, WNV, Rift Valley Fever (RVF), and Alkhurma virus (Alayed et al., 2018). While there is a single report on the presence of ZIKV in the southwestern region (Khatar et al., 2013), no significant investigation on the seropositivity of ZIKV in southwestern Saudi Arabia has been conducted, despite widespread reporting of congenital disabilities and anomalies in these regions (Tambo et al., 2019). Additionally, these mountainous regions within Saudi Arabia, known for their vegetation, have been associated with mosquito populations and disease transmission (de Noronha et al., 2018).

Therefore, in this study, we aimed to screen for ZIKV and DENV IgG and IgM antibodies in asymptomatic pregnant women and those attending outpatient (OP) clinics for regular health checkups or exhibiting mild to severe clinical symptoms of systemic illness. Although our primary focus was on ZIKV, the presence of DENV in these areas prompted us to investigate the coexistence of both viruses. However, reports suggest that ZIKV may be permissive via the placenta in the first trimester (Kerkhof et al., 2020). Thus, regardless of their clinical presentation, we randomly screened pregnant women in all three trimesters.

MATERIALS AND METHODS

Study Design
The study was conducted at the Department of Microbiology and Clinical Parasitology, College of Medicine, King Khalid University. Blood samples were collected from pregnant women attending the outpatient department (OPD) at Maternity and Children's Hospital, Abha, after providing a bilingual oral and written explanation of the study. Ethical clearance was obtained from the Ethics Committee at the College of Medicine, King Khalid University, with reference number REC # 2017-05-24. Our prospective study extended from 2019 to the winter of 2020. The study investigation was aligned with the World Health Organization (WHO) clinical criteria for ZIKV infection (Petersen et al., 2016) and utilized IgG and IgM enzyme-linked immunosorbent assay (ELISA). Based on clinical observations, the samples were divided into two groups: Group 1, which did not fulfill the clinical symptoms of the WHO classification, and Group 2, which fulfilled the clinical symptoms of WHO ZIKV.

The serology results of ZIKV IgM and IgG were then used to classify the samples based on clinical observations. In cases where there was a discrepancy between the clinical symptomology and ELISA results, the closest cross-reacting antibodies, specifically DENV, were checked to eliminate any samples that tested positive for DENV.

Sample Collection
Aseptically, whole blood samples (5 ml) were collected using sterile disposable syringes and red-top vacutainer tubes. The labeled tubes were left to stand for 30 min at room temperature to allow clot formation. Serum was obtained by centrifuging the clotted tubes at 2200 RPM (revolutions per minute) for 10 min. The clear serum was then transferred into new multiple aliquots, labeled, and stored at -80°C until further testing.

Zika IgM and IgG ELISA
Serum samples were screened for anti-Zika IgG/IgM using commercially available indirect ELISA kits from EUROIMMUNE (Lubeck, Germany). Briefly, 100 µl of positive and negative controls and 1:101 diluted serum samples were added to coated ELISA plates and incubated for 60 min at 37°C. This was followed by three cycles of manual washing and the addition of 100 µl of anti-IgG/IgM peroxidase conjugate. The plates were then incubated for 30 min at room temperature, followed by three washes. Subsequently, 100 µl of the substrate was added and incubated for 15 min at room temperature. The reaction was stopped by adding 100 µl of stop solution, and the absorbance was measured at dual wavelengths of 450 and 630 nm using an ELISA reader (Huma reader, Wiesbaden, Germany). The results for Zika IgG were calculated as RU/ml, obtained from a standard curve generated by the ELISA reader. A patient’s unit <16 was considered negative, while >22 was considered positive.

For Zika IgM antibodies, results were calculated as a ratio from the following equation:

\[
\text{Ratio} = \frac{\text{Extinction of calibrator}}{\text{Extinction of patient sample}}
\]

The patient’s serum is considered negative if the ratio is < 0.8 and positive if the ratio is ≥ 1.1.

Dengue IgM and IgG ELISA
Serum samples were screened for anti-DENV IgG/IgM antibodies using commercially available indirect ELISA kits from HUMAN (Wiesbaden, Germany). In brief, 100 µl of positive and negative controls, in duplicate, and diluted serum samples (1:101) were added to coated ELISA plates and incubated for 30 min at 37°C. After three manual washes, 100 µl of anti-IgG/IgM conjugate was added to all corresponding wells, except the blank, and incubated for 30 min at room temperature. The plates were washed three times, and 100 µl of substrate solution was added to all wells, followed by incubation for 15 min at room temperature in the dark. The reaction was stopped by adding 100 µl of stop solution, and the resulting color was measured at dual wavelengths of 450 and 630 nm using an ELISA reader (Huma reader, Wiesbaden, Germany). The patient’s results for DENV IgG and IgM were calculated as units per milliliter (unit/ml) using the following equation:

\[
\text{DENV IgG/IgM antibody units} = \frac{\text{Patient absorbance} \times 10}{\text{Cut of value (COV)}}
\]

COV for IgG /IgM = Mean negative control+3.5.
A patient is considered negative if their units are < 9 U/ml, while a patient is considered positive if their units are ≥ 11 units.

**Statistical Analysis**

The mean, standard deviation, and significance of risk factors were calculated using Fisher’s Exact test, Mann-Whitney U test, and Chi-square test, with the data analyzed using SPSS software (Version 20). Correlation and regression analyses assessed the positive correlation between the seroconverters overlap. A significance level of P < 0.05 was considered statistically significant.

**RESULTS & DISCUSSION**

The information regarding group 1 and group 2 individuals was compared based on gestational age and type of delivery to assess any potential association with ZIKV seropositivity in pregnant women (Table 1). The observations indicate that among Group 1, comprising individuals who did not exhibit classical ZIKV clinical presentations or have detectable ZIKV IgG or IgM titers, a total of 78.8% were distributed as follows: 15.8% among 35–46 weeks, 65.5% in 20–34 weeks, and 18.7% in 13–19 weeks (Table 1). Among the remaining 46%, the maximum symptoms were observed at 20–34 gestational weeks but without any correlation to ZIKV serology. In Group 2, 58.6% of the samples from individuals showed antibodies of IgG or IgM antibodies to ZIKV (Table 1), indicating exposure to ZIKV. However, only six samples had titers above the cutoff to be considered serologically positive. Nonetheless, no statistical significance was observed regarding gestational age or type of delivery (Table 1).

Among the cases screened for clinical symptoms (Table 2), according to the WHO criteria for ZIKV infection diagnosis, headache was a common symptom, followed by rashes (4.3%), arthritis (6.5%), and fever (21.7%). Only four individuals (8.7%) exhibited symptoms that met the WHO criteria for ZIKV infection and were classified as pregnant women at high risk who fulfilled the symptomatic ZIKV infection criteria set by the WHO (Pan American Health Organization, 2017). Differentiating between ZIKV-positive and -negative pregnancies based on symptoms alone was impossible due to overlapping symptoms and the lack of suitable sensitive laboratory tests. Fever was reported by the WHO as a major symptom, without any statistical significance compared to the 8.7% who fulfilled the WHO criterion. The frequency of Group 1 (78.8%, n = 171) in the current study was consistent with previous investigations in Yap Island (Lanciotti et al., 2008) and French Guiana (Pena et al., 2019), which reported 81% and 77% asymptomatic cases, respectively. Only 4.34% of ZIKV patients reported rashes, which contradicts the findings of the Yap Island research that observed rashes in 90% of ZIKV cases (Lanciotti et al., 2008).

The major findings suggest that skin pigmentation among different populations played a significant role in the contradictory results related to rashes. Among all individuals (n = 46), only 4 (8.7%) ZIKV cases fulfilled the WHO criterion, with fever being a prominent symptom in 10 (21.7%) cases, followed by arthritis in 3 (6.5%) cases and rashes in 2 (4.34%) cases. These symptoms have been reported by several researchers (Lanciotti et al., 2008; Duffy et al., 2009; Waehre et al., 2014; Flamand et al., 2017; Hoen et al., 2018), along with headaches (Centers for Disease Control and Prevention, 2019; Redivo et al., 2020; Halani et al., 2021). In this study, 54.34% of ZIKV cases reported severe headaches compared to 61.40% of non-ZIKV cases.

Out of the 217 serum samples obtained from pregnant women, 1.8% (n = 4) and 1.0% (n = 2) tested positive for ZIKV IgM and IgG, respectively (Table 3, Figure 1a and b). These results corresponded to 13% of the group 2 individuals (n = 46) who fulfilled the WHO criteria for ZIKV infection. Comparing our seropositivity rates of 1.8% for ZIKV IgM and 1.0% for IgG among pregnant women, a study conducted in

### Table 1. Demographic characteristics of pregnant women and the mode of delivery show clinical symptoms associated with ZIKV infection

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group 1 (n=171)</th>
<th>Group 2 (n=46)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35-42</td>
<td>27 (15.8%)</td>
<td>14 (30.4%)</td>
<td>0.07</td>
</tr>
<tr>
<td>20-34</td>
<td>112 (65.5%)</td>
<td>27 (58.6%)</td>
<td></td>
</tr>
<tr>
<td>13-19</td>
<td>32 (18.7%)</td>
<td>5 (10.8%)</td>
<td></td>
</tr>
<tr>
<td>Type of Delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-section</td>
<td>84 (49%)</td>
<td>16 (34.7%)</td>
<td>0.69</td>
</tr>
<tr>
<td>Vaginal Delivery</td>
<td>87 (51%)</td>
<td>30 (65.2%)</td>
<td></td>
</tr>
</tbody>
</table>

*p-value < 0.05 – Significant.

### Table 2. Groups 1 and 2 show signs and symptoms as per the WHO criteria for clinical diagnosis of ZIKV infection

<table>
<thead>
<tr>
<th>Variables</th>
<th>Signs</th>
<th>Group 1 (n=171)</th>
<th>Group 2 (n=46)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO Criteria</td>
<td>Rash</td>
<td>25 (14.61%)</td>
<td>2 (4.34%)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>Conjunctivitis</td>
<td>8 (4.67%)</td>
<td>1 (2.17%)</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>Arthritis</td>
<td>18 (10.52)</td>
<td>3 (6.5%)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Fever</td>
<td>11 (6.43%)</td>
<td>10 (21.7%)</td>
<td>0.55</td>
</tr>
<tr>
<td>Other Symptoms</td>
<td>Lymphadenopathy</td>
<td>2 (1.17%)</td>
<td>1 (2.17%)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Myalgia</td>
<td>2 (1.17%)</td>
<td>4 (8.7%)</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Headache</td>
<td>105 (61.40%)</td>
<td>25 (54.34%)</td>
<td>0.89</td>
</tr>
<tr>
<td>Fulfilled any one of the WHO criteria</td>
<td></td>
<td>62 (36.25%)</td>
<td>16 (34.7%)</td>
<td>0.20</td>
</tr>
<tr>
<td>Not fulfilled WHO criteria</td>
<td></td>
<td>109 (63.74%)</td>
<td>30 (65.21%)</td>
<td>0.20</td>
</tr>
<tr>
<td>ZIKV Serology (IgM + IgG) *</td>
<td></td>
<td>–</td>
<td>41/46**</td>
<td>–</td>
</tr>
<tr>
<td>DENV Serology (IgM + IgG) *</td>
<td></td>
<td>124/171***</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*The samples which showed the ab titer above the cut off and high titer either in ZIKV or DENV. The double positive sample was considered under ZIKV due to its clear positive titer by PRNT.

** 5 samples out of 46 did not show any antibody titer to ZIKV and DENV.

***47 out of 171 did not show any antibody titer to DENV and ZIKV.
the Najran region reported higher rates of 5.85% and 2.68% for ZIKV IgM and IgG, respectively (Alayed et al., 2018). This slight increase in seropositivity may be attributed to the larger sample size and longer duration of their study. Another study carried out in north central Nigeria reported anti-ZIKV-positive rates of 4% for IgM and 3% for IgG, which were slightly higher than our findings (Mathe et al., 2018). Since cross-reactivity among Flavi viruses is common, and the seroprevalence of dengue fever was unknown in our area, we assessed the samples for DENV IgM and IgG. The results showed seropositivity of 1.3% (n = 3) for both DENV IgM and IgG (Table 3, Figures 1a and b).

It was important to investigate cross-reactivity with DENV because there have been no reported cases of ZIKV in this part of Saudi Arabia. The results for DENV were as expected, with a seropositivity rate of 1.3%. This further supports the challenging nature of interpreting ZIKV serology, particularly in regions like ours where DENV is either not reported or is endemic. These findings align with previous studies highlighting the cross-reactivity of ZIKV serology with other flaviviruses, especially DENV (Balmaseda et al., 2017; Premkumar et al., 2018; Low et al., 2021). Diagnostic laboratories must be aware of this cross-reactivity, particularly in countries where molecular diagnosis is not feasible and there are limited laboratory facilities. Serological testing using IgM ELISA becomes the mainstay of diagnosis in such cases (Musso & Gubler, 2016). However, studies suggest using a highly sensitive test followed by a more specific one to address the cross-reactivity among flaviviruses (Malone et al., 2016; Michelson et al., 2019).

Table 3. Comparison of seropositivity prevalence to ZIKV and DENV among pregnant women

<table>
<thead>
<tr>
<th>Test</th>
<th>Abs</th>
<th>Positivity (%) and n</th>
<th>ZIKV + DENV IgM Double Positive (%) and n</th>
<th>ZIKV + DENV IgG Double Positive (%) and n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIKV</td>
<td>IgM</td>
<td>1.8 (4)</td>
<td>0.4 (1)</td>
<td>NIL</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>1.0 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV</td>
<td>IgM</td>
<td>1.3 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>1.3 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Comparison of seropositivity prevalence to ZIKV and DENV among pregnant women.

None of the samples tested positive for both ZIKV IgM and IgG or DENV IgM and IgG. However, we did observe that 0.4% (n = 1) of the total 217 individuals or 14% (n = 1) out of the seven individuals who tested positive for ZIKV IgM (n = 4) or DENV IgM (n = 3) were positive for both ZIKV and DENV IgM (Table 3). When examining the overlapping serotiters of ZIKV IgM and IgG with DENV IgM and IgG, respectively, a few samples showed very high overlapping titers, with one sample being positive for both ZIKV and DENV IgM. However, no other samples showed positivity or a statistically significant positive correlation between high titers of ZIKV and DENV IgM (r (8) = 0.07, p > 0.05) or IgG (r (22) = 0.90, p > 0.05) values. It is worth noting that some of the positive samples for ZIKV IgG displayed high serotiters for DENV IgG, indicating the presence of cross-reactivity. This observation showed a near-positive correlation value of 0.90, although statistical significance was not achieved (Figures 2 and 3).

Furthermore, high-titer samples positive for ZIKV were subjected to confirmation through plaque reduction neutralization tests (PRNTs). One sample (n = 1) was tested by the Arboviral Diseases Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80521, and was found to be positive. This particular sample showed a high titer for ZIKV IgG; however, it did not meet the criteria for positivity according to the kit standards. Unfortunately, obtaining a second or paired sample for follow-up was impossible. These results indicate the ongoing prevalence of ZIKV infection, highlighting the need for testing both asymptomatic and symptomatic pregnant women and further monitoring of the fetus (Duffy et al., 2009; Melo et al., 2016). Screening for the incidence of ZIKV becomes particularly challenging using serology in populations where evidence of the presence or pre-exposure of ZIKV and DENV is not periodically documented. This cross-reactivity complicates the determination of ZIKV incubation and antibody prevalence, further confounded by other poorly investigated flaviviruses.

**CONCLUSION**

Many pregnant women exhibited clinical symptoms associated with ZIKV, with the highest occurrence observed during the 20–34 gestational weeks. IgM and IgG antibodies against ZIKV were detected in pregnant women in and around Abha. Approximately 13% of the samples showing clinical symptoms based on the WHO criteria for ZIKV exhibited either ZIKV IgM or IgG antibodies.

![Figure 1](image-url)  
**Figure 1.** a. Results of Zika and dengue IgM ELISA. b. Results of Zika and dengue IgG ELISA.
Figure 2. Overlapping serotiters of both Zika and dengue IgM. The inner panel shows selected high-titer samples overlapping.

Figure 3. Overlapping serotiters of both Zika and dengue IgG. The inner panel shows selected high-titer sample overlapping.
However, no significant correlation was found between clinical signs and ZIKV serology. The study revealed significant overlapping of ZIKV antibodies with DENV antibodies in the samples, indicating cross-reactivity among flaviviruses. Only one sample with a higher titer of ZIKV IgG was confirmed positive through PRNT. These findings highlight the need for broad screening of flavivirus-neutralizing antibodies. It is important to note that vector screening was not attempted to validate the presence of ZIKV, although there is a continuous inflow of travelers from ZIKV-endemic areas. Therefore, the current observations suggest the necessity for broader, multicentric studies nationwide, including vector screening, to obtain a clearer and more comprehensive understanding of the prevalence of Zika and to prepare for managing potential silent epidemics.

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Declaration of Conflict of Interest

Authors have no conflict of interest to declare

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