**Virologica Sinica**

**Supplementary Data**

**Screening for Arboviruses in Healthy Blood Donors: Experience from Karachi, Pakistan**

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**Materials and Methods:**

**Study setting and design**

This study was conducted at the blood bank and the clinical microbiology sections of the Department of Pathology and Laboratory Medicine, Aga Khan University. This was a descriptive (cross-sectional) study conducted on blood donors who presented to the blood bank from 1st July 2018 to 31st December 2018. The sampling method employed was consecutive (total enumerative) sampling. Three hundred and sixty healthy blood donors were screened for DENV, WNV and JEV.

**Inclusion & exclusion criteria**

All blood donors above 18 years of age who presented to the blood bank were included in the study. Blood donors who met any of the following criteria were deferred and hence, excluded from the study: less than 18 years of age, history of anaphylaxis, anaemia viz. a haemoglobin level of less than 13.5 g/dL for males and less than 12.0 g/dL for females, history of any antibiotic intake in the past two weeks, blood donation within past 4 months, blood recipient within past one year and history of any coagulation factor deficiency.

**Sample size calculation**

Sample sizes for this study were determined for detecting PCR positivity and seropositivity for arboviruses in donor blood samples using the WHO sample size software. A study conducted by Simmons et al. (Simmons et al., 2016) based on high incidence of CHIKV and frequency of viraemic blood donations, reported 161 (9.7%) positive for CHIKV RNA. We estimated that a minimum sample size of 210 donor blood samples would be needed to detect a 9.7% PCR positivity for arboviruses, keeping a 95% confidence interval and 4% margin of error.

Furthermore, a study conducted by Ranjan et al. (Ranjan et al., 2016) based on high seroprevalence of DENV infection in blood donors, reported 116 cases (58%) positive for anti-DENV IgG and 27 cases (13.5%) positive for anti-DENV IgM. We estimated that a minimum sample size of 280 donor blood samples will be needed to detect a 13.5% seropositivity of IgM antibodies for arboviruses, keeping 95% confidence interval and 4% margin of error. Thus, a minimal sample size of 280 donor blood samples was found to be adequate for detecting seropositivity and PCR positivity for arboviruses in donor blood samples in our study population.

**Sample collection**

For the purpose of ELISA and nucleic acid screening, 10 mL whole blood was collected in Gel tubes from each of the blood donors. After collection the blood was allowed to clot at room temperature for 15–30 minutes. The clot was removed by centrifuging at 1,000–2,000 ×*g* for 10 minutes in a refrigerated centrifuge. The resulting supernatant (serum) following centrifugation was transferred into a clean polypropylene tube by pipetting. The samples were maintained at 2–8 °C while handling. The serum was then apportioned into 0.5 mL aliquots and stored at –20 °C.

**Sample testing**

All the samples obtained were tested for nucleic acid detection and serum IgM antibodies. ELISA testing was performed using commercially available Detect™ IgM Capture ELISA kits (Inbios International, USA) kits. However, for DENV, two different kits were used: 191 samples were tested with DENV Detect™ IgM Capture ELISA kit (Inbios International, USA) and 169 samples were tested with Standard™ E Dengue IgM ELISA kit (SD Biosensor, Republic of Korea). This was due to shortage of kits during outbreak season after the monsoons. The decision for selecting Standard™ E Dengue IgM ELISA kit for ELISA testing of the remaining 169 samples was based on the similarity of the two kits. Both DENV Detect™ IgM Capture and Standard™ E Dengue IgM ELISA kits had similar controls, virus-specific recombinant antigens and antibodies and enzyme substrate. The incubation steps and interpretation methodology employed was also similar among the kits.

ELISA was performed in accordance with the manufacturer’s instructions. Briefly, serum samples along with negative and positive controls were diluted with the sample dilution buffer and incubated in microtiter wells coated with anti-human IgM antibodies, followed by incubation with virus-specific recombinant antigens. The wells were treated with a virus-specific monoclonal antibody labelled with the enzyme horseradish peroxidase HRP tetramethyl benzidine (TMB) substrate with recommended incubation time and washing cycles. An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by absorbance measurement at 450 nanometers.

The mean absorbance values of the positive and negative controls were calculated from the absorbance values of their duplicates/triplicates respectively, which were found to be within the acceptable range. Furthermore, the cut-off value was calculated using the following formula: cut-off value = mean absorbance of negative control + 0.400. Once the cut-off value was obtained, the index value for each sample was calculated by dividing the sample absorbance by the cut-off value.

For nucleic acid detection, the samples were amplified using real time reverse transcriptase polymerase chain reaction (RT-PCR). Nucleic acid extraction was performed using QIAamp Viral RNA Mini kit (Qiagen, Germany) as per the manufacturer’s protocol. The extracted viral RNA was reverse transcribed to cDNA using iScrpitTM cDNA Synthesis kit (Bio-rad, USA). The primer and probe sequences used for the amplification process of cDNA for each arbovirus are listed in Supplementary Table S3.

Specific primers for each virus were added to the respective reaction mixtures. As per manufacturer’s recommendation, one-tenth of the reaction volume was used for downstream PCR. The complete reaction mix was incubated in a thermal cycler using the following protocol: priming for 5 minutes at 25 oC, reverse transcription for 20 minutes at 40 oC and RT inactivation for 1 minute at 90 oC.

**Data recording and statistical analysis**

Baseline, demographic data and clinical information regarding blood donors was collected on a standardized study proforma. Information regarding donor’s age, gender, location, blood group and any history of fever were recorded. In addition, the blood products prepared and transfused from the donated blood of each of the study subjects were recorded in the blood bank data base. The recipients of blood and blood products were followed after transfusion for the occurrence of any transfusion reactions for the duration of their hospital stay.

Demographic and laboratory data (ELISA and PCR results) collected on hard copies of the study questionnaires were entered into Microsoft Excel (Microsoft Corporation; version 16.0.12026.20174 / September 17, 2019). Frequencies and proportions were computed for donors belonging to different districts of Karachi, their gender, age groups, and blood groups were tabulated according to positive and negative results on serology and PCR assay results. Clinical information including the presence of fever in the study subjects at the time of blood donation and occurrence of any transfusion reactions in recipients of products from the donated blood were also entered. Entry verification of data during transfer from hard copies to the statistical software was done by twice matching of each of the variables entered. This data was transferred to Statistical Package for Social Science program (SPSS) (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) for the purpose of statistical analysis. Statistical analysis was performed to assess any significance of association of donor’s age, gender, blood group and location in the Karachi City districts with the results of ELISA and PCR positivity by calculating odds ratio with their confidence intervals. Where values were too small, *P* values were calculated using Fisher exact test.

**References**

Ranjan, P., Natarajan, V., Bajpai, M., Gupta, E., 2016. High seroprevalence of dengue virus infection in blood donors from Delhi: a single centre study. J Clin Diagn Res 10:DC08–DC10.

Simmons, G., Brès, V., Lu, K., Liss, N.M., Brambilla, D.J., Ryff, K.R., Bruhn, R., Velez, E., Ocampo, D., Linnen, J.M., Latoni, G., Petersen, L.R., Williamson, P.C., Busch, M.P., 2016. High incidence of chikungunya virus and frequency of viremic blood donations during epidemic, Puerto Rico, USA, 2014. Emerg Infect Dis 22:1221–1228.

**Supplementary Table S1.** Demographic, geographical characteristics and laboratory data of the healthy blood donors positive for IgM antibodies (n = 26) for arboviruses screened at the Aga Khan University Karachi Sindh Pakistan (Jul–Dec 2018).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Donor Serial #** | **Age (years)** | **Gender** | **Blood group** | **Town/ City** | **Blood components transfused** | **Transfusion reaction(s) in recipients** | **RNA-PCR** |
| DENV IgM Positive blood donors: | | | | | | | |
| 1 | 41 | Male | B+ | Karachi | FFP, PRBC & Platelets | None | -ve |
| 2 | 25 | Male | A+ | Karachi | FFP, PRBC & Platelets | None | -ve |
| 3 | 56 | Male | B+ | Malir Town/ Karachi | PRBC & Platelets | None | -ve |
| 4 | 28 | Male | A+ | Karachi | PRBC & Platelets | None | -ve |
| 5 | 24 | Male | B+ | Gulshan Town/ Karachi | PRBC & Platelets | None | -ve |
| 6 | 31 | Male | B+ | Gulshan Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 7 | 33 | Female | B- | Jamshed Town/ Karachi | PRBC & Platelets | None | -ve |
| 8 | 22 | Male | B- | Gulshan Town/ Karachi | PRBC & Platelets | None | -ve |
| 9 | 39 | Male | B+ | North Karachi Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 10 | 50 | Male | B+ | Gulshan Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 11 | 22 | Male | AB+ | Gulshan Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 12 | 35 | Male | O+ | Quetta | FFP, PRBC & Platelets | None | -ve |
|  | | | | | | | |
| WNV IgM Positive blood donors | | | | | | | |
| 1 | 29 | Male | A+ | Gulshan Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 2 | 37 | Male | O+ | Karachi | FFP, PRBC & Platelets | None | -ve |
| 3 | 30 | Male | B+ | North Karachi Town/ Karachi | PRBC & Platelets | None | -ve |
| 4 | 36 | Male | O+ | North Karachi Town/ Karachi | PRBC & Platelets | None | -ve |
| 5 | 36 | Male | O+ | North Karachi Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 6 | 29 | Male | B+ | Liaquatabad Town/ Karachi | PRBC & Platelets | None | -ve |
| 7 | 22 | Male | O+ | Gulshan Town/ Karachi | None | None | -ve |
| 8 | 25 | Male | A+ | Orangi Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
| 9 | 24 | Male | O+ | Gulistan-e-Johar/ Karachi | PRBC & Platelets | None | -ve |
| 10 | 25 | Male | A+ | Gulistan-e-Johar/ Karachi | PRBC & Platelets | None | -ve |
| 11 | 24 | Male | B+ | Karachi | PRBC | None | -ve |
| 12 | 29 | Male | AB+ | Malir Town/ Karachi | FFP, PRBC & Platelets | None | -ve |
|  | | | | | | | |
| DENV & WNV IgM Positive blood donors | | | | | | | |
| 1 | 42 | Male | B+ | Malir Town/ Karachi | PRBC & Platelets | None | -ve |
|  | | | | | | | |
| DENV & WNV & JEV IgM Positive blood donors | | | | | | | |
| 1 | 25 | Male | O+ | Gulshan Town/ Karachi | FFP, PRBC & Platelets | None | -ve |

**Supplementary Table S2**. Distribution of the blood donors according to geographical location.

|  |  |  |  |
| --- | --- | --- | --- |
| **Province** | **District/ City** | **Number of donors n (%)** | **IgM positive cases n (%)** |
| **Sindh** | Karachi | 292 (81.1) | 25 (96.2) |
|  | Hyderabad | 6 (1.7) | 0 (0) |
| Thatta | 22 (6.1) | 0 (0) |
| Tharparkar | 2 (0.6) | 0 (0) |
| Sukkur | 4 (1.1) | 0 (0) |
| Tando Allahyar | 7 (1.9) | 0 (0) |
| Shikarpur | 1 (0.3) | 0 (0) |
| Kashmore | 1 (0.3) | 0 (0) |
| Total | 335 (93.1) | 25 (96.2) |
|  | | |  |
| **Baluchistan** | Quetta | 10 (2.8) | 1 (3.8) |
|  | Gwadar | 2 (0.5) | 0 (0) |
| Sibbi | 1 (0.3) | 0 (0) |
| Khuzdar | 3 (0.8) | 0 (0) |
| Hab Chauki | 1 (0.3) | 0 (0) |
| Swat | 1 (0.3) | 0 (0) |
| Not known | 1 (0.3) | 0 (0) |
| Total | 19 (5.3) | 1 (3.8) |
|  | | |  |
| **KPK** | Peshawar | 2 (0.5) | 0 (0) |
|  | Koshistan | 1 (0.3) | 0 (0) |
| Total | 3 (0.8) | 0 (0) |
|  | | |  |
| **Punjab** | Islamabad | 1 (0.3) | 0 (0) |
|  | Lahore | 1 (0.3) | 0 (0) |
| Attock | 1 (0.3) | 0 (0) |
| Total | 3 (0.8) | 0 (0) |

**Supplementary Table S3:** Primer and probe sequences used for RT-PCR and sequencing reactions

|  |  |  |
| --- | --- | --- |
| **Virus Type** | **Primer or Probe** | **Sequence (5**′ **– 3**′**)** |
| DENV-1 | DEN-1-NS1 forward | CAAAAGGAAGTCGYGCWATA |
|  | DEN-1-NS1 reverse | CTGAGTGAATTCTCTCTRCTRAAC |
| DEN-1-NS1 probe | FAM-CATGTGGYTGGGAGCRCGC-BHQ-1 |
| DENV-2 | DEN-2-NS1 forward | CAGGYTATGGCACYRTCACRAT |
|  | DEN-2-NS1 reverse | CCATYTGCAGCARCACSATCTC |
| DEN-2-NS1 probe | FAM-CTCYCCRAGAACGGGCCTMGACTTCAA-BHQ-1 |
| DENV-3 | DEN-3-NS1 forward | GGACTRGACACACGCACCCA |
|  | DEN-3-NS1 reverse | CATGTCTCTACCTTCTCGACTTGYCT |
| DEN-3-NS1 probe | FAM-ACCTGGATGTCGGCTGAAGGAGCTTG-BHQ-1 |
| DENV-4 | DEN-4-NS1 forward | TYRTYCTAATGATGCTRGTCG |
|  | DEN-4-NS1 reverse | TCCACCYGAGACTCCTTCCA |
| DEN-4-NS1 probe | FAM-ATGCGTAGGAGTRGGRAACA-BHQ-1 |
|  | | |
| WNV | WNV-NS2A forward | CCTTTTCAGYTGGGCCTTCTG |
|  | WNV-NS2A reverse | CAGTGTAVGTVATRCCCCCAA |
| WNV-NS2A probe | FAM-AGCCAAGATCAGCATGCCAGC-BHQ-1 |
|  | | |
| JEV | JEV-NS forward | CCTTTTCAGYTGGGCCTTCTG |
|  | JEV-NS reverse | CAGTGTAVGTVATRCCCCCAA |
| JEV-NS probe | FAM-TGACCATTCCTGCGGTTTTGGGG-BHQ-1 |