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Electronic Supplementary Material

Sero-epidemiological Survey of Crimean-Congo Hemorrhagic Fever among the Human Population of the Punjab Province in Pakistan

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Supplementary Materials and Methods

Ethical statement

Following approval vide letter no. IERB/129 dated September 26, 2016, sampling and necessary procedures were conducted according to international ethical research guidelines.

Study area

This study was carried out in the selected districts of the Punjab Province in Pakistan. The Punjab Province (31° N, 72° E) is the second-largest (205,344 km²) and most populous Province of Pakistan, with a population of 101.6 million. The Province shares a border with India to its south-east, Azad Kashmir to the north-east, the Khyber Pakhtunkhwa Province and federal capital area of Islamabad to the north, Sindh to the south-west, and Baluchistan to the west. Lahore is the largest city and capital of the Province. The Punjab Province has a diverse landscape, comprising of fertile alluvial plains with five rivers (Indus, Jhelum, Chenab, Ravi, Sutlej), hilly areas, salt range (Pothohar Plateau), and deserts (Thal and Cholistan). Most of the region experiences hot summers and

foggy winters with a temperature range of –10° C to 50°C. Taken together, the environment of the Punjab Province is very suitable for agriculture, livestock rearing, and perpetuation of a vast range of biological vectors.

Data collection

Over a period of 8 months (from October 2016 to May 2017), blood samples (n = 1052) were collected on a convenient basis as aseptically as possible from clinically suspected inhabitants visiting the primary health-care centers in each district. The suspected individuals had a history of persistent fever (>38°C) (https://prepare.ersnet.org/lrmedia/2018/pdf/515.pdf). The districts included Lahore, Chakwal, Jhelum, Rajanpur, Dera Ghazi Khan, Mianwali, Attock, Bahawalpur, Rahim Yar Khan, and Rawalpindi. Among these, seven districts (Rawalpindi, Chakwal, Attock, MianWali, Dera Ghazi Khan, Rajanpur, and Lahore) had had previous cases of CCHF (either clinical or antigen-based or both), while three (Jhelum, Rahim Yar Khan, Bahawalpur) were without any previously reported cases of CCHF. All the individuals included in the study were aged >18 years. After obtaining the participants' informed consent, information such as age, gender, occupation, history of tick bites, and knowledge about CCHF was collected. A total of 3–5 mL of blood was drawn from a peripheral vein in a gel-clot activator vacutainer (Improvacuter, Germany), centrifuged at 2000 *g* for 10 minutes and stored at –80^{o}C until further use.

Immunological assay

Serum samples were tested for the presence of anti-CCHFV IgG using an ELISA kit (VectoCrimea - CCHF - IgG ELISA, Vector Best, Novosibirsk, Russia), according to the manufacturer's instructions. Briefly, a 1:100 diluted serum sample (100 µL) was added in a 96-well plate and incubated at 37 °C for 1 hour along with positive and negative controls. The excess of antibodies was washed, followed by the addition of 100 µL of anti-human IgG HRP to detect the anti-CCHFV IgG in the serum sample. The plate was then further incubated at 37 °C for 30 minutes. The unbound HRP labelled anti-human IgG was washed, followed by addition of 100 µL of TMB substrate. The plate was incubated at room temperature in the dark for 25 minutes and the color intensity was measured at 450 nm using a 96-well microplate reader (Rayto, Hamburg, Germany) model RT 2100 C. The optical density (OD) of each sample was compared with a cut-off OD that was determined with the addition of 0.2

in the OD value of the negative control. A test sample was considered positive for CCHFV-IgG if the $OD_{sample} \geq OD_{cut\ off}$.

Statistical analysis

The obtained data were analyzed using the GraphPad Prism version 6.01 software ~~(Swift 1997)~~. Pearson's Chi-square test was used to evaluate the association between the occurrence of CCHF in patients and its potential categorical predictors. All variables with a P-value equal to or less than 0.20 in univariate analysis were included in the binary logistic regression as explanatory variables. P-values of < 0.5 were considered statistically significant.