

Dengue virus infection in Croatia: seroprevalence and entomological study

Iva Pem-Novosel¹, Tatjana Vilibic-Cavlek^{2,3}, Ira Gjenero-Margan¹, Bernard Kaic¹,
Andrea Babic-Erceg⁴, Enrih Merdic⁵, Alan Medic⁶, Miljenko Ljubic⁷,
Djana Pahor⁸, Marijan Erceg¹

¹Epidemiology Department, Croatian National Institute of Public Health, Zagreb, Croatia;

²Virology Department, Croatian National Institute of Public Health, Zagreb, Croatia;

³School of Medicine University of Zagreb, Zagreb, Croatia;

⁴Molecular Diagnostic Department, Croatian National Institute of Public Health, Zagreb, Croatia;

⁵Biology Department, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia;

⁶Epidemiology Department, Zadar County Institute of Public Health, Zadar, Croatia;

⁷Epidemiology Department, Dubrovnik County Institute of Public Health, Dubrovnik, Croatia;

⁸Epidemiology Department, Primorje-Gorski Kotar County Institute of Public Health, Rijeka, Croatia

SUMMARY

During 2011-2012, a total of 1,180 sera samples were collected from residents of seven Croatian counties located on the Adriatic Coast and four counties in northeastern Croatia and tested for the presence of dengue virus (DENV) IgG antibodies using ELISA. Reactive samples were further tested by IFA. Seven samples (0.59%) tested positive for DENV antibodies. Seroprevalence rates by county varied from 0-2.21%. The highest seropositivity rate (2.21%) was found in the Dubrovnik-Neretva County where autochthonous dengue cases were recorded in 2010. Additionally, 3,699 mosquitoes were collected from 126 localities along the Adriatic coast in August-September, 2011. *Aedes albopictus* was the most prevalent species (81.37%). No evidence of DENV RNA was detected by RT-PCR among 1,748 female mosquitoes.

KEY WORDS: Dengue virus, Seroprevalence, *Ae. albopictus*, Croatia.

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Dengue is one of the most important emerging arthropod-borne viral infectious diseases in the world, posing a major public health problem worldwide. It is caused by one of the four dengue virus serotypes (DENV 1-4) of the genus *Flavivirus*, from the family *Flaviviridae*. DENV is transmitted to humans through the bites of infected mosquitoes of the genus *Aedes*, most often *Aedes (Ae.) aegypti* and *Ae. albopictus* (WHO 2009).

Ae. albopictus originated in Southeast Asia and has spread during the last 30-40 years to North,

Central and South America, parts of Africa, northern Australia and Europe. Since its first appearance in Albania (1979), *Ae. albopictus* has been reported in 20 European countries (Medlock *et al.*, 2012).

In Croatia, *Ae. albopictus* was recorded for the first time in 2004 in the area surrounding Zagreb. Within two years, *Ae. albopictus* was further identified throughout the territory of the Adriatic coast and has become permanently established in the coastal areas of Croatia (Klobucar *et al.*, 2006). In September 2010, the first autochthonous case of dengue fever was recorded on the Pelješac peninsula. Croatia is the second country in Europe, after France, where the transmission of autochthonous dengue infection has been recorded (Gjenero-Margan *et al.*, 2011). In Croatia, the prevalence of DENV infection has not been widely investigated since 1980. A small serosurvey in

Corresponding author

Tatjana Vilibic-Cavlek, MD, PhD

Virology Department

Croatian National Institute of Public Health

Rockefellerova 12, Zagreb, Croatia

E-mail: tatjana.vilibic-cavlek@hzjz.hr

north-western Croatia was carried out in 2007 (Golubic' and Dobler, 2012). The aim of this seroepidemiological and entomological study was to estimate the prevalence of dengue virus (DENV) infection in Croatia among humans and mosquitoes.

During 2011-2012, 1,180 sera samples taken from residents of seven Croatian counties located on the Adriatic coast (n=904) and four counties in northeastern Croatia (n=276) were tested for the presence of DENV-specific IgG antibodies. The residents included 525 (44.5%) males and 655 (55.5%) females aged from 2 to 87 years. Serum samples were obtained from de-identified banks of samples in the County Public Health Institutes taken from patients coming in for routine testing (preoperative check-ups, physical examinations, patient contacts, antenatal screening and couples undergoing medically assisted reproduction) with no symptoms of acute febrile disease. None of them reported any illness consistent with dengue fever during 2010.

Serologic tests were performed by the National Reference Laboratory for Arboviral Disease at the Virology Department, Croatian National Institute of Public Health (CNIPH) using a commercial enzyme-linked immunosorbent assay (ELISA; Euroimmun, Lübeck, Germany). Positive and borderline samples were further tested by a commercial indirect immunofluorescence assay (IFA; Euroimmun, Lübeck, Germany). Reactive samples were additionally tested for West Nile virus (WNV; Euroimmun, Lübeck, Germany) and Chikungunya virus (CHIKV; Euroimmun, Lübeck, Germany) IgG antibodies to rule out cross-reactions. Samples from the northeastern part of Croatia were also analyzed for tick-borne encephalitis virus (TBEV).

Mosquitoes were collected in 126 localities on the Adriatic Coast, from Umag to Dubrovnik, in the period of highest mosquito activity, i.e. from August 16-September 24, 2011. The sampling of mosquitoes was carried out using Biogents Sentinel traps baited with the standard BG-Lure attractant.

Mosquitoes were identified by species and gender (Becker *et al.*, 2010). Females (n=1,748) were grouped into 46 pools of up to 40 specimens and tested for DENV RNA using a qualitative real-time reverse transcriptase-polymerase

chain reaction (RT-PCR). Viral RNA was extracted using the automatic vacuum extraction method - QIAextractor (Qiagen, Germany). The RT-PCR reactions were performed according to the manufacturer's instructions (SuperScript III Platinum One-Step RT-PCR system, Invitrogen). Pan-dengue primers and probe targeting 3'NC region were retrieved from the database at the National Center for Biotechnology Information (forward primer AGGACYAGAGGTTA-GAGGAGA; reverse primer CGYTCTGTGC-CTGGAWTGAT; probe FAM-ACAGCATATTGACGCTGGGARAGACC-TAMRA) (Leparc-Goffart *et al.*, 2009). Amplification and detection reactions were carried out using an ABI Prism 7500 detector (Applied BioSystems). The 40th cycle was used as a threshold (Ct>40). The study was approved by the Ethics Committees of the CNIPH and the County Public Health Institutes. Seven of the 1180 samples were found positive, with an overall seroprevalence rate of 0.59% (95% CI=0.15-1.03). One participant showed borderline results for DENV-IgG antibodies in the ELISA test. There were 5/904 positive samples (0.55%; 95% CI=0.07-1.04) from the Adriatic Coast and 2/276 (0.72%; 95% CI=0-1.73) from the northeastern part of Croatia. Using IF, all samples were confirmed as positive. Seroprevalence rates by county varied from 0-2.21%. The seropositive participants from the Adriatic Coast are residents of the Dubrovnik-Neretva County, Primorje-Gorski Kotar and Zadar County. The participants from northeastern Croatia are residents of the Osijek-Baranja County (Figure 1).

A total of 3,699 mosquitoes were collected along the Croatian littoral. Three mosquito species were found in the analyzed material: *Ae. albopictus*, *Culex (Cx.) pipiens* and *Culiseta (Cs.) longiareolata*. The most prevalent species was *Ae. albopictus* species (3010/81.37%) followed by *Cx. pipiens* species (688/18.59%). Only one specimen of the species *Cs. longiareolata* was found. No evidence of dengue viral RNA was detected by RT-PCR.

Seroepidemiological studies of DENV in endemic regions have shown prevalence rates of up to 91.6% (Braga *et al.*, 2011, Muhammad Azami *et al.*, 2011). There are very few data on the prevalence of DENV antibodies in Europe and the Mediterranean region. One Ital-



FIGURE 1 - Geographical distribution of dengue seropositive cases in Croatia.

ian study reported only a sporadic presence of DENV antibodies in the inhabitants of the Turin Province (Pugliese *et al.*, 2007). A Turkish study conducted in Central and Northern Anatolia found that 0.9% of blood donors were positive to DENV IgG antibodies (Ergünay *et al.*, 2010). There are few published seroprevalence studies on DENV in Croatia. One study was conducted back in 1980 in a limited area of northeastern Croatia bordering Hungary. Neutralizing antibodies against DENV type 1 and hemagglutination inhibition antibodies on a sample of 380 young healthy persons were documented in 2.1% and 3.9% cases, respectively, (Ropac *et al.*, 1988). Additionally, a small serosurvey of DENV on 300 samples of blood donors was performed in north-western Croatia in 2007. No samples tested positive to DENV antibodies (Golubić and Dobler 2012).

In 2010, autochthonous dengue fever cases were recorded in Croatia. Nucleotide sequence analysis showed that the viruses detected in patients from Pelješac belong to DENV genotype 1 (Kurolt *et al.*, 2013).

Having confirmed in October 2010 a second autochthonous case of dengue fever in a resident of the same settlement where a German patient had stayed on holiday, 112 sera samples were collected from inhabitants of the Pelješac peninsula and the island of Korčula. DENV-specific

antibodies were found in 5.4% of the samples (Gjenero-Margan *et al.*, 2011). No autochthonous dengue cases have been reported in Croatia since 2010. However, sporadic imported cases have continuously been notified (Vilibić-Cavlek *et al.*, 2012). Results of the present study demonstrate that 0.55% of Croatian coastal inhabitants have been exposed to DENV. The highest seropositivity rate (2.21%) was found in Dubrovnik-Neretva County, the same county where autochthonous dengue cases were reported in 2010. In northeastern Croatia, in the same geographic areas where the 1980 study was performed, the seroprevalence was 0.72%. An important aspect of dengue control is vector surveillance and control. Detection of DENV positive mosquitoes is useful for monitoring infection rates within the vector mosquito population and provides an early warning signal to predict a possible upcoming outbreak (Conceição *et al.*, 2010). Even in endemic countries in the middle of an outbreak, it is hard to find positive mosquitoes. The best method is to sample mosquitoes around the houses with known DENV cases during the time of transmission (Getis *et al.*, 2003; Chansang and Kittayapong, 2007; Guedes *et al.*, 2010). None of the tested mosquito pools in this study were found positive for DENV RNA.

The study has some limitations. The ideal method for serologic confirmation of DENV is the plaque-reduction neutralization test. However, we found no cross-reactions with WNV in ELISA and CHIKV antibodies in IFA. Two DENV reactive samples from patients residing in TBE endemic regions were also negative for TBEV in ELISA.

Despite these limitations, the results of this study showed a low DENV prevalence rate (0.59%) within the tested Croatian population. Vector control measures, dengue surveillance and health education should be performed on a continual basis to maintain the current favorable epidemiological situation. These results indicate that there is no widespread dengue infection in the Croatian population and that the risk of a dengue hemorrhagic fever outbreak is very low. However, seroepidemiological studies should be conducted regularly to identify a possible increased risk and take timely and appropriate measures.

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