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SEROPREVALENCE AND ENTOMOLOGICAL STUDY ON CHIKUNGUNYA VIRUS AT THE CROATIAN LITTORAL

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During 2011–2012, a total of 1008 serum samples from randomly selected inhabitants of seven Croatian counties located on the Adriatic Coast were tested for the presence of chikungunya virus (CHIKV) IgG antibodies using indirect immunofluorescence assay.

Nine participants (0.9%) from four counties were found to be seropositive to CHIKV. Seroprevalence varied from 0.5% to 1.8% between counties. Additionally, a total of 3,699 mosquitoes were captured in 126 localities from August 16 to September 24, 2011. Three mosquito species were found: *Ae. albopictus* (3010/81.4%), *Cx. pipiens* (688/18.6%) and only one specimen of the *Cs. longiareolata*. Female mosquitoes ($N = 1,748$) were pooled. All pools tested negative for CHIKV RNA using a real-time RT-PCR.

Keywords: Chikungunya virus, seroprevalence, Croatia, entomology

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Introduction

Chikungunya fever is a tropical arboviral disease caused by chikungunya virus (CHIKV) that belongs to the family *Togaviridae*, genus *Alphavirus*. Chikungunya is endemic in Africa, Southeast Asia and on the Indian subcontinent [1]. Its global emergence started in 2004. A large outbreak originated in Kenya has rapidly spread throughout numerous islands of the Indian Ocean (2005–2006), India (2006–2007) and parts of Southeast Asia (2006) [2–5]. After a few years of relative dormancy, CHIKV transmission restarted in 2009 and 2010. Many of these epidemics continued into 2012 [6]. In 2013, the first documented outbreak of CHIKV in the Americas was recorded (Caribbean Islands) [7].

CHIKV is maintained by two different transmission cycles. In Africa, the virus is maintained in a sylvatic cycle between non-human primates as the main reservoirs and different species of forest-dwelling mosquitoes *(Aedes, Mansonia, Culex)* [8]. In Asia, transmission of CHIKV occurs in an urban cycle involving humans and mosquitoes of the *Aedes* genus *(Ae. aegypti*, *Ae. albopictus)* [9].

In the last three decades, *Ae. albopictus* has rapidly expanded from Asia and Africa to the Americas and Europe [10, 11]. In Europe, travel-associated cases of chikungunya fever have been continuously recorded [12]. During the summer of 2007, a chikungunya outbreak involving more than 200 cases was reported in the Ravenna Province, north-eastern Italy after virus introduction from India which was the first transmission of chikungunya fever within Europe [13, 14]. This outbreak raised concern because the vector *Ae. albopictus* has already been introduced in many European countries, including Albania, Belgium, Bosnia and Herzegovina, France, Greece, Italy, the Netherlands, Serbia, Montenegro, Slovenia, Spain and Switzerland [15]. In addition, in September 2010, autochthonous transmission of CHIKV with two confirmed cases was recorded in the French Riviera [16].

For the first time in Croatia, *Ae. albopictus* was registered in Zagreb, the capital of Croatia, in October 2004 [17]. From September to October 2005, *Ae. albopictus* was discovered in several places in the Istria peninsula [18]. Since Italy has been known for many years as a country with a high concentration of *Ae. albopictus* [19], its spread to Croatia as a neighbouring country was expected. Within two years, additional records of *Ae. albopictus* presence were made in many Croatian places along the Adriatic Coast [20–21].

We conducted a seroprevalence study on CHIKV among inhabitants of the Croatian counties located on the Adriatic coast and entomologic study for the presence of CHIKV RNA in mosquito samples.

Materials and Methods

During 2011–2012, a total of 1008 serum samples were tested for the presence of CHIKV IgG antibodies. The samples were randomly selected from de-identified banks of samples taken from participants aged 3–88 years coming for routine testing at 7 Croatian public health institutes located on the Adriatic Coast. None of them reported symptoms of recent febrile disease. CHIKV IgG antibodies were detected using a commercial indirect immunofluorescence assay (Anti-Chikungunya virus IgG IIFT, Euroimmun, Lübeck, Germany). IgG titer ≥10 was considered positive. Reactive samples were further tested for CHIKV IgM antibodies (Anti-Chikungunya virus IgM IIFT, Euroimmun, Lübeck, Germany) as well as for potential cross-reactivity with dengue virus (DENV; Anti-Dengue virus IgG ELISA, Euroimmun, Lübeck, Germany) and West Nile virus (WNV; Anti-West Nile virus IgG ELISA, Euroimmun, Lübeck, Germany).

In addition, entomologic investigation was performed from August 16 to September 24, 2011, in the period of the highest mosquito activity. A total of 3,699 mosquitoes were captured at 126 locations along the entire Adriatic Coast. Sampling was carried out using Biogents Sentinel traps baited with the standard BG-Lure attractant. Collected mosquitoes were separated by species and gender. Female mosquitoes ($N = 1{,}748$) were pooled (46 pools of up to 40 specimens) and analyzed for CHIKV RNA using a qualitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). For nucleic acid isolation, an automated system QIAxtractor (QIAGEN) was used. Real-time RT-PCR was performed using a single-tube RT-PCR kit according to the manufacturer's instructions (Invitrogen SuperScript™III Platinum® One-Step Qualitative Kit). The amplification and detection were performed with 7500 Real Time PCR System machine (Applied Biosystems). Each tube contained a 25-µl reaction mixture which included 5 µl of isolated RNA, 0.2 µM forward primer (CCGAAAGGAAACTTCAAAGCAACT), 0.2 µM reverse primer (CAGATGCCCGCCATTATTGAT) and 0.1 µM probe (FAM-GGGAGGTGGAGCATG-MGB). The reaction mixtures were exposed to a 30 min 50 °C RT step, a 2 min of Taq activation at 95 °C and 50 cycles consisting of 95 °C for 15 s and 55 °C for 32 s.

The study was approved by the Ethics Committee of the Croatian National Institute of Public Health.

Results

Nine samples (0.9%, 95%CI = 0.4–1.7) were found positive for CHIKV IgG antibodies. CHIKV IgM antibodies were not documented in any IgG seropositive

Table I. Prevalence of chikungunya virus IgG antibodies among counties

County	Tested *N* (%)	CHIKV IgG positive *N* (%)	95%CI
Overall	1008 (100)	9 (0.9)	0.4–1.7
Istria	236 (23.4)	4 (1.7)	0.5–4.3
Primorje-Gorski Kotar	112 (11.1)	2 (1.8)	0.2–6.3
Lika-Senj	99 (9.8)	0	0.0–3.7
Zadar	114 (11.4)	2 (1.8)	0.2–6.3
Sibenik-Knin	108 (10.7)	0	0.0–3.4
Split-Dalmatia	200 (19.8)	1 (0.5)	0.0–2.8
Dubrovnik-Neretva	139 (13.8)	0	0.0–2.6

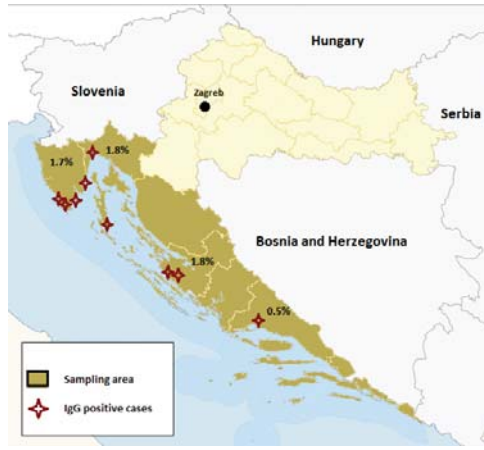

Figure 1. Geographical distribution of chikungunya virus IgG positive cases

sample. No one sample showed cross-reactive antibodies with DENV and WNV. Five (55.5%) positive samples were from male and four (45.5%) from female subjects from four Croatian counties. The median age of seropositive participants was 36.7 years (range 15–77 years). The seroprevalence varied from 0.5% to 1.8% among counties (Table I). Geographical distribution of seropositive cases is presented in Figure 1. Four participants were residents of the Istrian peninsula, two participants were residents of north Croatian coast and Veli Lošinj island while three participants were from Middle Dalmatia.

Entomologic investigation revealed three mosquito species. Out of 3,699 mosquitoes collected, 3010 (81.4%) were *Ae. albopictus*, 688 (16.6%) were *Cx. pipiens*

and only one specimen was *Cs. longiareolata*. All tested mosquito pools were negative for CHIKV RNA.

Discussion

CHIKV is a re-emerging virus of public health importance causing major outbreaks in Africa and Southeast Asia [2, 4, 7, 22, 23]. In endemic regions, the seroprevalence rates reach 75% [5, 24]. With an increase in global travel, the risk for CHIKV spreading outside of endemic areas has heightened. Imported CHIKV infections in travellers have been regularly reported in several European countries [12]. However, autochthonous transmission was documented only in Italy and France [13, 14, 16]. In Europe, there are few published studies on the prevalence of CHIKV. An Italian study conducted in the two neighbouring villages to Emilia-Romagna Region where the outbreak of chikungunya occurred in 2007 showed a prevalence of 10.2% [25]. An entomological survey carried out on August 21 and 22, 2007 in the same area demonstrated CHIKV RNA in one pool of mosquitoes collected in Castiglione di Ravenna [26]. Moreover, CHIKV antibodies were documented in 1.3% German aid workers, with the highest seroprevalence reported in those who had resided in Benin (5.7%) and Thailand (5.5%) [27].

In Croatia, autochthonous transmission of some mosquito-borne arboviruses such as dengue virus (2010) [21, 28], West Nile virus (2012–2013) [29, 30] and Usutu virus (2013) [31, 32] has been documented. Seroepidemiological studies on dengue and West Nile virus showed prevalence rates of 0.6% and 1%, respectively [33]. In addition, CHIKV IgG antibodies were sporadically detected in travellers from endemic areas (data from the National Reference Laboratory for Arboviruses, Croatian National Institute of Public Health). However, there are no data on the seroprevalence of CHIKV. The results of this study showed that 0.9% of inhabitants of the Croatian littoral have been exposed to this virus. Since autochthonous transmission of chikungunya is not documented in Croatia so far, seropositive persons have most likely been exposed to CHIKV abroad.

The presence of *Ae. albopictus* is well known for several years along the entire Croatian littoral. *Ae. albopictus* was the most prevalent mosquito species detected in this study (81.4%), however, CHIKV RNA was not found in any of the tested pools.

In conclusion, the results of this study indicate a low CHIKV prevalence rate (0.9%) among inhabitants of the coastal Croatian region. Although Croatia is not endemic country for CHIKV, current data including recent autochthonous Italian and French CHIKV cases indicate that indigenous transmission of CHIKV

is possible. Permanent vector control measures should be regularly performed as the risk of chikungunya fever is definitely present, particularly in regions with established *Ae. albopictus* population.

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Conflict of Interest

None.

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