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Detection of West Nile Virus – Lineage 2 in *Culex pipiens* mosquitoes, associated with disease outbreak in Greece, 2017

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ABSTRACT

During July-October 2017 a WNV outbreak took place in the Peloponnese, Southern Greece with five confirmed deaths. During routine monitoring survey in the Peloponnese, supported by the local Prefecture, we have confirmed the presence of all three *Culex pipiens* biotypes in the region, with a high percentage of *Culex pipiens/molestus* hybrids (37.0%) which are considered a highly competent vector of WNV. *Kdr* mutations related to pyrethroid resistance were found at relatively low levels (14.3% homozygosity) while no mosquitoes harboring the recently identified chitin synthase diflubenzuron-resistance mutations were detected in the region. As an immediate action, following the disease outbreak (within days), we collected a large number of mosquitoes using CO_2 CDC traps from the villages in the Argolis area of the Peloponnese, where high incidence of WNV human infections were reported. WNV lineage 2 was detected in 3 out of 47 *Cx. pipiens* mosquito pools (detection rate = 6.38%). The virus was not detected in any other mosquito species, such as *Aedes albopictus*, sampled from the region at the time of the disease outbreak. Our results show that detection of WNV lineage 2 in *Cx. pipiens* pools is spatially and chronologically associated with human clinical cases, thus implicating *Cx. pipiens* mosquitoes as the most likely WNV vector. The absence of diflubenzuron resistance mutations and the low frequency of pyrethroid (*kdr*) resistance mutations indicates the suitability of these insecticides for *Cx. pipiens* control, in the format of larvicides and/or residual spraying applications respectively, which was indeed the main (evidence based) response, following the disease outbreak.

1. Introduction

During July-October 2017, a West Nile Virus (WNV) outbreak unraveled in Southern Greece, in the Peloponnese region, the largest peninsula in Greece covering 21 439 km^2 and hosting 1 100 071 people. Until October 25, 2017 the outbreak has resulted in five human fatalities and a total of forty-eight confirmed WNV-infection cases in Greece. Twenty-eight of these cases (58.3%) presented West Nile neuroinvasive disease (WNND) with severe clinical manifestations. The suspected prefectures of exposure were Argolis (municipalities of Argos-Mykines and Nafplio) and Arcadia (municipality of North Kynouria) (HCDCP, 2017). WNV is thought to circulate in Europe at least since the 1960s and WNV Lineage 2 is responsible for one of the deadliest outbreaks in Europe occurring in Northern Greece during the summer of 2010, resulting in 262 clinical human cases and 35 fatalities. Successive outbreaks occurred over the next four years mainly in Central and Northeastern Greece (Gossner et al., 2017; Patsoula et al., 2016; Hernandez-Triana et al., 2014).

WNV is an RNA virus belonging to the Flaviviridae family, which is transmitted mainly by the mosquito species *Culex pipiens*. *Aedes albopictus* can also transmit the virus, but is considered a secondary vector (Hernandez-Triana et al., 2014; Fortuna et al., 2015; Giatropoulos et al., 2012). Birds are amplifying hosts of WNV while mammals, including humans, are incidental and “dead-end” hosts. Among the three *Cx. pipiens* biotypes, *Cx pipiens pipiens*, *Cx. pipiens molestus* and the hybrid form *Cx. pipiens pipiens/molestus*, the latter is considered a highly competent vector of WNV due to its opportunistic biting behavior which maintains the virus among avian hosts and favors its

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transmission to humans. Mosquito surveillance following the major disease outbreak in 2010, recorded the presence of all three *Cx. pipiens* biotypes in Central and Northern Greece including the hybrid form, which could be a key factor for the establishment of WNV transmission in Greece (Fortuna et al., 2015; Giatropoulos et al., 2012; Fotakis et al., 2017; Gomes et al., 2013; Patsoula et al., 2017).

The main way to control mosquitoes and diseases transmitted by mosquitoes, particularly in the absence of effective vaccines as in the case of WNV, is the reduction of the vector's population size by chemical control. This includes the use of mosquito adulticides, primarily pyrethroids in the form of insecticide residual sprays (IRS), or fogging, or treated material and the application of larvicides, such as diflubenzuron and *Bacillus thuringiensis* (Bt), at the mosquito breeding sites (Zhang et al., 2016; Guidi et al., 2013). However, insecticide resistance against both pyrethroids and diflubenzuron has been reported in *Cx. pipiens* and has been linked to mutations in the voltage-gated sodium channel (*kdr* mutations) and chitin synthase gene, respectively (Fotakis et al., 2017; Grigoraki et al., 2017; Douris et al., 2016).

Routine epidemiological surveillance for WNV is essential for confronting the virus in Greece and the whole of Europe (Gossner et al., 2017). Vector monitoring is a central component of WNV surveillance and a prerequisite towards an integrated approach that could produce early and reliable alarms for potential disease outbreaks (Gossner et al., 2017). During surveillance, it is important to both identify the vector and detect mutations associated to insecticide resistance in order to efficiently apply the most suitable insecticide.

Here, we report the identification of WNV lineage 2 in *Cx. pipiens* mosquito pools from the Argolis area of the Peloponnese, at the time when severe human clinical infection cases were reported. The biotype composition and insecticide resistance profile of *Cx. pipiens* mosquitoes from the Peloponnese region is also reported, to support evidence based management activities.

2. Materials and methods

2.1. Routine entomological monitoring and genotyping for species ID and resistance mutations

Routine entomological monitoring was conducted in the Peloponnese, supported by the local Prefecture since 2016, with CO_2 (dry-ice)-baited CDC traps. Specimens were preserved at −80 °C until testing. Ethical approval was not sought since no personal data of human cases were needed for the study. Following morphological analysis to species level, *Cx. pipiens* female adults were used for the molecular analysis. Genomic DNA (gDNA) was extracted from single mosquitoes using the DNAzol protocol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Biotype analysis was performed with the use of a PCR diagnostic assay (Bahnck and Fonseca, 2006) which relies on polymorphisms of the 5′ flanking region of the microsatellite locus CQ11 specific for *pipiens* and *molestus* alleles (primers in Suppl. Table 2). The thermal cycler conditions were: 95 °C–5 min, followed by 40 cycles × [94 °C–30 s, 54 °C–30 s, 72 °C–40 s] and a final extension at 72 °C–5 min. DNA fragments were analyzed by electrophoresis on a 2.0% w/v agarose gel. The presence and frequency of the target-site knock down resistance (*kdr*) mutation L1014F (TTA to TTT) on the voltage gated sodium channel was detected with an allele specific PCR assay (Martinez-Torres et al., 1999) (primers described in Suppl. Table 2). The PCR conditions were: 95 °C −5 min followed by 40 cycles × [94 °C–30 s, 48 °C–30 s, 72 °C–1 min] and a final extension at 72 °C–10 min. DNA fragments were analyzed by electrophoresis on a 1.5% w/v agarose gel. The presence of chitin synthase C-terminus target site resistance mutations spanning the 1043 position was detected with a PCR reaction as described in (Grigoraki et al., 2017) (primers in Suppl. Table 2). The PCR conditions were: 95 °C for 5 min followed by 30 cycles × [94 °C −30 s, 60 °C–30 s, 72 °C–1 min] and a final extension of 72 °C–10 min. PCR products were purified using a commercially available kit (PCR clean-up, Macherey Nagel, Dueren, Germany) and sequenced (Macrogen Sequencing Facility, Amsterdam) using the primer 5′- ACGTTTGCGGGTGTGATGTC-3′. Direct sequencing protocols for *kdr* and diflubenzuron resistance mutations were additionally applied in five randomly selected *Cx. pipiens* mosquito pools (cDNA samples) used for WNV detection. For the *kdr* analysis the PCR conditions were as described above, but with the use of Cgd1 and Cgd2 primers (Suppl. Table 2). PCR products were purified from a 2.0% w/v agarose gel using a commercially available kit (DNA extraction from agarose gel, Macherey Nagel, Dueren, Germany) and sequenced (Macrogen Sequencing Facility, Amsterdam) using the primer Cgd1. For the detection of diflubenzuron resistance mutations, we followed the respective protocol described above. The mutant allele frequency in the mosquito pools was calculated by analyzing the heights of fluorescence peaks from the electropherogram (Bioedit) as described in (Van Leeuwen et al., 2008).

2.2. Targeted mosquito sampling, following WNV incidence

From July 28, 2017 till August 19, 2017 mosquitoes were collected using CO_2 (dry-ice)-baited CDC traps in the prefectures of Argolis and Arkadia in the Peloponnese, where the highest incidence of WNV infection human cases were reported. Mosquitoes were counted and identified to species level using morphological characteristics (Becker et al., 2010) and were pooled based on mosquito species and sampling location in N = 47 pools (average = 18 mosquitoes per pool, range = 5–32) (more details are presented in Suppl. Table 1). Samples were preserved at −80 °C until analysis. Routine entomological monitoring survey was conducted in the Peloponnese, supported by the local Prefecture since 2016, with CO_2 (dry-ice)-baited CDC traps. Ethical approval was not sought since no personal data of human cases were needed for the study.

2.3. Total RNA extraction from mosquito pools

Total RNA was isolated using the TRI reagent protocol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The concentration and purity of the extracted total RNA for each pool was determined by spectrophotometry using a NanoDrop 2000c spectrophotometer (Thermo Scientific) and its integrity was confirmed *via* agarose gel electrophoresis (1.0% w/v).

2.4. Molecular detection of Flaviviruses

Conventional one-step Reverse Transcriptase-PCR (RT-PCR) was performed to assess the presence of Flaviviruses, based on the method described by Kuno G. et al. (Kuno et al., 1998). A ready to use RT-PCR master mix was used (FTD, Luxembourg) with 1 μM of primers (described in Suppl. Table 2) and 2.0 μg of total RNA in a final reaction volume of 20 μL. The RT-PCR thermal protocol for the Pan-Flavivirus assay was the following: 50 °C–30 min, 95 °C–3 min, 40 cycles × [95 °C–15 s, 55 °C–20 s, 72 °C–60 s], 72 °C–10 min. Electrophoreses of PCR products was performed on 1.5% w/v agarose gels.

2.5. Molecular detection of WNV

Detection of WNV was performed by a multiplex Real Time one step RT-PCR TaqMan assay that simultaneously detects and differentiates WNV-lineage 1 and WNV-lineage 2 (Del Amo et al., 2013; Jimenez-Clavero et al., 2006). Reactions were performed in the CFX Connect™ Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using a RT-PCR master mix (FTD, Luxembourg), 500 nM primers, 300 nM probes (Suppl. Table 2) and 2.0 μg of total RNA in a total reaction volume of 10 μL. The thermal cycle parameters were: 50 °C–15 min, 95 °C–3 min, and 45 cycles × [95 °C–3 s and 60 °C −30 s]. Samples were amplified in triplicates and each run always included a non-template control and a