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Abstract

West Nile virus (WNV) is one of the most widely distributed mosquito-borne viruses in the world. In North Africa, it causes human cases of meningoencephalitis with fatalities in Algeria and in Tunisia, whereas only horses were affected in Morocco. The aims of this study were to detect WNV in mosquitoes and to determine seroprevalence of WNV in Moroccan horses by the detection of IgG antibodies. A total of 1,455 mosquitoes belonging to four different species were grouped by collection site, date, and sex with 10 specimens per pool and tested for 38 arboviruses using a high-throughput chip based on the BioMark Dynamic array system. Out of 146 mosquito pools tested, one pool was positive for WNV. This positive pool was confirmed by real time RT-PCR. The serosurvey showed that 33.7% (31/92) of horses were positive for competitive enzyme-linked immunosorbent assay (cELISA) test. The flavivirus-sphere microsphere immnoassay (MIA) test, targeting three flaviviruses (WNV, Usutu virus (USUV) and Tick borne encephalitic virus (TBEV)) showed that 23 sera out of 31 were positive for WNV, two for USUV, two for USUV or WNV, and four for an undetermined flavivirus. Virus neutralization tests with USUV and WNV showed that 28 of 31 sera were positive for WNV and all sera were negative for USUV. This study reports, for the first time, the detection of WNV from *Culex pipiens* mosquitoes in Morocco and its circulation among horses. This highlights that the detection of arboviruses in mosquitoes could serve as an early warning signal of a viral activity to prevent future outbreaks in animals and humans.

Keywords: Mosquito-borne-viruses; *Culex pipiens*; Horses; WNV; RT-PCR; Serological technics

1. Introduction

Arboviruses (Arthropod-borne viruses) circulate primarily within an enzootic cycle among wild animals, and spillover transmission to humans and domestic animals have been more frequently reported since two decades (Weaver and Reisen, 2010). Some viruses such as dengue (DENV), chikungunya (CHIKV) and Zika, do not longer need to amplify in animal species to trigger urban epidemic cycles and produce extensive epidemics (Weaver et al., 2018). Factors contributing to these changes are diverse but all point out the preponderant role of human activities in creating ecological niches suitable to vector-borne pathogens, worsened by climate changes offering larger regions suitable to mosquito development (Sutherst, 2004). Among these arboviruses, West Nile virus (WNV; *Flaviviridae*, *Flavivirus*) is maintained in nature in an enzootic transmission cycle between birds and *Culex* mosquitoes. It also infects humans and other animals causing a serious disease and death (Hayes, 2001; Murgue et al., 2001). Birds are considered to be the most important hosts of WNV because they can develop a sufficient viremia to infect mosquitoes after a blood supply (Komar et al., 2003). Humans and horses develop weak and short-term viremia and are considered as dead end hosts (Bowen and Nemeth, 2007). Since its discovery in 1937 in Uganda (Smithburn, 1942), WNV has circulated in Africa mainly associated with mild symptoms (Benjelloun et al., 2016; Sule et al., 2018). The first outbreak describing neuroinvasive symptoms in humans was reported in Israel in 1951 (Murgue et al., 2001). After a silence of more than 30 years, a new emergence, in Romania in 1996 then in Italy and in France respectively in 1998 and in 2000. Since 2004, new strain of the line2 circulates in Europe (Lecollinet et al., 2020). In 1999, WNV was first detected in the Unites States, in New York (Nash et al., 2001) and then spread to the West coast, to Canada and to Central and South America (Banet-Noach et al., 2003; Charrel et al., 2003).

Currently, WNV is present in Africa, the Middle East, Europe, Asia, Australia and America and has become the most widely distributed of the encephalitic flaviviruses (Chancey et al., 2015). It possesses one of the highest potentials for re-emergence in North Africa; WNV circulates actively in the region based on two lines of evidence: (i) repeated reports of WNV outbreaks in this region and (ii) the vector *Cx. pipiens* widely present. WNV has been circulating for a very long time in the Mediterranean region (Murgue et al., 2001) affecting mainly humans and horses (Benjelloun et al., 2016; Johnson et al., 2018; Papa, 2019). In Morocco, several outbreaks of WNV have been reported: (i) in 1996 with 94 equine cases including 42 deaths and only one human case (El Harrack et al., 1997; Tber Abdelhaq, 1996),

(ii) in September and October of 2003 when WNV circulated among horses in Kenitra (Schuffenecker et al., 2005), (iii) in 2010 in horses (World Animal Health Information Database, 2010 ; Benjelloun et al., 2017; Durand et al., 2016; El Rhaffouli et al., 2013). The two strains of WNV (outbreaks in 1996 and 2003) belonged to clade 1a of lineage 1 (schuffenecker et al., 2005). The principal vector of WNV in Europe, Northern USA and North Africa is *Culex pipiens* which is the most widespread vector in temperate regions including North Africa (Amraoui et al., 2012b; Romo et al., 2018). Moreover, two coexisting *Culex pipiens* forms (*pipiens* and *molestus*) are distinguished in temperate areas and correspond to different behavioral, physiological and genetic forms (Harbach, 2012). The form *pipiens* is ornithophilic while *molestus* bites humans and other mammals (Fonseca et al., 2004). These two forms have been proven to hybridize in some regions such as the United States (Fonseca et al., 2004), South Europe (Gomes et al., 2009) and North Africa (Amraoui et al., 2012a; Krida et al., 2015). Hybrids show intermediate trophic preferences assigning them the role of bridge vector for a viral transmission from animals to humans (Fonseca et al., 2004). Targeted surveillance for WNV within mosquito populations offers an opportunity to detect virus prior to the emergence of disease in equine species or human populations (Calzolari et al., 2012). To date, no virological data concerning WNV in mosquitoes from Morocco are available. The aims of this study are: (i) Evaluation of the circulation of mosquito-borne arboviruses in Morocco using a new high-throughput tool based on the BioMark ™ Dynamic matrix system capable of screening a large panel of arboviruses in a single experiment. (ii) Detection of WNV past circulation in equids through the confirmation of anti-WNV antibodies in horse serum samples by three serological techniques: cELISA, MIA and microneutralization test (MNT).

2. Materials and methods

2.1. Study sites

The three regions selected for the study are: (i) Mohammedia, a large city located on the coast of the Atlantic Ocean, at 24 km northeast from Casablanca (33°40’25.3”N7°26’42.5”W), where WNV cases were reported in horses in 2010; (ii) Moulay Bouselham 34°52'28.7"N6°17'14.5"W, where WNV cases were reported in horses in 2010 and (iii) Tanger 35°46’44.3N5°50’50.1W located on the North of Morocco, where WNV cases were

reported in horses in 1996“. Sites of mosquito captures were identified based on records of past circulation areas for WNV (at a period corresponding to a season of high potential transmission risk with high densities of mosquitoes, presence of migratory birds, case reports of meningoencephalitis …).

2.2. Mosquito collection

Adult mosquito collections were conducted over four consecutive nights in September and October 2018 using six CDC light traps (Communicable Disease Centers of Atlanta, USA) supplemented with dry ice as a source of CO2 (from 7:00 pm to 7:00 am) in three regions (Figure 1). The traps were placed approximately 1.5 meters above ground. Traps were dispersed inside houses and near stables and lakes. Live mosquitoes were transported in cages to the laboratory to be identified using identification keys (Brunhes et al., 2000). The identification was carried out on living mosquitoes asleep by the cold on a freeze pack.

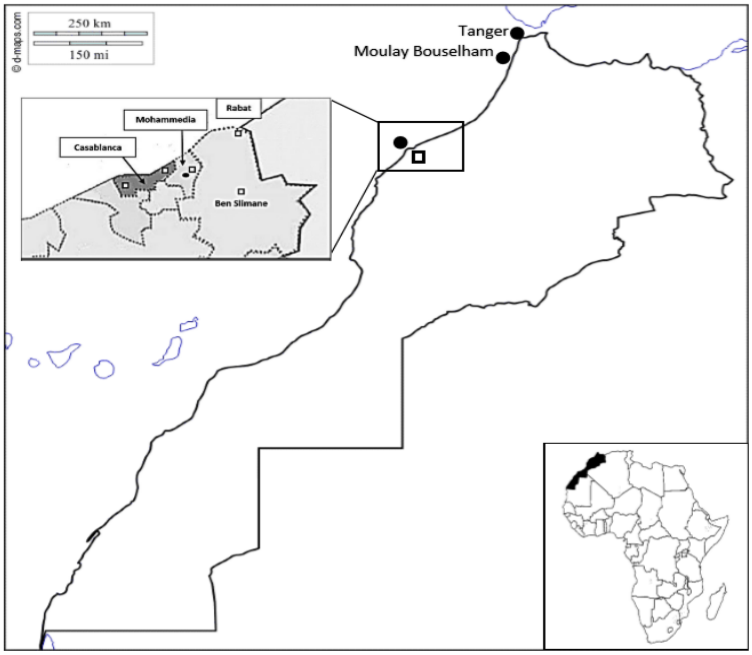

Fig.1. Map of the study area showing the location of mosquito collection cities (●) (Tanger, Moulay Bouselham, Mohammedia) and horse serum collection sites (□) (Casablanca, Mohammedia, Benslimane).

2.3. Mosquito dissection and RNA extraction

After morphological identification, mosquitoes were dissected on a freeze pack to isolate the abdomen from the remaining parts of the body. Abdomens of the same species were grouped

in pools of 10 individuals and the rest of mosquito bodies were stored individually at -80°C until RNA extraction.

Total RNAs were extracted from each pool using Nucleospin RNA kit according to the manufacturer's instructions. Pools were grinded in 350 µL Lysis Buffer and 3.5 µL β-mercaptoethanol using piston stirrer. Total RNA per pool was eluted in 60 µL of RNase free water and stored at −80 ◦C until use.

2.4. Reverse Transcription and cDNA Pre-Amplification

RNAs were transcribed to cDNA by reverse transcription using the qScript cDNA Supermix kit according to the manufacturer's instructions (Quanta Biosciences, Beverly, USA). For cDNA pre-amplification, the Perfecta Preamp Supermix (Quanta Biosciences, Beverly, USA) was used according to the manufacturer's instructions. These two procedures were performed according to the protocols described by Moutailler et al., 2019.

2.5. High-throughput real-time PCR system

A high throughput epidemiological surveillance method based on a microfluidic system (Fluidigm, South San Francisco, CA, USA) was used according to the protocol described by Moutailler et al., 2019. This technic is more sensitive than real time PCR. This method allows to perform real-time PCR using 96.96 chips, resulting in 9216 individual reactions. The primer sequences / probe sets used in this study are available in Moutailler et al. 2019. A negative water control has been included for each chip. A positive control with cDNA (virus reference material) or DNA (Plasmid) was used. To determine if inhibitors could be present in the sample, DNA from the Escherichia coli strain EDL933 was added to each sample as an internal inhibition control, using gene-specific primers and probes E. coli eae (Nielsen & Andersen, 2003). Data were acquired on the BioMarkTM real-time PCR system and analyzed using Fluidigm real-time PCR analysis software to obtain Ct values (Michelet and al., 2014).

2.6. Real time PCR

RNA from WNV positive mosquito pool was screened by real-time PCRs on a StepOne Instrument (Applied Biosystem, Thermo Fisher Scientific, Illkirch, France). Real-time RT-PCR assay targeting a different segment of the WNV genome (5'UTR and part of the capsid gene) (Linke et al., 2007) was performed in a final volume of 25 µL using 5µL of RNA and AgPath-ID™ One-Step RT-PCR Reagents (Thermo Fisher Scientific, France) containing

primers and probes at 400 nM and 200nM respectively. Thermal cycling conditions were as follows: 45°C for 10 minutes, 95°C for 10 min, 45 cycles at 95°C for 15 s and 60°C for 1 min.

2.7. Blood sampling and recovery of horse sera

A total of 92 horses were sampled in five different equestrian clubs, located in the region of Casablanca-Mohammedia-Benslimane (area where was declared the last WNV epizootic in Morocco in 2010) (figure1). Horses sampled were more than 2 years old, male and female, and of different breeds; they showed no clinical signs associated with West Nile disease. Each blood sample was collected in dry tubes and transported at +4 ° C to the laboratory where samples were centrifuged. The sera were aliquoted, separated and stored at -20 ° C until use.

2.8. Serological test

2.8.1. Competitive enzyme-linked immunosorbent assay (cELISA)

The serological diagnosis was made using a cELISA test (ID Screen® West Nile Competition ELISA Kit, ID Vet, France) to give an indication of the presence or absence of anti-WNV antibodies in horses sera. Analysis and interpretation were performed according to the manufacturer's instructions. The optical density was observed and the S/N ratio (optical density of the sample / optical density of the negative control * 100) was calculated. Samples with a S/N ratio of 40% or less were considered positive while those with a S/N ratio comprised between 40 and 50% were considered doubtful, and those with a S/N ratio higher than 50% were considered negative. This test has been used to give an indication of the presence or absence of anti-WNV antibodies in sera, but cross-reactions with other flaviviruses may occur (Beck et al., 2017).

2.8.2. Microsphere immunoassay

Flavivirus microsphere immunoassay (MIA) was performed on cELISA positive samples as described by Beck et *al*. (2015). Briefly, recombinant soluble ectodomain of WNV envelope (E) glycoprotein (WNV.sE) and the recombinant E domains III (rEDIIIs) of WNV, USUV and TBEV containing virus-specific epitopes were covalently bound to fluorescent beads following the protocol previously described in (Beck et al., 2015; Vanhomwegen et al., 2017). Reactivity against WNV.sE is indicative of the presence of anti-flavivirus antibodies (as with the IDVET cELISA), and WNV.EDIII, TBEV.EDIII and USUV.EDIII allows to distinguish between these flaviviruses.

The cut-offs of WNV.sE, WNV.EDIII, and TBEV.EDIII antigens were found to be 17, 54 and 61, respectively, as described in (Beck et al., 2015). For USUV, due to no positive horse serum against this disease, the cut-off was determined from the mean of median of fluorescence (MFI) values of 66 negative horse sera plus 3 standard deviations of the mean. The 66 sera used to determine the cut-off were sampled in Poland (35 sera) and Ireland (31 sera) and were all found negative with the ID screen West Nile competition kit (IDVet). The MIA results were interpreted according to the following algorithm : (i) a serum was considered positive for WNV (or alternatively for USUV) if it reacted against WNV.sE and WNV.EDIII (of alternatively USUV.EDIII) (ii) In case of positive reactions with several rEDIIIs for viruses belonging to the Japanese encephalitis serocomplex (i.e. USUV and WNV) an animal was considered infected with a specific flavivirus if the corresponding bead coupled to rEDIII generated an MFI at least two-fold greater than that generated with the other beads. If a 2 fold difference could not be achieved, the animal was considered to be infected with WNV or USUV (iii) since TBEV belongs to another serocomplex, a serum was considered positive for TBEV when the TBEV.EDIII MFI was above the cut off (iiii) finally, a sample was considered positive for an undetermined flavivirus if it reacted with WNV.sE but not with any of the EDIIIs.

2.8.3. Seroneutralization tests

Flaviviruses identified by MIA and all undetermined ELISA-positive flavivirus samples were investigated using MNT against WNV and USUV.

Neutralizing antibody titers against WNV and USUV were determined by MNT on Vero cells in 96-well cell culture plates, following the protocol described in (Beck et al., 2015), using WNV strain IS-98-ST1 (Genbank ID AF481864.1, provided by P. Després, IPP) and USUV strain France 2018. The MNT results were interpreted according to the following rules: (i) A serum was considered positive if cells were protected at the 1:10 serum dilution for WNV and USUV. Owing cross-neutralization between flaviviruses especially in the same serocomplex, we identified the infecting flavivirus by considering the virus with the highest neutralization capacity, and with neutralization titers that differ by at least a four-fold factor (Beck et al 2015).

3. Results

3.1. High-throughput screening of mosquito-borne viruses

A total of 1,455 mosquitoes were analyzed. 670 mosquitos were collected from Mohamedia region, all belonging to *Culex pipiens* species, and 780 mosquitoes collected from regions of northern Morocco (Moulay Bouselham-Larache (n = 595) and Tangier (n = 190)) belonging to *Aedes detritus* species (n = 320), *Aedes caspius* (n = 110), *Anopheles maculipennis* (n = 5) and *Culex pipiens* species (n = 160). Among tested pools (146), one pool from Mohammedia area was positive for WNV (see table 1). On the other hand, all the pooled mosquitoes from Tanger and Moulay Bouselham regions were negative (Table 1).

3.2. Real time RT-PCR

The positive pool for WNV was confirmed by a real time RT-PCR targeting a different fragment of the WNV genome.

3.3. Detection of anti-WNV antibodies in horses

In order to determine the WNV seroprevalence in 92 horses, we used three techniques: cELISA which detects anti-flavivirus antibodies, the MIA test which allows to distinguish between the three flavivirus: WNV, USUV and TBEV, and the MNT which allows the confirmation of the presence of anti-WNV antibodies and anti-USUV antibodies.

The seroprevalence by cELISA was 33.7% (31/92). The MIA test showed that 25.0% (23/92) were positive for WNV, 2.1% (2/92) were positive for USUV, 4.3% (4/92) were positive for undetermined flavivirus and 2.1% (2/92) were positive for both WNV and USUV (Table 2).

The MNT showed that 30.4% (28/92) were positive for WNV with titers between 20 to superior to 320. The results of the two methods were in accordance for the 23 MIA WNV positives. Conversely, 2 samples positive for USUV, 2 positives for both WNV and USUV and one for undetermined flavivirus by MIA were found WNV positive by MNT. All sera were negative for USUV in MNT (Table 3).

3. Discussion

This study reports, for the first time, the detection of WNV in *Culex pipiens* mosquitoes and high seroprevalence rate of WNV infection in horses from Morocco (30% by MNT test). We used the new high-throughput screening method, which examines 38 arboviruses (94 different

263 genotypes/serotypes) in a single experiment (Moutailler et al., 2019). In Morocco, WNV was
264 isolated and sequenced only once during the epizootic of 2003. The viral isolation was
265 performed from a cerebral biopsy of a dead horse (Schuffenecker et al., 2005). In our study,
266 WNV-infected mosquitoes were only found in Mohammedia region and not in Moulay
267 Bouselhame region, probably owing to the low number of specimens examined: 160
268 individuals in Moulay Bouselhame compared to 670 tested in Mohammedia region. We
269 reported a low *Cx. pipiens* infection rate (0.15% (1/670), a result in agreement with other
270 investigations (Engler et al., 2013): 1.2% for *Cx. pipiens* in Tunisia (Monastiri et al., 2018),
271 0.56% for *Cx. perexiguus* in Algeria (Benbetka et al., 2018), 0.24% for *Cx. interrogator* and
272 0.28% for *Cx. nigripalpus* in Chiapas, México (Ulloa et al., 2009). Anti-flavivirus antibodies
273 have been detected by cELISA in 34% (31/92) of horse sera and 100% (31/31) of cELISA
274 positive sera were found reactive to the WNV.sE bead by MIA. These two independent
275 methods corroborated the detection of anti-flavivirus antibodies in horses (Beck et al., 2015).
276 To improve the specificity of flavivirus serological screening, flavivirus positive samples
277 were tested by MIA using WNV.EDIII, USUV.EDIII and TBEV.EDIII antigens which
278 contain virus-specific-epitopes (Beasley et al., 2004; Beck et al., 2015) and by MNT against
279 WNV and USUV. A significant proportion of horses (23/28) were found to be WNV positive
280 by the two technics. Two samples were found positive against USUV in MIA but positive for
281 WNV in MNT. Such discordant results could originate from a lower specificity of the
282 flavivirus EDIII MIA technique. The fact that these sera were tested by MIA with WNV and
283 USUV nonstructural protein 1 (NS1) and were found positive for WNV (data not given)
284 supports this hypothesis. We did not identify the flavivirus involved in 9.7% (3/31) of horse
285 infections evidenced by positive cELISA reactions. As already mentioned by several authors
286 (Beck et al., 2015), WNV cELISA tests lack specificity in the diagnosis of WNV infection. Of
287 31 sera tested positive for anti-WNV IgG by ELISA, only 28 had specific neutralizing
288 antibodies. Benjelloun and his collaborators studied seroprevalence of WNV in different
289 regions of Morocco in 2011. They found a seroprevalence rate of WNV similar to our study
290 35% and 31% respectively by cELISA and MNT (Benjelloun et al., 2017). Another study
291 carried out with 297 military working horses and 231 dogs in Morocco have described,
292 respectively, a rate of 60% and 62% of seroprevalence by cELISA (Durand et al., 2016). This
293 high rate of WNV seroprevalence has also been reported by other team in Tunises and Algeria
294 (Ben Hassine et al., 2014; Lafri et al., 2017). USUV and WNV have been shown to co
295 circulate and share the same vectors (Zannoli and Sambri, 2019); however, we did not detect
296 USUV either in mosquitoes nor in horse sera, while USUV has been shown to circulate in

Tunisia and in Morocco (Ayadi et al., 2019; Ben Hassine et al., 2014; Figuerola et al., 2009). For the sera found positive with the ELISA test and negative in WNV and USUV MNTs (3/31), such reactions could correspond to low WNV antibody responses under the detection MNT threshold or to animals infected with other flaviviruses (yellow fever virus, …). Recently, cELISA positive and WNV MNT negative horses on French pacific islands turned out to be Dengue and Zika-infected. The serological results highlighted the importance of taking into account the risk of circulation of other arboviruses such as dengue, chikungunya and zika viruses, since the vector of these arboviruses, *Ae. albopictus,* was recently established in Morocco (Bennouna et al., 2017) and their ability to transmit these viruses efficiently has been experimentally proven (Amraoui et al., 2019).

Our results are in agreement with many arguments for active circulation of WNV in this area of Morocco that are: (i) high abundance of *Cx. pipiens* mosquitoes in a region hosting migratory birds (Jourdain et al., 2007), and (ii) their ability to experimentally transmit WNV (Amraoui et al. 2012). The water reservoirs present in this area would constitute ideal ecological niches for repeated contacts between domestic or migratory birds and mosquitoes, thus allowing the amplification of the virus in an enzootic cycle (Zeller and Murgue, 2001). WNV has been responsible for sporadic outbreaks of disease in countries around the Mediterranean sea since the 1960 (Hubálek and Halouzka, 1999). These have involved infections in humans and/or horses (Zeller et al., 2004). All outbreaks were reported between July and September. The principal vector of WNV in Europe and USA is *Culex pipiens* (Esteves et al., 2005; Kilpatrick et al., 2005). These countries have favorable conditions for maintaining the WNV transmission cycle such as environmental factors and climatic conditions (Rogers and Randolph, 2006). These conditions support virus circulation, and when the density of vector mosquitoes is enhanced and sensitive hosts are available, outbreaks are observed. The high prevalence of antibodies among horses also suggest that substantial circulation of the virus may have occurred during the previous seasons. The observed titer of the positive sera is probably due to re-infections during the previous 2010 outbreak or a prolonged and repeated exposure to viruses in endemics areas. These results suggest that WNV was present in Morocco in 2018 without resulting in disease outbreaks among humans or horses, as opposed to in 1996, 2003 and 2010, when cases did occur (El Harrack et al., 1997; El Rhaffouli et al., 2012; Schuffenecker et al., 2005). Absence of outbreaks despite the circulation of the virus could be explained by the acquisition of a

protective immunity after the first infection by humans and horses and, possibly by the lack of entomological virus surveillance and asymptomatic underreported cases.

Our results strongly suggest that WNV remains silent and spillover events to humans occurs only under favorable ecological conditions. Therefore, understanding ecological factors and environmental conditions leading to WNV outbreak is of major importance. Persistence of WNV in over wintering *Cx. pipiens* is an important mechanism in the maintenance of this arbovirus (Nasci et al., 2001). Thus, WNV is likely to cause future sporadic and foreseeable outbreaks not only in Morocco but along the migratory flyways of birds between Africa and Europe. Virus circulation becomes permanent and is probably maintained by mosquito vector movements and reintroduction of migratory birds.

This study brings important data about an active WNV circulation in mosquitoes in Morocco. It is suggested that public health authorities should implement WNV surveillance activities and setting up an entomological surveillance as an early alert system around the Mediterranean Basin to prevent future outbreaks is highly needed.

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Table 1: Mosquito species, number of mosquitoes collected and virus detected by high-throuput chip based on the BioMark Dynamic arrays system.

Collection Site	« GPS » coordinates	Biotope	Mosquito Species	Number of mosquitoes screened	Number of pools	Virus Detected through Microfluidic System	Type of confirmation Performed
Mohammedia	33°38’22.4N 7°26’08.8W	Peri - urban	*Culex pipiens*	670	67	WNV*	WNV confirmed by Real time RT-PCR
Tanger	35°46’44.3N 5°50’50.1W	Urban	*Culex pipiens*	190	19	_	_
Moulay bouselham	34°52'28.7"N 6°17'14.5"W	Rural	*anoh detritus,* *Aedes caspius,* *culex pipiens,* *Anophele maculipennis*	320 110 160 5	32 11 16 1	_	_
Total				**1455**	**146**		

WNV., West Nile virus; CHIKV., Chikungunya virus. (-) no virus detected and no confirmation performed. *WNV detected in one pool of *Cx.pipiens*.

Table 2: cELISA flavivirus results and WNV, USUV and TBEV results by MIA

Number of samples	cELISA			MIA confirmation of positive cELISA results				
Number of horses sampled	**negative**	**Doubtful**	**Positive**	**WNV**	**USUV**	**TBEV**	**Flavivirus**	**WNV or USUV**
92	61	0	31(33.7%)*	23 (25%)*	2(2.1%)*	0	4 (4.3%)*	2 (2.1%)*

* The percentage was based on total sample numbers (assuming all cELISA negative would also be negative by MIA)

Table 3: MNT confirmation of positive cELISA against WNV and USUV

Number of samples	USUV	undetermined flavivirus	WNV	Distribution of WNV positive samples by titer (%)					
31	0	3	28 (30.4%)	I:20	I:40	I:80	I:160	I:320	>1:320
				3 (3.3%)*	2 (2,3%)*	1 (1%)*	11 (11,9%)*	4 (4.3%)*	7 (7.6%)*

* The percentage was based on total sample numbers (assuming all cELISA negative would also be negative by MNT)