THE WEST NILE VIRUS (WNV) MOLECULAR DETECTION IN VECTORS: FROM THE BENCHTOP TO THE FIELD - DEPLOYABLE LABORATORY

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ABSTRACT

The WNV molecular detection has been well documented and studied in Romania since the occurrence of the urban outbreak in 1996. A lot of molecular methods (classical Reverse-Transcription PCR, Real-Time PCR, sequencing) were used and described during this time.

The presence of WNV in Romania was established by integrative studies (entomological, serological and molecular approaches). Nowadays it is well known that WNV is present in different localities along the Danube River, including Tulcea and the Danube Delta itself.

The WNV is a flavivirus with a single positive strand RNA genome of approximately 11000 nucleotides, the virus is transmitted by mosquitoes and the reservoir is represented by birds, the virus causing encephalitis and meningoencephalits in humans.

Due to the advances in equipment platform and reagent chemistry we are able today to investigate WN viral-load in mosquitoes (vectors) in a field – deployable laboratory.

The present work describes how a benchtop method for WNV detection (developed in 2005 by our team) was modified for field purposes in conjunction with the use of Mini8 Plus™-thermocycler and MD-BOX-LAB from Coyote-Bioscience Co. Ltd.

The result pleads for: 1) LWW is subject to continual improvement and adaptations and 2) using magnetic beads for RNA extraction to avoid prolonged centrifugation steps.

Keywords: Deployable, WNV, LWW, Vectors, PCR.

REZUMAT

Detecția moleculară a virusului West Nile a fost bine documentată și studiată în România odată cu epidemia urbană din 1996. Numeroase metode moleculare (clasica revers-transcriere PCR, PCR în timp real, secvențierea) au fost folosite cu această ocazie. Studiile integrate (entomologice, serologice și moleculare) au stabilit prezența permanentă a virusului în România. Astăzi este bine cunoscut că virusul West Nile este prezent în diferite localități situate de-a lungul Dunării, inclusiv în Tulcea și Delta Dunării. Virusul West-Nile este un flavivirus cu un genom ARN de 11000 de nucleotide reprezentat de un singur lanț pozitiv de ARN, virusul este transmis de țânțari, iar rezervorul său este reprezentat de păsări, virusul provoacă encefalite și meningoencefalite la om.

Datorită noutăților apărute la echipamentele de biologie moleculară, precum și a celor privind utilizarea diferiților reactivi, astăzi este posibil să investigăm încărcătura virală în țânțari (vectorii virusului) într-un laborator mobil, direct pe teren.

Lucrarea de față descrie cum o metodă folosită în laborator pentru detecția virusului West Nile (dezvoltată în 2005 de echipa noastră) a fost modificată pentru lucrul pe teren, în conjuncție cu utilizarea echipamentelor nou achiziționate: termociclerul în timp real Mini8 Plus™ și laboratorul mobil MD-BOX-LAB, produse de Coyote-Bioscience Co.Ltd.

Rezultatele obținute pledează pentru: 1) laboratorul mobil/de teren de biologie moleculară este un subiect care suportă permanent îmbunătățiri și 2) utilizarea bilelor magnetice pentru izolarea ARN, fapt ce ar reduce considerabil timpul pentru pașii de centrifugare din protocol.

Cuvinte-cheie: Laborator mobil, WNV, LWW, Vectori, PCR.

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INTRODUCTION

The West Nile is an arbovirus (flavivirus) with a single positive strand RNA genome of approximately 11000 nucleotides (10960 nt) [1]. The virus is transmitted in most of the cases by mosquitoes (culicidae/vectors) and the reservoir is represented by birds. The virus causes encephalitis and meningoencephalitis (with clear cerebrospinal fluid) in humans.

In 1996, Bucharest and suburbs were affected by a meningoencephalitis outbreak (453 clinical cases and 9% fatalities) caused by WNV [2-4].

The WNV molecular detection has been well documented and studied in Romania since the urban outbreak in 1996 occurred. A lot of molecular methods (classical Reverse-Transcription PCR, Real-Time PCR, sequencing) were used and described during this time [5-8].

The concept of Laboratory Without Walls (LWW) has become more popular for disease outbreak causative agent identification.

It is a modular field application of molecular biology that provides clinical laboratory support in resource limited areas and in remote locations [9].

Related with the LWW concept, the present work describes how a benchtop method for WNV detection (developed in 2005 by our team) was modified for field purposes in conjunction with the use of Mini8 Plus™-thermocycler and MD-BOX-LAB from Coyote-Bioscience Co. Ltd. (www.coyotebio.com).

MATERIAL AND METHODS

Sample: Field mosquitoes (in pools, up to 50 mosquitoes) homogenized in sterile PBS pH = 7.0 (30 μL/1 mosquito) with a deployable grinder (included in the MD-BOX-LAB) were centrifuged (13000 rpm/1 min.) and 100 μL supernatant was used for RNA QIAmp Viral RNA Mini Kit according to the manufacturer's protocol.

RNA quantification was made using a MAESTROGEN micro-volume Spectrophotometer (deployable device too). Total RNA concentrations between 20-700 ng/L were used for Reverse-Transcription (RT) PCR.

cDNA from the viral WN RNA was synthetized using GeneAmp®RNA PCR kit (with MuLV reverse transcriptase) with 1 μL RNA template (in different dilutions in ultrapure DEPC treated water) in presence of WN-2 primer (reverse; Table 1). The RT-conditions were: 3 min at 50°C followed by 15 min at 95°C, followed by 5 min. at 5°C.

First amplification (408bp fragment)/ Classical PCR without Fluorescence Detection

After RT, in the same tube was added a PCR mixture containing the GeneAmp®Core Kit up to 100 μL (final volume). The PCR amplification of a 408 bp fragment was achieved using WN-1 and WN-2 primers first described by Shi *et al.* in 2000 (Table 1) [10]. The thermocycler parameters were: 1 min initial denaturation at 95°C and then 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 1.00 min. followed by a final extension of 7 min at 72°C. The amplification was performed in a Coyote Mini8 Real-Time PCR deployable Platform without the fluorescent data acquisition.

Second amplification (104bp fragment)/ Real-Time WNV PCR Detection

After RT-PCR, the amplification products were diluted (in ultrapure DEPC treated water) and 1 μL was added to the nested PCR mixture (final volume 50 μL) containing SYBR Green I (SYBR™Green PCR Master Mix) and WN-3

Table 1 - The primer sequences used in this study for obtaining the 408bp and 104bp amplicons

Primer name/code	Primer sequence
WN-1	5'-TTGTGTTGGCTCTCTTGGCGTTCTT-3'
WN-2	5'-CAGCCGACAGCACTGGACATTCATA-3'
WN-3	5'-CAGTGCTGGATCGATGGAGAGG-3'
WN-4	5'-CCGCCGATTGATAGCACTGGT-3'

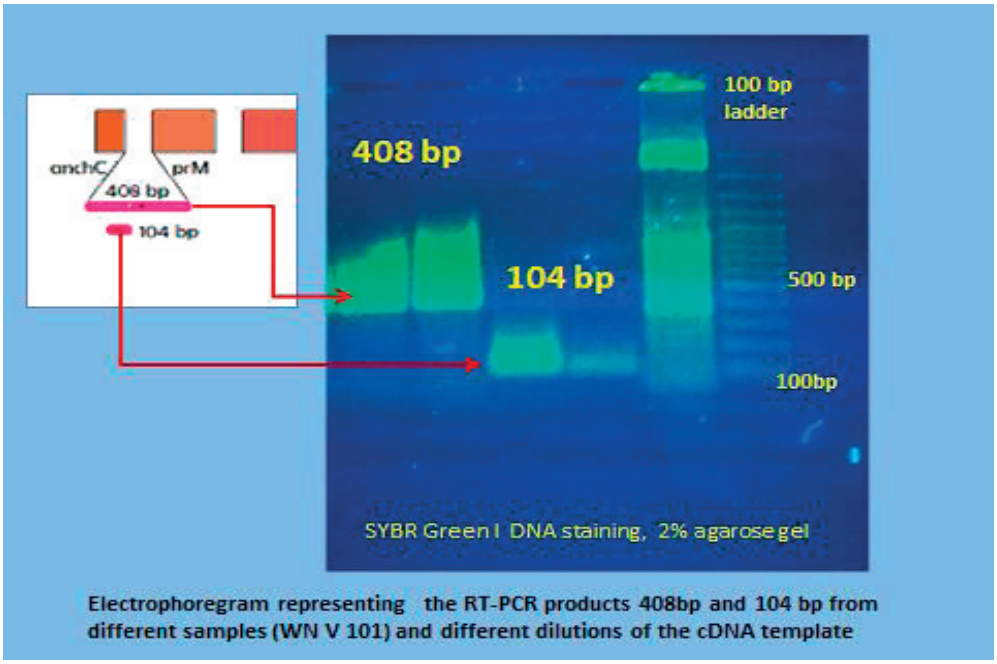

Fig. 1. Electropherogram representing the RT-PCR products 408bp and 104 bp from different samples and different dilutions of cDNA template

and WN-4 primers (Table 1). The amplification was performed in a Coyote Mini8 Real-Time PCR deployable Platform with the fluorescent data acquisition on FAM/SYBR channel and with the Melting curve analysis.

RESULTS

The products of the expected 408 bp cDNA, which contained the 3′ region of the C gene and the 5′ region of the *prM* gene were observed (Fig. 1) in all experimental variants (different dilutions: 1, 10^{-1}; 10^{-2} in ultrapure DEPC treated water). The nested PCR products of the expected 104 bp from the 408 bp amplicon (as template) were observed also in all experimental variants (different dilutions in ultrapure DEPC treated water; 1, 10^{-2}; 10^{-3}) (Fig. 1). The intensities of the DNA

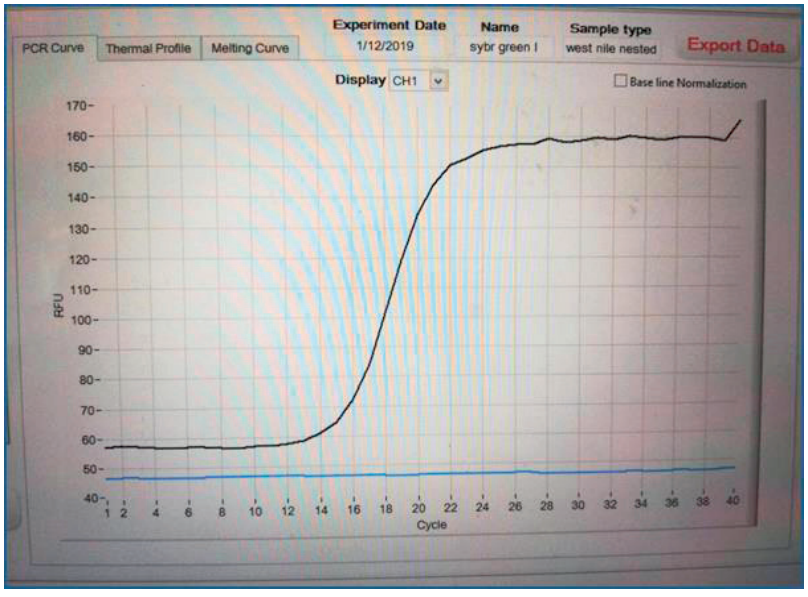

Fig. 2. The Relative Fluorescence Units VS Amplification cycles plot in non-logarithmic view using the Ch 1 (FAM/ SYBR Green) detection channel from the Mini8 Coyote Cycler platform

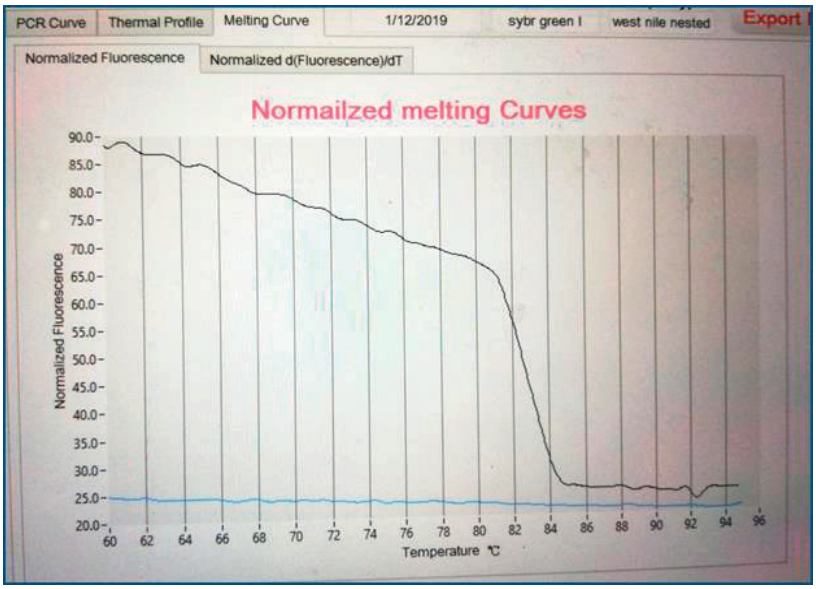

Fig. 3. The Melting temperature (Tm) plot in presence of the fluorescence DNA intercalating dye (SYBR Green) from the Mini8 Coyote Cycler platform

bands (during agarose gel electrophoresis and SYBR Green I gel staining) decreased with decreasing amounts of input WNV cDNA. No DNA product was amplified from the PCR mixture without WNV cDNA, the same result was obtained for the bands representing the 104 bp. amplicon (Fig. 1).

For Real-Time PCR approach the positive sample was well represented in classical curves of the fluorescence acquisition data (on SYBR/FAM channel) (Fig. 2).

Using the SYBRGreen I DNA dye the amplification product can be analyzed by melting curve in both classical normalized data (Fig. 3) or by peaks of normalized data (Fig. 4).

The negative sample was represented always as a flat line (Figs. 2-3) or small peaks. (Fig. 4).

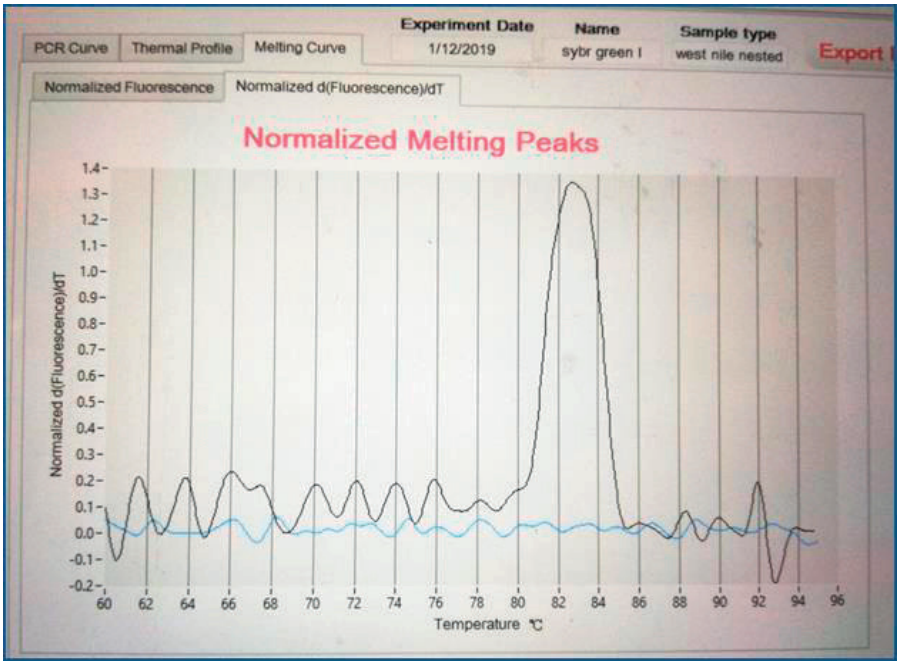

Fig. 4. The Normalized Melting Peaks (Second derivative) plot in presence of the fluorescence DNA intercalating dye (SYBR Green) from the Mini8

DISCUSSION

Nowadays, LWW has already been used for aquisition of valuable molecular data while the investigators were still on outbreak location.

In Australia, a LWW was used to demonstrate the presence of bacteria (*Burkholderia pseudomallei*) in the field samples, other approaches were made for Influenza A virus identification, NW-Australia in 2009; Australian arboviruses in 2010 and other arboviral viruses panel in Sri-Lanka rainforest [9].

The present paper describes the first Romanian LWW approach for WNV detection using a mobile Reverse-Transcription platform.

Due to the versatility of the RNA extraction kit the input material could be: mosquitoes homogenate, mouse brain homogenate, avian cloacal swabs; human, avian, mammalian sera or human CSF (cerebrospinal fluid), so, theoretically the method can be used for all kinds of samples.

Because the RNA isolation using the QIAmp Viral RNA Mini Kit uses few centrifugation steps at high speed and is energy and time consuming, we intend to use magnetic beads for RNA isolation kit, which is more suitable for use under the field conditions. SYBR Green I nucleic acid dye is more sensitive than Ethidium Bromide for electrophoresis and as intercalating dye in PCR products bring valuable data about the melting temperature of presence of specific or/and nonspecific amplicons.

CONCLUSION

The results plead for: 1) LWW is subject to continual improvement and adaptations 2) using magnetic beads for RNA extraction to avoid prolonged centrifugation steps.

In conclusion, the Shi method [10] adapted for our workflow conditions can lead to identification of WNV in a multi-level manner (RT-PCR and then nested PCR in classical way or/and Real-Time PCR) in a field deployable laboratory.

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The MD-BOX-LAB including the mobile Real-Time PCR device and the MAESTROGEN micro-volume Spectrophotometer were purchased by the Romanian Ministry of Defence in 2018.

Conflict of interests: There is no conflict of interests to declare.

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