European Network for Neglected Vectors and Vector-Borne Infections

To: MC Chair, Andrei Mihalca and STSM coordinator, Dušan Petrić.

Short Term Scientific Missions (STSM) report

STSM grantee: PhD Tatiana Sulesco
Title of the STSM: Molecular survey for mosquito-borne parasites in field collected mosquitoes from Moldova.
COST Action: TD1303, WG 1.
Reference code: COST-STSM-ECOST-STSM-TD1303-121015-063575.
Host institution: Bernhard Nocht Institute for Tropical Medicine, Department of Molecular Parasitology, Hamburg, Germany.
Host professor: Egbert Tannich (e-mail tannich@bnitm.de)
Period: from 12/10/2015 to 27/11/2015

1 Purpose of the STSM

The aim of the Short Term Scientific Missions was the molecular detection of mosquito-borne filarial parasites (*Dirofilaria repens* and *D. immitis*) in field collected mosquitoes from Moldova and analyses of the spatial distribution, habitat preferences, seasonal abundance as well as human biting activity of the parasite-carrying mosquito species. *Dirofilaria repens* and *Dirofilaria immitis* are known to cause human infections in Moldova. Therefore, knowledge about prevalence of the parasites in respective mosquito vectors is a prerequisite for risk assessments. Accordingly, molecular studies have been performed during the STSM to determine potential mosquito species involved in circulation of these zoonotic filarial species in Moldova.

The objectives of the STSM were:

1. Study of modern techniques and specific approaches in molecular screening of mosquito samples for emerging mosquito-borne parasites and pathogens (RNA/DNA extraction techniques, conventional PCR, real-time PCR, DNA sequence analysis, experience of work in the laboratories under different biosafety levels).
2. Assessment of prevalence of filarial nematode (*Dirofilaria spp*.) parasites and the estimation of minimum mosquito infection rates in different wild-caught mosquito species, collected in Moldova using different trappings techniques in various ecosystems.

The research performed within this STSM contributed to the TD1303 COST Action objectives of "One Health" concept in the ecology of vector-borne diseases (WG1).

2 Description of the work carried out during the STSM

A previous study conducted in Moldova between 2010 and 2015 allowed participant to collect and identify the mosquito species across the country (Fig. 1). The mosquitoes were collected from human volunteers, man-made shelters or cattle sheds using Center for Disease Control (CDC) Miniature Light Traps model 512 (John W. Hock Company). These mosquitoes were used for further molecular screening for *Dirofilaria spp*. in the host laboratory according to the Work Plan previously provided.

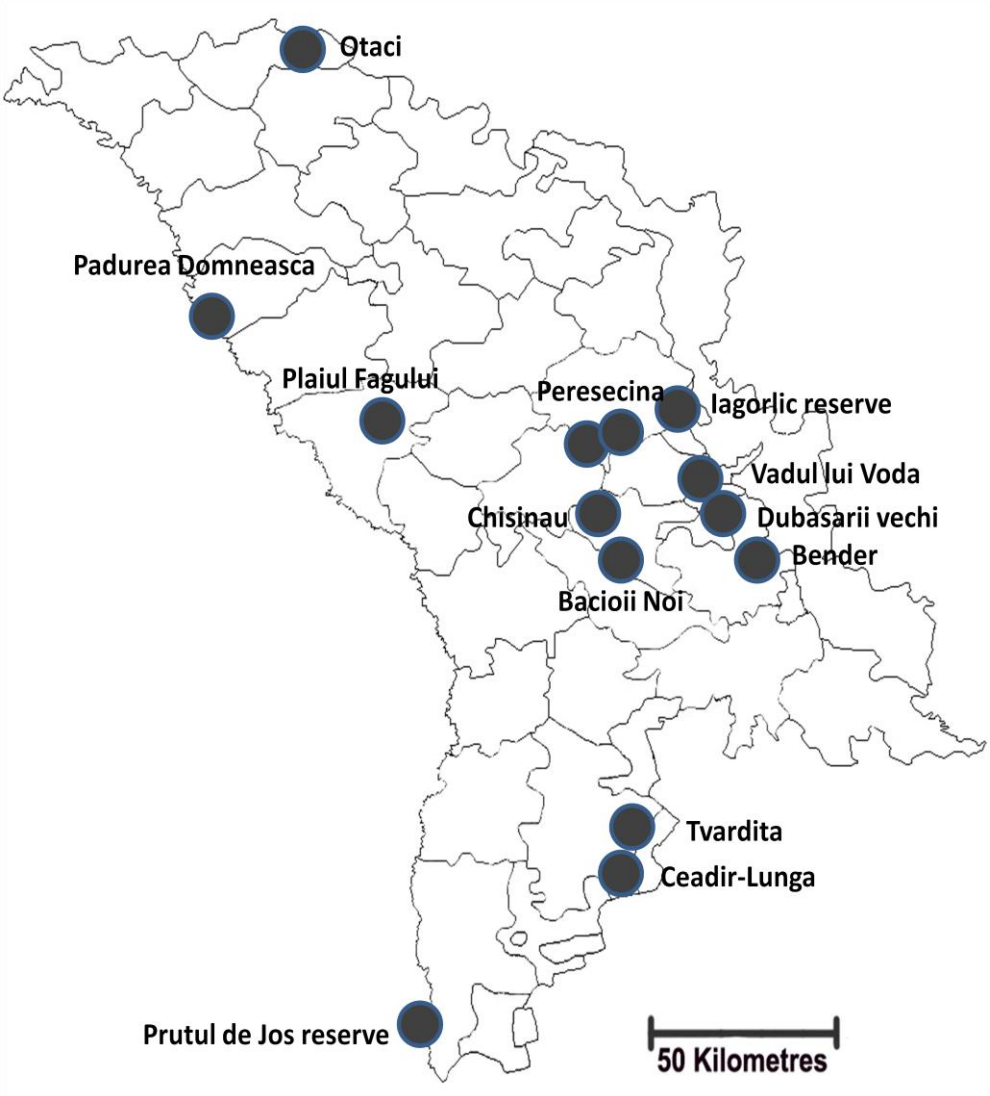


Fig. 1. Location of mosquito trapping sites in Moldova.

A total of 4,488 female mosquitoes belonging to six genera (*Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, and *Uranotaenia*) were pooled by species, date and collection site, comprising between one to 35 specimens per pool. In total, 347 mosquito pools were obtained. Each pool was homogenized by a TissueLysser (Qiagen, Hilden, Germany) for 3 min at 50 oscillation/s in the presence of two stainless steel beads (5 mm) and 1 ml of cell culture medium (high-glucose Dulbecco's modified Eagle's medium [DMEM]). The suspensions were clarified by centrifugation for 5 min at 13,000 g. The supernatant was used for simultaneous RNA/DNA extraction by means of the MagMAX™ Pathogen RNA/DNA Kit using the MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Thermo Fisher Scientific Inc., CA, USA) or QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

The extracted DNA was analyzed by a real-time PCR assay for the detection of *D. immitis* or *D. repens*, targeting ~ 100 bp fragment of the mitochondrial genome or the 16S rRNA gene using two separate sets of primers and probes (Table 1).

Table 1.

Primers and probes designed for *D. repens*/*immitis* detection

Gene	Organism	Primer	Sequence (5'-3')	Length
16S rRNA	*D. immitis*	Imm**F**	CTA TAT GTT ACC TTA ATT GG	20 bp
16S rRNA	*D. immitis*	Imm**T**	**ROX** – GTA GCT AGT AAG TTT ACC TTG – **BHQ2**	21 bp
16S rRNA	*D. immitis*	Imm**R**	CTT AAC CAT TAT CTT AGA TCA G	22 bp
COI	*D. repens*	Rep**F**	GAG ATG GCG TTT CCT CGT G	19 bp
COI	*D. repens*	Rep**T**	**JOE** – GTT GCT TTG TTA ATG GTT TAT C – **BHQ1**	22 bp
COI	*D. repens*	Rep**R**	GAC CAT CAA CAC TTA AAG	18bp

F – forward primer, **R –** reverse primer, **T –** Taqman Probe.

The real-time PCR was performed using the Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Australia). The reaction mixture (20 µl) contained 10 µl of 2x HotStarTaqH Master Mix Kit (Qiagen, Germany), 25 mM $MgCl_2$, 4 pmol / µl forward primer, 24 pmol / µl reverse primer, 16 pmol / µl probe RepT, or 0.8 pmol / µl probe ImmT, 1mg/ml BSA, 4.45 µl H_2O, and 2 µl of extracted DNA (except no-template controls).

The thermo profile consisted of:

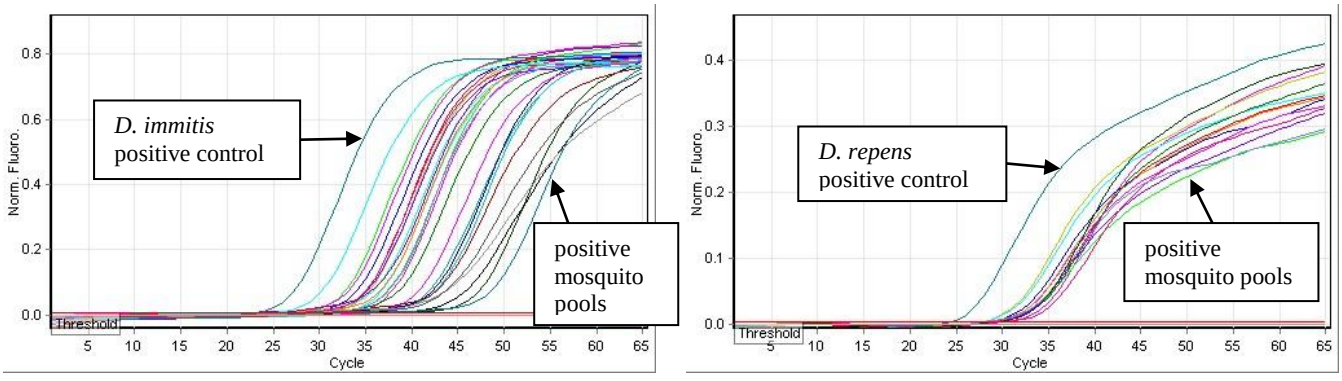
Initial denaturation:	15 min – 95°C	
Denaturation:	15 sec – 95°C	65 cycles
Annealing:	30 sec – 61°C	
Extension:	30 sec – 72°C	
	30 sec – 40°C	

Fluorescence signals were registered during the extension step of the reaction and the data were analyzed by Rotor-Gene^{TM} 6000 software version 6.1.8.1. (Corbett Research, Australia) (Fig. 2). As a specificity control, PCR amplicons of all samples with positive signals in the real-time PCR were subjected to DNA sequencing on both strands using the same sets of primers that were used in the real-time PCR. Sequences were edited and aligned using MacVector software. For species identification all sequences generated were compared with sequences from public data bases (GenBank).

3. Description of the main results obtained

A total of 347 pools comprising 4,488 female mosquitoes from 22 species or species complexes in six genera were examined for the presence of *D. immitis* and *D. repens* DNA, respectively, by real-time PCR (Table 2). Using the set of primers for *D. repens,* filarial DNA was detected in 103 pools, representing 15 mosquito species or species complexes. Thirty two and 28% percent of real-time PCR positive pools were from *An. maculipennis s.l.* and *Culex pipiens/torrentium* specimens, respectively. Thirty percent (n = 29) of positive pools were already sequenced and analyzed. The sequences obtained showed 99% to 100% identity to *D. repens* sequences in public data bases.

Using the set of primers for *D. immitis*, 40 pools were positive for filarial DNA by the real-time PCR, representing eight mosquito species or species complexes. Up to now, 48% (n = 19) of PCR fragments from positive pools were already sequenced and analyzed. The sequences obtained showed 99% and 100% identity to *D. immitis*. Sequencing of the remaining positive pools for *D. repens/immitis* is currently under investigation. The results will be updated as far as new data are obtained. Nevertheless, the data generated so far clearly indicate substantial infestation of *Dirofilaria immitis* and *Dirofilaria repens* in Moldova.



A **B**

Fig. 2. Example of quantitation data for cycling *D. immitis* (A) and *D. repens* (B).

This is the first study of mosquitoes collected in Moldova and examined for *D. immitis* and *D. repens* infection, which provides new data of mosquito species naturally infected with *D. immitis/repens* in

the country. The frequent detection of *D. immitis* and *D. repens* in field collected *An. maculipennis s.l.* and *Culex pipiens /torrentium,* along with their seasonal abundance and widespread distribution in the country suggest their central role as vectors of mosquito-borne zoonotic filarial nematodes in Moldova.

During the STSM applicant was also trained to detect arbovirus RNA (families Flaviviridae, Bunyaviridae, Togaviridae and Rhabdoviridae) in field collected mosquitoes.

Foreseen publications/articles resulting from the STSM

The results obtained during the STSM will be publicated in peer-reviewed journals.

Table 2. Mosquito species and pools examined.

	species	no. mosquitoes tested	no. pools tested	real-time PCR positive pools (no. specimens) for *D. repens*	no. pools (no. specimens) sequenced for *D. repens*	real-time PCR positive pools (no. specimens) for *D. immitis*	no. pools (no. specimens) sequenced for *D. immitis*
1	*An. maculipennis s.l.*	956	61	33 (598)	13 (263)	17 (326)	8 (158)
2	*An. claviger*	3	1	0	0	0	0
3	*An. hyrcanus*	2	1	1 (2)	0	1 (2)	0
4	*An. plumbeus*	4	2	0	0	0	0
5	*Ae. cinereus*	1	1	0	0	0	0
6	*Ae. vexans*	314	33	6 (66)	2 (33)	0	0
7	*Ae. geniculatus*	26	10	3 (5)	1 (2)	1 (5)	0
8	*Ae. annulipes*	51	10	3 (25)	2 (6)	1 (9)	0
9	*Ae. behningi*	2	2	0	0	1 (1)	1 (1)
10	*Ae. cantans*	15	5	2 (7)	1 (4)	0	0
11	*Ae. caspius*	26	13	3 (4)	0	0	0
12	*Ae. cataphylla*	3	1	0	0	0	0
13	*Ae. dorsalis*	7	1	0	0	0	0
14	*Ae. flavescens*	1	1	1 (1)	0	0	0
15	*Ae. riparius*	9	4	3 (1)	1 (6)	0	0
16	*Ae. sticticus*	24	7	2 (7)	0	0	0
17	*Cx. modestus*	203	25	7 (54)	1 (35)	2 (4)	0
18	*Culex pipiens /torrentium*	2662	133	29 (532)	6 (57)	16 (273)	10 (174)
19	*Cs. longeariolata*	4	4	2 (2)	0	0	0
20	*Cs. annulata*	38	13	4 (12)	1 (5)	1 (1)	0
21	*Cq. richiardii*	18	11	4 (7)	1 (1)	0	0
22	*Ur. unguiculata*	119	8	0	0	0	0
	total	**4488**	**347**	**103 (1323)**	**29 (412)**	**40 (621)**	**19 (333)**

Dr. Tatiana Sulesco 

BNITM
Bernhard Nocht Institute for Tropical Medicine

Bernhard Nocht Institute for Tropical Medicine
P.O. Box 30 41 20, D-20324 Hamburg, Germany

Prof. Dr. Egbert Tannich
Head of Department
Molecular Parasitology
Bernhard-Nocht-Str. 74
20359 Hamburg
Tel +49 40 42818-477
Fax +49 40 42818-512
E-Mail tannich@bnitm.de

Hamburg, November 27, 2015

To whom it may concern

Letter of confirmation on the successful execution of the mission of Dr. Tataina Sulesco

Herewith, I confirm the participation of Dr. Tatiana Sulesco in the Short Term Scientific Mission Grant (EurNegVec TD1303 COST Action), organized by the Bernhard Nocht Institute for Tropical Medicine, Department of Molecular Parasitology, Hamburg, Germany, from 12th October 2015 to 27th November 2015. I confirm that the aims of the project proposal entitled "Molecular survey for mosquito-borne parasites in field collected mosquitoes from Moldova" have been successfully accomplished.

Sincerely yours,

Prof. Dr. Egbert Tannich