COST Action TD1303

European Network for Neglected Vectors and Vector-Borne Infections

(EurNegVec)

STSM Report

Detection of co-infections with tick-borne pathogens in tick and host samples (dogs, wild carnivores and rodents) and typing of *Anaplasma phagocytophilum* isolates by Multi Locus VNTR (Variable Number Tandem Repeat) Analysis (MLVA).

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BASIC DATA:

Name: Matei Ioana Adriana

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Topic: Detection of co-infections with tick-borne pathogens in tick and host samples and typing of *Anaplasma phagocytophilum* isolates by MLVA.

Contact: Dr. Muriel Vayssier-Taussat / Dr. Nadia Haddad

Host institution: INRA, École Nationale Vétérinaire d'Alfort, UMR BIPAR ENVA Anses UPEC USC INRA, Maisons-Alfort, France

PURPOSE OF THE STSM

Anaplasma phagocytophilum is small, pleomorphic, Gram-negative, obligate intracellular bacterial organism associated with *Ixodes* tick species with a worldwide distribution on the northern hemisphere (Stuen, Granquist and Silaghi, 2013). Multiple genetic variants of the bacterium have been indentified and characterized (von Loewenich et al. 2003; Scharf et al., 2011; Hunh et al., 2014) and sub-populations/sub-cycles within the species are now being discussed. Despite of numerous studies the ecology and epidemiology of different *A. phagocytophilum* genetic variants are not completely elucidated. To understand the circulation of *A. phagocytophilum* genetic variants and to identify their reservoir hosts are required discriminant genetic markers. For this aim a new approach of MLVA for *A. phagocytophilum*

has been recently developed (Dugat et al. 2014). The MLVA technique is a popular typing approach for studying the epidemiology of pathogenic agents, due to several advantages: high discriminatory power, robustness, repeatability, inter-laboratory portability and speed (Lindstedt, 2005).

Only few scattered data concerning the epidemiology of *A. phagocytophilum* in Romania are available and no data are available regarding the *A. phagocytophilum* genetic variants circulating in the country. The first molecular identification of this pathogen in Romania was in 2012, when Păduraru et al. (2012) identified it in *I. ricinus* collected from roe deer (*Capreolus capreolus*) and goats in 2 sites in Eastern Romania. Subsequently, several other reports on its presence or prevalence were published in dogs (Hamel et al., 2012; Mircean et al., 2012) and wild boars (*Sus scrofa*) (Kiss et al., 2014), as well as in ticks (Matei et al. 2015) and ticks collected from livestock (Ioniță et al., 2013), hedgehogs (*Erinaceus roumanicus*) (Dumitrache et al., 2013), tortoises (*Testudo graeca ibera*) (Paştiu et al., 2012) and birds (Mărcuțan et al., 2014).

With this view, the aim of this STSM was to evaluate the genetic variability of *Anaplasma phagocytophilum* by Multi Locus VNTR (Variable Number Tandem Repeat) Analysis (MLVA) in different tick samples. The second aim was to indentify the co-infection of some tick-borne pathogens in different mammal samples from Romania and Africa. The scientific mission was carried out according to the Work Plan previously provided with slight modifications. The study was framed into the TD1303 COST Action objectives of WG 2. This STSM aimed also the strengthening the collaboration and networking between scientist from participating COST countries. In addition the STSM to the ANSES, Faculty of Veterinary Medicine from Maison-Alfort (France) allowed the applicant to learn new laboratory techniques and to gain experience in the laboratory research.

DESCRIPTION OF THE ACTIVITIES CARRIED OUT DURING THE STSM

I. Typing of *Anaplasma phagocytophilum* isolated from different tick samples:

A selection of representative tick DNA samples was made. The ticks chosen for this study were questing *Ixodes ricinus* (n=60), *Ixodes ricinus* (n=100) collected from hedgehogs (*Erinaceus roumanicus*) and *Hyalomma aegyptium* (n=80) collected from tortoises (*Testudo graeca ibera*). In all this ticks samples, *Anaplasma phagocytophilum* DNA was previously detected targeting

msp 2 gene (Paştiu et al., 2012; Dumitrache et al., 2013) and *ank* A gene (Matei et al., 2015). For the MLVA we used 5 VNTR (Table 1) tested previously by Dugat et al. (2014) following the same protocol for four of the VNTR (APV A, APV B, APV C and APV D) and a modified annealing temperature for the APV E. The VNTR amplification was conducted in a volume of 25 µl, containing 5 µl purified DNA, 4 µl 5X high fidelity amplification buffer, 200 µM each dNTP, 0.5 µM each primer (Eurofins MWG Operon, Ebersberg, Germany) and 0.4 units of Phusion DNA polymerase (Fisher Scientific, Waltham, USA). An initial denaturation step at 98°C for 30 s was followed by 35 cycles of denaturation at 98°C for 10 s, annealing at optimal temperature (56°C for APV A, APV B, APV C and APV D, and 59°C for APV E) for 30 s and extension at 72°C for 1 min. After 35 cycles, there was a final extension step at 72°C for 10 min. PCR products were separated by long electrophoresis (8h) on 1% SeaKem LE agarose gels (Ozyme, Saint Quentin en Yvelines, France) in TBE buffer (Lonza, Basel, Switzerland), and stained with ethidium bromide for imaging.

VNTR	Primers	BU Length (bp)	Gene	Genome localization
APVA	F: CAACGCGAGCACGTCATCATCAGAA	201	*APH_0032*	28845-29712
	R: CACACTGACGTTACCGTGCTCGAAG			
APVB	F: GGGGGTATGACGAGTGTGGTAGCAA	114	*rpe*	53792-54393
	R: CCTTACTGCACACCGTACACGCAAA			
APVC	F: CCTACGGGGTGTCTTGCGTCCTA	189	*APH_0351*	340359-340834
	R:CTGCGCGAGTTTATGTGCAACT			
APVD	F: ATAGTGTGCAAGGCGCTAGTAATG	123	*virB6-3*	376959-377498
	R: TGTCGGACTATGCTTTTCACCATT			
APVE	F: CGACCTATGATCGCAGTGTA	20 21	*APH_0215*	214596-214852
	R: GTAGCAAGGTAACCACTACCA			

Table 1. Characteristics of the selected APVs and the corresponding forward and reverse primers

II. Developing a similar MLVA approach for *Ehrlichia canis*:

a. Computer analysis of repetitive DNA sequences for use as VNTR candidates:

The genomic DNA sequence of the *E. canis* Jake strain (Reference Sequence NC_007354) [http://www.ncbi.nlm.nih.gov/genome/] was screened for repetitive DNA sequences using the tandem repeats database developed by Le Flèche et al. (2001) [http://minisatellites-rec.igmors.u-

psud.fr/GPMS/]. The following criteria were applied to potential VNTR candidates: 1. total length between 100 and 2000 bp; 2. unit length between 100 and 500 bp; 3. basic unit (BU) copy number between 2 and 6000 and 80 to 100% BU identity. BLASTN analysis of the repeat sequences excluded VNTRs present in other available genomes.

b. Primer design for VNTR candidates:

The tandem repeats database described by Le Flèche et al. (2001) [http://minisatellites-rec.igmors.u-psud.fr/GPMS/] also provided 500 bp of flanking sequences both upstream and downstream of each VNTR candidate locus.

BLASTN analysis of these flanking sequences was used to design forward and reverse primers. Primers were designed ensuring that no annealing would occur with available pathogen genomes which can be found in carnivore's blood or with available host genomes and with the paired primer, or itself (OligoCalc).

III. Identification of *Anaplasma phagocytophilum* co-infections with several TBPs from different mammalian samples:

For this study were selected several *Anaplasma/Ehrlichia* positive DNA samples. In total, 34 dog samples from Romania and Africa, 25 wild carnivore samples from Romania, 12 rodent samples and 5 *R. rossicus* were take in account. Only thus 5 *R. rossicus* samples were not positive for *Anaplasma/Ehrlichia* infection. The co-infections were identified using a high-throughput Real-Time PCR system described by Michelet et al. (2014). The pre-amplification was made in a final volume of the 5 µl containing 2.5µl TaqMan PreAmp Master Mix (Applied Biosystems, France), 1.2µl pooled primers mix and1.3µl DNA, with one cycle at 95◦C for 10min, 14 cycles at 95◦C for 15s and 4 min at 60◦C. At the end of pre-amplification the reactions were diluted 1:10.

For the amplifications a 6 µl sample mix was prepared per sample, containing 3µl TaqMan® Gene expression MasterMix (Applied Biosystems, Foster City, CA), 0.3µl sample Loading Reagent (Fluidigm PN85000746) and 2.7µl of diluted pre-amplified DNA. A TaqMan® primer assay was prepared for each target, containing18 µM of each primer and 4 µM of probe. 3 µl of these primer assays were mixed with equal volumes of Dynamic Array (DA) assay loading reagent (Fluidigm PN85000736) to make assay mixes (9 µM primers and 2 µM probe). 5 µl of sample mixes, prepared as described, were then loaded into each sample in let of the dynamic array chip and 5µl of assay mixes were loaded into assay inlets. The chip was then placed on the IFC Controller HX for loading and mixing. After approximately 45 min the chip was ready for

thermal cycling and detection of the reaction products on the Biomark. PCR cycling comprised of 2 min at 50◦C, 10 min at 95◦C, followed by 40 cycles of 2-step amplification of 15s at 95◦C, and 1 min at 60◦C. Data were acquired on the BioMark™ Real-Time PCR System and analyzed using the Fluidigm Real-time PCR Analysis software to obtain crossing point (CP) values. For microfluidic tool evaluation on field samples, the assays were performed in duplicate. Two negative water controls were included per chip.

MAIN RESULTS

I. **Typing of *Anaplasma phagocytophilum* isolated from different tick samples:**

The amplification with all 5 selected VNTR was successfully performed for 59% (59/100) *Ixodes ricinus* collected from hedgehogs, 50% (30/60) questing *Ixodes ricinus* and 5% (4/80) *Hyalomma aegyptium* collected from tortoises. The electrophoresis was performed for all the amplification products of using the first 4 VNTR (APV A, APV B, APV C and APV D) and partial (35/59) for APV E (*Ixodes ricinus* collected from hedgehogs). The number of repetitions for each VNTR was calculated using the formula: (obtained product length-primers length)/unit length. The MLVA profiles and the distances between two MLVA profiles were established, the distance was the number of VNTRs with different numbers of repeats (distances thus varied between 0 and 5). From all the 59 samples, 16 distinct MLVA profiles were obtained. The partial results showed a high percentage (90% - 53/59) of ticks shared their MLVA profile with at least one other sample. For 86.4% (50/59) samples a single MLVA profile was obtained, while 13.6% of samples had two or more profiles. A high diversity was observed for the first 4 APV, the distance varied from 1 to 3 among 16 profiles and 5 of them showed a distance of 4. APV E seems to have the highest discriminatory power (10 different values), followed by APV B (7 different values) while APV A, C and D were similar (2 or 3 values).

The electrophoresis for remaining amplification products will be finished in the laboratory of the Faculty of Veterinary Medicine Cluj-Napoca, and the obtained MLVA profiles will be compared between the different tick samples. Also *Anaplasma phagocytophilum* DNA will be isolated from blood of the hedgehogs harboring the positive ticks and MLVA typing will be performed.

II. **Developing a similar MLVA approach for *Ehrlichia canis*:**

Following the computer analysis using the tandem repeats database developed by Le Flèche et al. (2001) [http://minisatellites-rec.igmors.u-psud.fr/GPMS/], 37 VNTR were indentified and the specific primers were designed.

III. **Identification of *Anaplasma phagocytophilum* co-infections with several TBPs from different mammalian samples:**

Despite the positive PCR results targeting either *16S rRNA* or *ankA* genes, *Anaplasma phagocytophilum msp2* gene was not amplified in any the tested samples. However, few pathogens were detected. The overall prevalence values for the detected pathogens are given in Table 2.

Species	*Bo_afla*	*Bo_sl23S*	*An_plgroEL*	*Eh_cadsb*	*Neo_mikgroEL*	*SFGgltA*	*Ba_hepap31*	*Fr_tufopA*	*Bab_ca18S*	*Bab_vohsp70*
Dog	0	0	14.71	5.88	0	0	0	0	0	0
Wild carn	0	0	0	4.00	0	4.00	0	4.00	8.00	0
Rodents	8.33	18.18	0	0	18.18	0	8.33	8.00	0	0
R. rossicus	0	0	0	0	0	0	0	20.00	20.00	40.00

Table 2. Prevalence of TBP's in different mammalian samples

FORESEEN PUBLICATIONS ARTICLES RESULTING FROM THE STSM

The results of this STSM will be published as a paper entitled "*Anaplasma phagocytophilum* genotypes circulating in Romania" by the end of 2015 in a peer-reviewed journal.

CONFIRMATION BY THE HOST INSTITUTION OF THE SUCCESSFUL EXECUTION OF THE STSM

I herein confirm the present report regarding the COST-ECOST-STSM-TD1303-200415-056593 at INRA, École Nationale Vétérinaire d'Alfort, UMR BIPAR ENVA Anses UPEC USC INRA, Maisons-Alfort, France.

1.06.2015 Dr. Muriel Vayssier-Taussat