European Network for Neglected Vectors and Vector-Borne Infections

STSM GRANTEE : PhD Ivona Laiu (Popovici)
HOST: PhDMuriel Vayssier-Taussat, ENVA
STSM Topic: Characterization of novel viruses carried by ticks
STSM type: Regular (from Romania to France)
COST Action: TD1303, WG 2
DURATION: 1 April- 30 June

SHORT SCIENTIFIC REPORT

CONTEXT AND OBJECTIVES

Ticks are arthropods considered to be vectors for diverse zoonosis agents such as viruses, bacteria and parasites. They are able to acquire and transmit the pathogen most commonly during their blood meal, but the inter stage transmission hypothesis was also incriminated. The incidence of tick-borne diseases (TBD) is increasing worldwide and some tick-borne pathogens—including many viruses (Tick borne encephalitis (TBE) virus, Kyasanur forest disease virus, Omsk haemorrhagic fever virus, Louping ill virus, Crimean-Congo haemorrhagic fever (CCHF) virus) have been reported in new geographical regions.

Due to advances in molecular biology, new species, strains or genetic variants of microorganisms are being detected in ticks, resulting in an ever- increasing list of pathogens capable of infecting animals and humans. For instance, more than one hundred tick-borne viruses have been identified worldwide. Among them, only few species are well known as the two major severe re-emerging viruses: TBEV and CCHFV. Others are often neglected because their pathogenicity for vertebrates is not well established and because few diagnostic tools are available. Therefore in order to prevent emerging tick-borne diseases, more effort is required to identify new or unexpected tick-borne pathogens, in particular viruses to identify their circulation within wildlife and to evaluate their importance in terms of human diseases.

In order to detect new microorganisms potentially pathogenic for humans and/or animals, the hosted laboratory apply Next Generation Sequencing (NGS) method combined with bioinformatics for the detection of new or unexpected pathogen in ticks. For that purpose a pool of 555 ticks were collected in the French Ardennes, a geographical area corresponding to a hot spot for ticks. The RNA extraction was performed for these samples. Transcriptome of a pool of 555 ticks were derived from pooled RNA retro-transcribed into cDNA, ligated and randomly amplified byWGA- whole genomic amplification. Sequencing has been performed to a depth of up to 130 million paired-end reads of 101 bases per pool allowing for robust de novo assembly. Using this strategy, the hosted lab has identified several new viral species in genus known to contain viruses transmissible to humans and/or animals belonging to *Bunyaviridae* family (*Nairovirus* and *Phlebovirus*) and *Reoviridae* family. Some of the

sequences encompass large parts of the virus genomes, while in other cases only a part of the genome was acquired. Contigs corresponding to Eyach virus, a Coltivirus genera member, were also observed.

The **Short Scientific Mission** intended to1/ better characterize both the coltivirus and the new Bunyaviridae viruses by filling in the gaps in the genome sequences and 2/ to analyse the effect of infection the Eyach virus, isolated from Ixodes ricinus ticks, in mice.

OBJECTIVES AND WORKING PLAN:

1/ Full-length genome sequence of new viruses: Some of the viral sequences corresponding to potentially new genera or species of viruses encompass large parts of the virus genomes, while in other cases only a part of the genome was acquired. To characterize new species of virus, full-length genome sequences will be derived using primers designed in the outer part of each contig obtained by NGS, in order to amplify the missing sequences (between these contigs).

This part includes:

-To design primers in the outer part of each Contig already obtained.
-To conduct RT and PCR on the pool of the 555 RNAs.
-To sequence and analyse PCR products to fill the gap
-To detect the positive samples among the 555 tested ticks

2/ Effect of Eyach virus infection on mice: This virus was first described in Germany, after in France as a potential pathogen for human central nervous system, specific antibodies being detected in patients with meningoencephalitis.

This part includes:

-To obtain viral inoculum on immunodeficiency mice
-To conduct experimental infections on immunity normal status mice
-To observe the behavior and health status modifications in mice used in our laboratory experiment
-To evaluate the EYACH virus presence in blood and organ samples collected from the mice used in our study

METHODS, RESULTS AND DISSCUSIONS

1 - Sequencing of the M Segment of the potentially new Phlebovirus and Nairovirus

NGS analysis of tick RNA extracts revealed 4 Contigs (8883, 8702, 1074, 9062) assigned to phlebovirus segment M and 2 Contigs (3246, 6797) for nairovirus segment M.

Using this results, several primers were designed for each Contig as described in table no 1:

Table no 1.

The primers designed for Bunyaviridae Contigs

CONTIG	Corresponding virus	Number of Forward Primers	Number of Reverse Primers	Total number of primers combinations
8883	phlebovirus	3	3	9
8702	phlebovirus	3	3	9
1074	phlebovirus	1	1	1
9062	phlebovirus	1	1	1
3246	nairovirus	2	2	4
6797	nairovirus	3	3	9

Using the classical PCR technique and the designed primers we tried to amplify the M segment of phlebovirus and nairovirus in the WGA treated pool of RNAs extracted from the 555 ticks. For testing the primers we used the Taq Core Kit from MP-BIO, 3 µl of cDNA and the following protocol (16,75µl $_dH_2O$; 2,5 µl tampon sans $MgCl_2$; 1,5µl $MgCl_2$ 25mM; 0,25 µlTaq Polimerase; 0,25 µl Primer Forward; 0,25 µl Primer Reverse)
The following amplification program was used:

Step 1: 94˚C for 4 minutes
Step 2: 94˚C for 35seconds
X°C for 30 seconds
72°C for 40 seconds
Step 3: repeat 39 times Step 2
Step 4: 72°C for 10 minutes
Hold 10°C,

where X represents the annealing temperatures adjusted for every pair of primers.

For phlebovirus, we amplified 2 fragments corresponding to the 8702 Contig, 5 fragments for the 8883 Contig and 1 amplicon for the 1074 Contig (table 2).

Table no 2

Table 2: Positive results obtained using specific designed primers for Phlebovirus segment M

CONTIG	Forward primer	Revers primer	Molecular weight
8702	PM 8702 F1	PM 8702 R2	400
	PM 8702 F1	PM 8702 R3	350
8883	PM 8883 F1	PM 8883 F2	100
	PM 8883 F2	PM 8883 F3	100
	PM 8883 F3	PM 8883 F4	150
	PM 8883 F2	PM 8883 R3	900
	PM 8883 F3	PM 8883 R1	200
1074	PM 1074 F1	PM 1074 R1	100-200

When positive results were obtained the amplification products were sent for sequencing.

For studying the new viruses prevalence we had chosen the pair PM 8702 F1(forward) with PM 8702 R2 (reverse). The RNAs extracted from ticks were reverse-transcripted in cDNA using the Superscript III First-Strand Synthesis system for RT-PCR, from Invitrogen and a three steps RT program. From the total of 555 cDNA samples tested we found only one positive sample (sample no 508, figure 1), which was sent for sequencing.

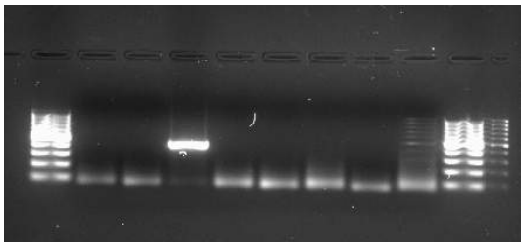


Figure 1. The 508 positive sample in agarose gel after electrophoresis

We aligned the nucleotide sequences of the 8702 Contig obtained by NGS technique, with that of thepositive results obtained by PCR after WGA amplification using the PM 8702 F1 and PM 8702 R2 primers and after cDNA amplification obtained in the 508 sample. Their identity was 100%. This confirms the amplification success of the segment M Phlebovirus after using the NGS Contig 8702 for primers design.

The fact that we found the presence of this Phlebovirus by detecting the M segment in such a small percentage of tick samples (0.18%) suggests that this virus does not make part of the usual tick microbiome.

For Nairovirus, we did not succeed to obtain amplicons using the designed primers.

2- Eyach virus: pathogenic effect on mice

* Production of Eyach virus:

Eyach Virus isolation was performed by intracranial inoculation of 2 mice 5 days old with 20µl inoculum which was at the second passage and prepared from tick grinding. Four days after inoculation the mice died and the brain was collected. After individually grinding the brain in 1ml DMEM with 10% BFS, the viral RNA extraction was performed on a fraction of the homogenate using the Nucleospin RNA II Kit, from Macherey-Nagel. Presence of EYACH virus was tested by the RT-qPCR (using LightCycler 480 RNA Master Hydrolysis ProbesKit and the 480 Multiwell Plate 96 LightCycler, both from Roche) with the following program: **Step 1**: 63°C for 3 minutes, **Step 2:** 95°C for 30 sec, Step 3: 95°C for 10 sec, 60°C for 30 sec, 72°C for 1 sec, **Step 4**: repeat Step 3 for 44 times, **Step 5**: 40°C for 3 seconds.

The results confirmed the presence of the EYACH virus in our samples of mice brains.

* Pathogenic effect of Eyach virus:

We then used this virus to infect intraperitonnally laboratory mice and to follow viremia for 21 days till the animals were killed and viruses detected in organs (spleen and brain).

For infection we used viral suspension prepared from the mice brains which was inoculated by intraperitoneal way in two lots of normal immune status mice OF1: one lot of 5 adult females (100µl per female) and another of 10 suckling mice (20µl per suckling mice). For negative control we used as well 5 adult females and 10 suckling mice inoculated with 100µl and 20µl PBS respectively in the same way as our subjects. Presence of the viral RNA was identified in blood and organs (brain and spleen) after RNA extraction (Nucleospin RNA II kit, Macherey-Nagel) followed by RT-qPCR.

Their health status, behavior and weight were observed for 21 days after inoculation. Blood samples were collected from the conjunctive and tail blood vessels in day 7 and from conjunctive in day 14 and day 21 post inoculation.

Results:

No particular behavior or health status modification was observed during this period.

We detected the EYACH virus in all blood samples collected at day 7, 14, 21 after inoculation from the animals (adults and suckling mice) infected with the virus while blood samples from negative controls were negative.

Comparing the results (table 3), the CP values obtained from the blood were lower at day 14 and 21 compared to day 7 suggesting that the virus replicated within the infected subjects.

Table no 3

The CP values for blood samples collected in day 7, 14 and 21

Day	Adults	Suckling mice	Plasmide
Day 7	34,71-37,64	31,78-34,96	8,04-8,14
Day 14	26,99-33,60	22,76-25,82	8,09-8,42
Day 21	26,97-33,42	24,30-28,84	8,32-8,32

Brain extracts of infected mice were also positive (table 4):

Table no 4

The CP values for brain samples

Sample	Adults	Suckling mice	Plasmide
Brain	33,88-40,03	24,81-31,31	8,09-8,62

This result indicates that Eyach virus is able to induce a viremia as well as infect the brain of laboratory mice without causing any damage (at least during the first 21st days

following infection). In the near future, we have planned to 1/ quantify viral particles in the blood and in the brain to insure the virus in indeed able to multiply in blood and 2/ to monitor the effect of infection in the long term.

All together these results demonstrated that Eyach virus isolated from *Ixodes ricinus* is able to infect laboratory mice and to colonize their brain without causing any immediate damage. This finding will lead us to investigate in the near future the role of rodents (and other animal) as reservoir of this virus in natural condition and the role of this virus in human or domestic animal unidentified tick borne diseases.

Valorization: a manuscript describing Eyach virus is under preparation (Detection and isolation of a neurotropic strain of the forgotten Eyach virus in France" Sara Moutailler, Elodie Devillers, Ivona Popovici, Muriel Vayssier-Taussat, Marc Eloit).

Personal consideration

This stage that took place within the BIPAR research unit between April 1st to June 30th, 2014 under the Mrs PhD Muriel Vayssier-Taussat coordination who gave me the opportunity to improve and update my virology knowledges as well as to learn new techniques and methods used in the research field. Thus I was trained to design primers, to do blast analyses on the diverse obtained data, including NGS and to use molecular detection methods adapted to tick borne viruses detection such RNA revers-transcription after RNA extraction, cDNA and RNA amplification by RT and PCR classic or real-time. In order to isolate viruses I was trained in working with laboratory animals consisting in handling, inoculation, collecting of pathological material, security measures etc. I also participated in collecting ticks by the flag method in the woods from recreation areas near Paris.

I consider that the Short Term Scientific Mission supported by COST in the WG 2 of the EurNegVec Action helped me in increasing my experience in the research field in general and in the tick-borne and new viruses in particular.

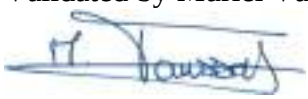
Acknowledgements

I would like to thank COST for funding the STSM TD1303-17088 and so giving me the opportunity to realize this stage.

I especially wish to thank Mrs PhD Muriel Vayssier-Taussat BIPAR research managerfor making me the honor to be my STSM host and who coordinated with professionalism, ability and enthusiasm this intership and to her team PhD. Sara Moutailler and Elodie Devillers who helped me in gaining many skills necessary in the research field.

Special thanks to Mr Prof PhD Marc Eloit, manager of the Laboratory for Pathogen Discovery, Pasteur Paris for availability and collaboration.

Validated by Muriel Vayssier-Taussat



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Maisons-Alfort, the 23th of July, 2014

Confirmation by the host institution of the successful execution of the STSM

I herein confirm the report of Ivona Laiu regarding the COST - STSM-ECOST-STSMTD1303 -010414-044112 in France.

Yours sincerely