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Prevalence of tick-borne viruses in *Ixodes ricinus* assessed by high-throughput real-time PCR

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Abstract (198 words)

Ticks are one of the principal arthropod vectors of human and animal infectious diseases. Whereas the prevalence of tick-borne encephalitis virus in ticks in Europe is well studied, there is less information available on the prevalence of the other tick-borne viruses (TBVs) existing worldwide. The aim of this study was to improve the epidemiological survey tools of TBVs by the development of an efficient high-throughput test to screen a wide range of viruses in ticks.

In this study, we developed a new high-throughput virus-detection assay based on parallel real-time PCRs on a microfluidic system, and used it to perform a large scale epidemiological survey screening for the presence of 21 TBVs in 18,135 nymphs of *I. ricinus* collected from five European countries. This extensive investigation has (i) evaluated the prevalence of four viruses present in the collected ticks, (ii) allowed the identification of viruses in regions where they were previously undetected.

In conclusion, we have demonstrated the capabilities of this new screening method that allows the detection of numerous TBVs in a large number of ticks. This tool represents a powerful and rapid system for TBVs surveillance in Europe and could be easily customized to assess viral emergence.

One-sentence summary (26 words)

Large scale epidemiological survey of 21 tick-borne viruses in 18,135 *Ixodes ricinus* allowed through the development of a new high-throughput tool based on microfluidic real-time PCR.

1. Introduction

Ticks are one of the principal arthropod vectors of human and animal infectious diseases and are able to transmit a wide range of bacteria, parasites and viruses (Gulia-Nuss *et al.*, 2016). Among the 900 tick species worldwide, *Ixodes (I.) ricinus* is the most widespread species in Europe, with the highest abundance. *I. ricinus* is able to engorge on many different vertebrate hosts (Rizzoli *et al.*, 2014, Egyed, 2017) and transmits several pathogens of medical and veterinary importance. These include the spirochetes from the *Borrelia* genus which are the cause of Lyme borreliosis and relapsing fever borreliosis, the bacteria *Anaplasma phagocytophilum* responsible for human granulocytic anaplasmosis (HGA), parasites from the *Babesia* genus responsible for piroplasmosis and tick-borne viruses (TBVs) including tick-borne encephalitis virus (TBEV), louping ill virus (LIV), uukuniemi virus (UUKV), kemerovo virus and eyach virus (EYAV) (de la Fuente *et al.*, 2008, Dantas-Torres *et al.*, 2012, Nuttall, 2014, Rizzoli *et al.*, 2014).

Whereas bacteria and parasites incidences in animals, humans, and ticks are well studied, there is less information available on viruses. Environmental changes, human travel and animal transportation have led to the emergence and/or the geographical expansion of several tick-borne pathogens (TBPs) worldwide including viruses (Dantas-Torres *et al.*, 2012, Lindgren *et al.*, 2012, Jore *et al.*, 2014, Vayssier-Taussat *et al.*, 2015). Several TBVs have already emerged in new territories, such as TBEV, LIV, powassan virus (POWV), deer tick virus, and Crimean-Congo hemorrhagic fever virus (CCHFV) (Hinten *et al.*, 2008, Mansfield *et al.*, 2009, Maltezou *et al.*, 2010), while novel arthropod-borne viruses are constantly being discovered (Yu *et al.*, 2011, McMullan *et al.*, 2012, Tokarz *et al.*, 2014, Yun *et al.*, 2014, Kosoy *et al.*, 2015). These trends highlight the importance of monitoring the distribution and prevalence of TBVs in European tick populations.

Whereas the prevalence of TBEV in ticks in Europe is well studied, there is less information available on the prevalence of other TBVs (Laaksonen *et al.*, 2017, Raileanu *et al.*, 2017, Rar *et al.*, 2017, Zajac *et al.*, 2017). More than 500 arthropod-borne viruses are currently recognized worldwide (Bichaud *et al.*, 2014) with at least 160 TBVs classified into nine different families: one DNA viral family, *Asfarviridae*, and eight RNA viral families, *Flaviviridae*, *Orthomyxoviridae*, *Reoviridae*, *Rhabdoviridae*, the newly recognized *Nyamiviridae* (order *Mononegavirales*), and the families *Nairoviridae*, *Phenuiviridae* and *Peribunyaviridae* in the new order, *Bunyavirales* (Nuttall, 2014, Kazimirova *et al.*, 2017).

Usually, TBV prevalence in ticks is estimated by RT-PCR or real-time RT-PCR (rRT-PCR) in assays which target specific viruses known (or suspected) to be present in the sample collection (Raileanu *et al.*, 2017). The disadvantage of this approach is the limited number of different viruses that can be tested, given the quantity of RNA required for one RT-PCR or rRT-PCR. The aim of this study was to improve the epidemiological survey tools of TBVs by the development of an efficient high-throughput test for screening of a wide range of viruses in ticks. For this purpose we developed a novel high-throughput surveillance method based on real-time PCR which is able to identify 21 major worldwide TBVs in parallel in one sample. This assay based on a microfluidic system (BioMark™ dynamic array system, Fluidigm) is capable of performing 2,304 individual real-time PCRs using 48.48 chips using very small volumes of RNA for each individual PCR reaction (Michelet *et al.*, 2014). In a single experiment, 47 ticks or pools of ticks can be tested for the presence of 21 viruses, as well as confirmation of the tick species. A similar system has already been successfully developed to screen 37 tick-borne bacteria and parasites (Michelet *et al.*, 2014). In the current study, we applied the new high-throughput assay to screen 18,135 *I. ricinus* collected from five European countries; Sweden, France, United Kingdom (UK), Denmark, and the Netherlands. As a result, we have shown that high-throughput real-time PCRs to screen TBVs in European ticks appeared effective, both in terms of specificity and sensitivity. This new development opens novel perspectives in detection capacities that could potentially be applied to TBVs surveillance and large scale epidemiological studies.

2. Materials and methods

2.1. Study area and tick collection

A total of 18,135 *Ixodes ricinus* nymphs, from 13 locations in 5 different countries were studied. Questing nymphs were collected using the flagging technique (Vassallo *et al.*, 2000) in one location in UK and in three different locations in each of the four other countries (Figure 1) with a maximum of 1500 nymphs per site. Collections at each site were originally pooled and stored as 49 pools of 25 nymphs each and 91 pools of just 3 nymphs each. For analysis of virus we later merged one large pool with two small pools resulting in 45 pool of 31 nymphs each per site, additional nymphs, if any, were not analyzed. In France, ticks were collected from Murbach (M) (47.918N; 7.146E), Autheux (A) (50.157N; 2.241E), and Senart forest (S) (48.677N; 2.484E) in May 2013. In Denmark, ticks were collected from Bidstrup (BIS) (55.560N; 11.897E) and Åbenrå (AAB) (55.052N; 9.383E) in June 2013 and in Kalø (KAL) (56.290N; 10.472E) in July 2013. In the Netherlands, ticks were collected from Austerlitz (Aus) (52.083N; 5.300E), Duin en Kruidberg (D) (52.430N; 4.615E), and Kuinderbos (K) (52.783N; 5.810E) in June 2013. In Sweden ticks were collected from Ramsvikslandets (RV) (58.420N; 11.250E), Morga hage (MH) (59.752N; 17.642E), and Hindens rev (HR) (58.573N; 12.914E) in May 2013. In the UK, ticks were collected from Richmond Park, Surrey (RP) (51.4427N; 0.2837E) in May 2013.

To confirm the presence of Eyach virus in the Netherlands, additional *I. ricinus* ticks were collected from five locations in the province of Overijssel, (close to 52.333N; 6.400E) in September 2015 and April 2016. A total of 291 adults and 1167 nymphs were collected and regrouped into 434 pools (2 adults/pool and up to 20 nymphs/pool).

2.2. RNA extraction

Ticks were morphologically identified to species level (Pérez-Eid, 2007) and preserved at -80°C. After washing once in 70% ethanol for 5 min and twice in distilled water for 5 min, pools were crushed in 300 µl of DMEM with 10% fetal calf serum and six steel balls using the homogenizer Precellys®24 Dual (Bertin, France) at 5500 rpm for 20 seconds or with one 5 mm steelball in the TissueLyser (Qiagen, Germany) at 30 Hz for 2 min.

RNA was then extracted using the Nucleospin RNA II extract kit (Macherey-Nagel, Germany) using 100µL of the homogenate. 200µL were conserved at -80°C for back-up. Total RNA per sample was eluted in 50 µl of RNase free water and stored at -80°C until further use. For the Swedish samples the extraction was performed in a Magnatrix 8000+ robot using the Vet Viral NA kit (NorDiag, Sweden) according to the manufacturer's instructions.

2.3. Assay design

Tick-borne viruses, their targeted genes and the corresponding primers/probe sets are listed in Table 1. For each pathogen or tick, primers and probes were specifically designed for this study, except for CCHFV for which previously published primers and probes were used (reverse complement were used for probes) (Wolfel *et al.*, 2007). Nairobi Sheep Disease virus (NSDV) was targeted with two different set of primers and probe to improve detection. Each primer/probe set was validated using a dilution range of several RNA positive controls or synthetic plasmids (with inserts corresponding to the targeted sequence) (Table 1) by real-time TaqMan RT-PCRs on a LightCycler® 480 (LC480) (Roche Applied Science, Germany). Real-time RT-PCR assays were performed in a final volume of 20 µl using the LightCycler® 480 RNA Master Hydrolysis Probes Mix 2.7X (Roche Applied Science, Germany), with primers and probes at 500nM and 250 nM respectively and 2 µl of control RNA. Thermal cycling conditions were as follows: reverse transcription (RT) at 63°C for 3 min, denaturation step at 95°c for 30 s, 45 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 1 s and one final cooling cycle at 40°C for 30 s. For positive controls where synthetic plasmids were used, primers and probes were tested by real-time TaqMan PCRs on a LC480 (Roche Applied Science, Germany). Real-time PCR assays were performed in a final volume of 12 µl using the LightCycler® 480 Probe Master Mix 1X (Roche Applied Science, Germany), with primers and probes at 200 nM and 2 µl of control DNA. Thermal cycling conditions were as follows: 95°C for 5 min, 45 cycles at 95°C for 10 s and 60°C for 15 s and one final cooling cycle at 40°C for 10 s.

2.4. Reverse Transcription and cDNA pre-amplification

RNAs were transformed to cDNA by reverse transcription using the qScript cDNA Supermix kit according to the manufacturer's instructions (Quanta Biosciences, Beverly, USA). Briefly the reaction was performed in a final volume of 5µL containing 1 µL of qScript cDNA supermix 5X, 1 µL of RNA and 3µL of RNase free water; with one cycle at 25°C for 5 min, one cycle at 42°C for 30 min and one final cycle at 85°C for 5 min.

For DNA pre-amplification, the Perfecta Preamp Supermix (Quanta Biosciences, Beverly, USA) was used according to the manufacturer's instructions. All primers were pooled to 200 nM final each. The reaction was performed in a final volume of 5 µL containing 1 µL Perfecta Preamp 5X, 1.25 µl pooled primers,

1.5 µL distilled water and 1.25 µL cDNA, with one cycle at 95°C for 2 min, 14 cycles at 95°C for 10 s and 3 min at 60°C. At the end of the cycling program the reactions were 1:5 diluted. Pre-amplified cDNAs were stored at -20°C until needed.

2.5. High-throughput real-time PCR

The BioMark™ real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using the 48.48 dynamic arrays (Fluidigm, USA). These chips dispense 48 PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble PCR reactions in individual chambers prior to thermal cycling resulting in 2,304 individual reactions.

Real-time PCRs were performed using FAM- and black hole quencher (BHQ1)-labeled TaqMan probes

with TaqMan Gene Expression Master Mix in accordance with manufacturer's instructions (Applied

Biosystems, France). Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 2-step amplification of 15 s 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 C_t values. See Michelet et al. 2014 for more detail (Michelet *et al.*, 2014).

Primers and probes were evaluated for their specificity against RNA reference materials in quadruplicate or duplicate, and used in duplicate to screen field samples. One negative water control was included per chip. *I. ricinus* RNA served to confirm the tested tick species and as a RNA extraction control. To determine if factors present in the sample could inhibit the PCR, *Escherichia coli* strain EDL933 DNA was added to each sample as an internal inhibition control, using primers and probe specific for the *E. coli eae* gene (Nielsen & Andersen, 2003).

2.6. EYAV detection in additional *I. ricinus* ticks from the Netherlands

RNA samples extracted from the 434 pools were screened for EYAV by classical real-time RT-PCR targeting the VP2 of the EYAV genome with specific primers and probes (Table 1). Real-time RT-PCR Taqman assays were performed in a final volume of 20 µl using the LightCycler® 480 RNA Master Hydrolysis Probes master mix (Roche Applied Science, Germany) at 1 X final concentration, with 0.5 µM specific primers and 0.25 µM probes, 3.25 mM manganese acetate [Mn(OAc)2] and 2 µl RNA. Positive and negative (water) controls were included in each run. Real-time RT-PCR thermal cycling conditions were as follows: 63°C for 3 min, 95°C for 30 s, 45 cycles at 95°C for 10 s then 60°C for 30 s, followed by cooling at 40°C for 10 s.

2.7. Validation of the results by RT-PCR, nested PCR and sequencing

Conventional RT-PCR followed (or not) by nested PCR using primers targeting different genes or regions than those of the BioMark™ system (Table 2), were used to confirm the presence of viral RNA in the field samples. Amplicons were sequenced by Eurofins MWG Operon (Germany), and then assembled using BioEdit software (Ibis Biosciences, Carlsbad). An online BLAST (National Center for Biotechnology Information) was used to compare results with published sequences listed in GenBank sequence databases.

2.8. Prevalence estimation

Prevalences were estimated assuming perfect sensitivity and specificity of pathogen detection using the online statistical program "Pooled prevalence for fixed pool size and perfect test" Method 2 (AusVet Animal Health Service http://epitools.ausvet.com.au/content.php?page=home). If all 45 pools of 31 nymphs were negative, prevalence was recorded as < 0.21%, because the 95% probability of sampling n (1395) negative ticks from a population with prevalence p is given as (1-p)n.

3. Results

Primer/probe sets were specifically designed to detect 21 TBV and two tick species (Table 1). Each set of primers/probe specifically identified their corresponding positive control samples (tick species or viral RNA or plasmid with the sequence of interest) via Taqman RT-real-time PCRs or Taqman real time PCRs on a LightCycler 480 apparatus, except for the two designs targeting NSDV. Resulting C_t values varied from 8 to 40 depending on sample type and nucleic acid concentration. To avoid sensitivity problems, a step of cDNA pre-amplification was included in the assay. The usefulness of such a pre-amplification step has already been demonstrated for the detection of 37 tick-borne pathogens (bacteria and parasites) using with the same system and allowed specific amplification of the targeted pathogen sequences [24]. Indeed, this step enabled detection of all positive controls via Taqman real time PCRs on a LC480 apparatus, again except for the two NSDV designs. Those designs were deleted from the chip for the rest of the analysis. The specificity of each primers/probe set was then evaluated using 34 TBV positive controls (different strains of each virus when possible or plasmid) on the BioMark™ system (Figure 2). Results demonstrated high specificity for each primer/probe set after pre-amplification (Figure 2). Indeed, 20 assays (on the 21 developed) were only positive for their corresponding positive controls. The assay for POWV showed cross-reactivity with deer tick virus.

A total of 18,135 nymphs, in 45 pools of 31 nymphs per site, from 13 European locations (five countries) were tested using the new assay on the BioMark™ system. Among the targeted viruses, 17 were not detected in ticks from any country (TBEV eastern subtype, TBEV Siberian subtype, langat, LIV, deer tick, POWV, meaban, West Nile, Kyasanur, Omsk, African swine fever, thogoto, dhori, Kemerovo, Colorado tick fever virus, dugbe, Schmallenberg). For each site, prevalences were estimated for each virus and are presented in Table 3.

1/ TBEV

TBE virus was detected in four pools from Sweden at the HR site with a prevalence of 0.30%. The four sequences obtained presented 100% identity, one sequence was deposited in GenBank (accession number: MH708169) and showed 98% homology with reference sequences from European subtype strains isolated in ticks (Italy, GenBank FJ159003), rodent (Finland, GenBank GU183380) and human sera (Czech Republic, GenBank KJ922515) (Table 4). The sequence presented only 96% homology with TBEV sequence isolated from *I. ricinus* ticks collected in Torö and Saringe areas, Sweden, in 2003 and 2009 respectively (GenBank DQ401140 and KF991106).

2/ UUKV

UUKV was detected in five pools in Sweden with a prevalence of 0.22% for HR site and 0.07% for MH and RV sites. The virus was also detected in two pools in the Netherlands (site K) with a prevalence of 0.15%. In total UUKV was detected in seven pools from Europe. Sequences obtained for the S segments (accession numbers: MH708178-MH708180 Sweden, MH708181-MH708182 the Netherlands) presented 99% (K site, the Netherlands) and 97% (HR site, Sweden) with Uukuniemi S segment (GenBank M33551 and KM114248, respectively). Sequences obtained for L segments (accession numbers: MH708173 the Netherlands, MH708174-MH708177 Sweden) presented between 94 to 97% homology with Uukuniemi L segment (GenBank D10759) (Table 4).

3/ EYAV

EYAV was detected in five pools from the Netherlands at the K site with a prevalence of 0.38%. To confirm the presence of the virus in the Netherlands, 291 adult and 1167 nymphal ticks were collected from five locations in the province of Overijssel. Among the 434 pools, only one nymphal pool was positive for EYAV (Prevalence 0.07% [0.002-0.403]). Sequences obtained from site K were 100% identical (accession number: MH708170) and presented 95 % homology with an Eyach virus - VP12 sequence available in GenBank (AF343061). Sequences obtained from Overijssel province (accession number: MH708171-MH708172) presented 99% and 97% homology, respectively, with two Eyach virus – VP1 sequences available in GenBank (AF282467 and AF343053) (Table 4).

4/ New-Nairovirus

One pool of ticks collected at the S site in France and two pools collected at the D site in the Netherlands were found positive for CCHF virus, with a prevalence of 0.07% and 0.15% respectively. A nested PCR targeting the N gene of CCHF virus was attempted on positive samples but failed to amplify a sequence of interest. However, a nested PCR targeting the S segment of viruses from the Nairovirus genus was tested on the three samples. An amplification product was obtained for one positive sample from D site (the Netherlands) when cDNAs were diluted at 1 to 5 before being assessed by nested PCR, and a band of 400 bp was visualized on an agarose gel for this sample (Figure 3). The PCR product was send for sequencing but the sequence obtained was not readable, so the sequence failed. Unfortunately no more RNA or tick homogenate were available to perform further analysis.

4. Discussion

In this study, we developed and tested in field a new high-throughput virus-detection assay based on microfluidic PCRs. Subsequently, we used this newly developed assay to perform a large scale epidemiological survey screening for the presence of 21 TBVs in 18,135 nymphs of *I. ricinus* collected from 13 European sites (one in UK, and three in France, Denmark, Sweden and the Netherlands). This large investigation has (i) evaluated the prevalence of four viruses present in the collected ticks, (ii) allowed the detection of UUKV for the first time in Sweden and UUKV and EYAV for the first time in the Netherlands, and (iii) detected a potential new-Nairovirus in ticks from France and the Netherlands.

Despite a preamplification step, two designs targeting NSDV were unable to detect the RNA positive controls used in this study. This failure suggests degradation of this RNA sample, unfortunately we were not able to obtain another positive control. Confirmation of the sensitivity of these two primer/probe sets needs to be undertaken in the future at least using a plasmid with the sequence of interest. Among the others sets of primer/probe, 13 allowed the detection of their targeted viral RNA with controls. For eight viruses, RNA positive controls were not available and plasmids containing targeted sequences have been used. For those viruses, and associated primer/probe sets, further evaluation of specificity is required.

In our study we screened 18,135 *I. ricinus* nymphs collected in 13 European sites for the presence of 21 TBVs and four viruses' prevalences were estimated (Table 3).

TBEV was only detected in one site in Sweden with a prevalence of 0.29%. TBEV causes severe central nervous system infection in 15,000 people in Europe and Asia each year (Dobler, 2010). Three subtypes of this virus exist: European (transmitted by *I. ricinus*), Siberian and Far Eastern (transmitted by *I. persulcatus*); which present a geographical distribution globally linked to their name (Mansfield *et al.*, 2009, Simmonds *et al.*, 2012, Nuttall, 2014). The distinctive characteristic of TBEV distribution inside each country is its focal pattern, each focus representing a hotspot of virus circulation (Dobler *et al.*, 2011, Suss, 2011). TBE is endemic in south-central Sweden, with the highest number of TBEV foci present in the Stockholm archipelago (Pettersson *et al.*, 2014). Prevalence of ticks for TBEV in our study is close to the one previously estimated in northern Europe (Denmark, Norway, Sweden and Finland). Indeed, Petterson et al, in 2014, evaluated Minimum infection rate (MIR) for TBEV in nymphal and adult *I. ricinus* at 0.28% and 0.23% for southern Sweden, with infection prevalence significantly lower in nymphs (0.10%) than in adult ticks (0.55%). They also estimated at a well-known TBEV-endemic locality, Torö island south-east of Stockholm, the TBEV MIR at 0.51% in nymphs and 4.48% in adults of *I. ricinus* (Pettersson *et al.*, 2014). Sequences of TBEV isolated in our study presented 98% and 96% homology with sequences of virus isolated in *I. ricinus* in Europe and Sweden, Torö 2003 and Saringe 2009 areas, respectively (Asghar *et al.*, 2017). In our study, TBEV was detected in the south-west of Sweden (HR site) in a recently affected TBE area. Indeed, TBEV has been spreading south west from the original focus in the Stockholm area during the last 20 years (Lundkvist *et al.*, 2011).

No TBEV or LIV infected ticks have been detected in our study in France, Denmark, UK, and the Netherlands even if TBE and LIV viruses are known to circulate in those countries (Laursen & Knudsen, 2003, Hansmann *et al.*, 2006, Fomsgaard *et al.*, 2009, Suss, 2011, Gilbert, 2016, Levy *et al.*, 2016, Jahfari *et al.*, 2017). Nevertheless, the prevalence of these viruses in ticks is usually low, and virus detection occurs mainly in local foci where the virus is known to circulate (Perez-Eid *et al.*, 1992, Fomsgaard *et al.*, 2009, Jeffries *et al.*, 2014, Jahfari *et al.*, 2017).

UUKV was detected in three sites in Sweden with a prevalence ranging between 0.07% and 0.22% and in one site in the Netherlands with a prevalence of 0.15%. UUKV was originally isolated at Uukuniemi (southern Finland) in 1979 from *I. ricinus* collected from cattle (Oker-Blom *et al.*, 1964). This virus is maintained in nature between its tick vector, *I. ricinus*, and its vertebrate hosts, forest rodents and birds (Hubalek & Rudolf, 2012). No animal or human

disease due to this virus has yet been reported (Hubalek & Rudolf, 2012). This virus is known to circulate in northern Europe (Norway and Finland), and eastern Europe (Hubalek & Rudolf, 2012). Nevertheless, to our knowledge, this is the first report of UUKV in ticks in Sweden and in the Netherlands, although the virus is known to be present in neighbouring and/or close countries. Birds and migratory birds may have played a role in the dispersion of the virus, and indeed several strains of UUKV have been isolated from immature *I. ricinus* collected on migratory passerines (Traavik, 1979). Sequences obtained during our study for L and S segments showed 94-97 % and 97-99% homology with UUKV L and S segments isolated from ticks in Finland and Czech Republic (GenBank D10759, KM114248, and M33551). Nevertheless it appears difficult to evaluate the genetic diversity of this virus in the field due to the few numbers of sequences available in GenBank (Mazelier *et al.*, 2016).

EYAV was detected from one site in the Netherlands with a prevalence of 0.38%. The presence of this virus in the Netherlands was confirmed by screening 291 adult and 1167 nymphal ticks collected from five locations in the province of Overijssel. Of these, one nymphal pool was positive for EYAV. Sequences obtained showed 95 to 99% homology with Eyach virus sequence (GenBank AF343061, AF282467-AF343053), nevertheless only few sequences are available in GenBank avoiding studies on the genetic diversity of this virus in field samples. EYAV was first isolated from *I. ricinus* ticks in Germany in 1972, followed by isolations from two tick species in France in 1981, *I. ventalloi* and *I. ricinus* (Rehse-Kupper *et al.*, 1976, Chastel *et al.*, 1984). This virus has subsequently remained undetected for the next 30 years, before being detected in two regions from France in 2010 and 2012 in *I. ricinus* ticks with a prevalence comprised between 0.07% and 5.26% (Moutailler *et al.*, 2016). EYAV has been indirectly linked to cases of encephalitis and polyradiculoneuritis in former Czechoslovakia (Malkova *et al.*, 1980), and Moutailler et al. have demonstrated the ability of EYAV to reach the brain of new suckling mice after intraperitoneal inoculation, indicating the ability of the virus to multiply in vertebrate hosts (Moutailler *et al.*, 2016). Nevertheless, until now, no viral RNA has been isolated from animals or humans, even if anti-Eyach virus antibodies have been identified in many animal species in France (European rabbit [*Oryctolagus cuniculus*], rodents, sheep, deer and mountain goats) (Chastel, 1998, Attoui *et al.*, 2002). Thus, specific detection of this virus in patients presenting with encephalitis could lead to an improved evaluation of its prevalence in humans in Europe.

CCHF-like virus was detected in one pool from France (S site) and two pools from the Netherlands (D site) with a prevalence of 0.07% and 0.15% respectively. A nested PCR specific to CCHF virus failed to confirm this result, although a nested PCR specific to *Nairovirus* genus produced one band at the expected size and was sent for sequencing. Unfortunately, no readable sequence was obtained to confirm our findings. The primers/probes set used in the microfluidic system was taken from the literature without modification and is used to detect CCHFV in patients (Wolfel *et al.*, 2007). However, our results suggest an ability of this primers/probes set to also detect other viruses from the *Nairovirus* genus in ticks, and tentatively named potential new-Nairovirus. CCHFV is a hemorrhagic virus with high public health concern in Europe, as this virus has emerged in numerous eastern and southern European countries in the last decades. Recently, fatal autochthonous human cases have been observed in Spain, but this virus is usually transmitted by *Hyalomma* spp. ticks (Maltezou *et al.*, 2010, Al-Abri *et al.*, 2017, Negredo *et al.*, 2017). Nevertheless, new *Nairovirus* sequences have been detected in ticks, e.g. *Ixodes ricinus,* in different studies, demonstrating the risk of misidentification of CCHFV presence in European ticks (Tokarz *et al.*, 2014, Xia *et al.*, 2015, Moutailler *et al.*, 2016, Shimada *et al.*, 2016). To conclude, CCHFV prevalence studies in ticks should always confirm their findings by sequencing to avoid misinterpretation, as Orkun and collaborators did in a large scale survey performed on different tick species collected from different areas in Turkey (Orkun *et al.*, 2017). As a consequence of our findings and to avoid misidentification of CCHF virus, a new set of primers/probe specific for this virus will be implemented in the PCR chip and the old set will be conserved as a Nairovirus genus specific one.

In our study 17 viruses were not detected in the 18,135 *I. ricinus* nymphs collected in 13 European sites. This result is not surprising as a large part of those viruses are not known to be present in Europe, e.g. deer tick virus, powassan virus, etc…, or not known to be transmitted by *I. ricinus* ticks, e.g. Kyasanur forest disease virus, African swine fever virus, dhori virus, etc….Nevertheless, a more extensive survey is needed to confirm our findings regarding the absence of European viruses usually transmitted by *I. ricinus*, e.g. Kemerovo virus, louping ill virus, etc…, in the five European countries studied in this project.

This study demonstrates the feasibility of high-throughput screening methods to enable the detection of numerous TBVs in ticks, often less studied than other tick-borne pathogens (TBPs) such as bacteria and parasites. Other high-throughput techniques (metagenomics methods) exist such as Whole Genome Sequencing (WGS, RNA sequencing) (Moutailler *et al.*, 2016) or resequencing array, but they are often time consuming, expensive and require specialized bioinformatics tools. Moreover, those techniques, often performed on pools, bear some weakness such as the lack of TBV prevalence estimation. Metagenomics microarray technology has also been developed to allow rapid simultaneous identification of all known viruses but also all virus families (within hours) in clinical samples (Erlandsson *et al.*, 2011, Rosenstierne *et al.*, 2014, Fridholm *et al.*, 2016). This technique should be tested on tick samples to investigate its ability to be used for epidemiological surveys. Nevertheless, the main advantage of the microfluidic system- based real-time PCR is that new sets of primers and probes targeting newly emergent viruses can easily be added to the assay, in contrast to arrays with fixed panels of probes. As an example, the recent emergence of Heartland and Bourbon viruses in USA (McMullan *et al.*, 2012, Kosoy *et al.*, 2015) and Severe fever with thrombocytopenia syndrome virus (SFTSV) in Asia (Yu *et al.*, 2011, Yun *et al.*, 2014) has led us to add primer/probe sets specific for these viruses to our panel of primers/probe sets, and are currently being used in novel large scale epidemiological surveys of TBVs in ticks.

In conclusion, our study describes a real-time RT-PCR approach based on a microfluidic system allowing multiple assays in parallel. The method is designed to specifically identify TBVs in European ticks. We demonstrated the capabilities of this new screening method that allows the detection of numerous TBVs in numerous tick and/or host samples, and the identification of viruses in regions where they were previously undetected. This tool represents a powerful, and a more rapid system compared to classical real-time PCRs, for TBVs surveillance in Europe and could be easily customized to assess viral emergence.

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The authors declare they have no conflict of interest.

6. Author contributions

FP, AA, CJ, vdWFJ, dKA, SH, MK, KK, BR, MS obtained the CoVetlab funding and designed the experiments. GM, ML and MS wrote the paper. GM, ML, NA, DE performed the experiments. AA, UK, CJ, vdWFJ, vSSC, JS, SH, MKL, KK, BR collected ticks and extracted RNAs. GM, ML, NA, DE, DS, FP, AA, UK, CJ, HB, vdWJF, dKA, vSSC, JS, SH, MK, FAR, KK, BR, and MS reviewed the manuscript. MS supervised the manuscript.

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Figure1. Sampling areas of *Ixodes ricinus* in Europe. Three sites for France, Denmark, Sweden and the Netherlands and one site in the UK.

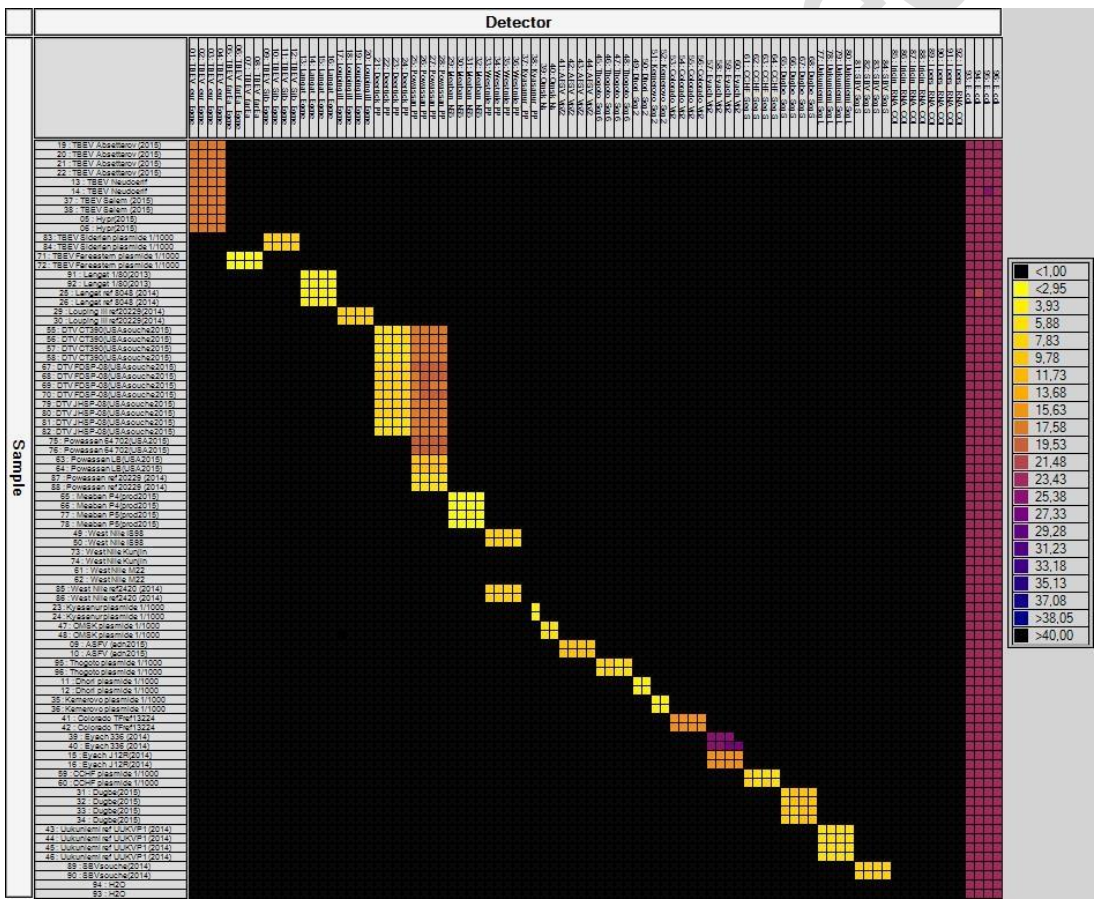

Figure 2. BioMark™ dynamic array system specificity test (96.96 chip). Each square corresponds to a single real-time PCR reaction, where rows indicate the pathogen in the positive control and columns represent the targets of each primer/probe set. C_t values for each reaction are indicted in color; the corresponding color scale is presented in the legend on the right. The darkest shade of blue and black squares are considered as negative reactions with C_t > 30.

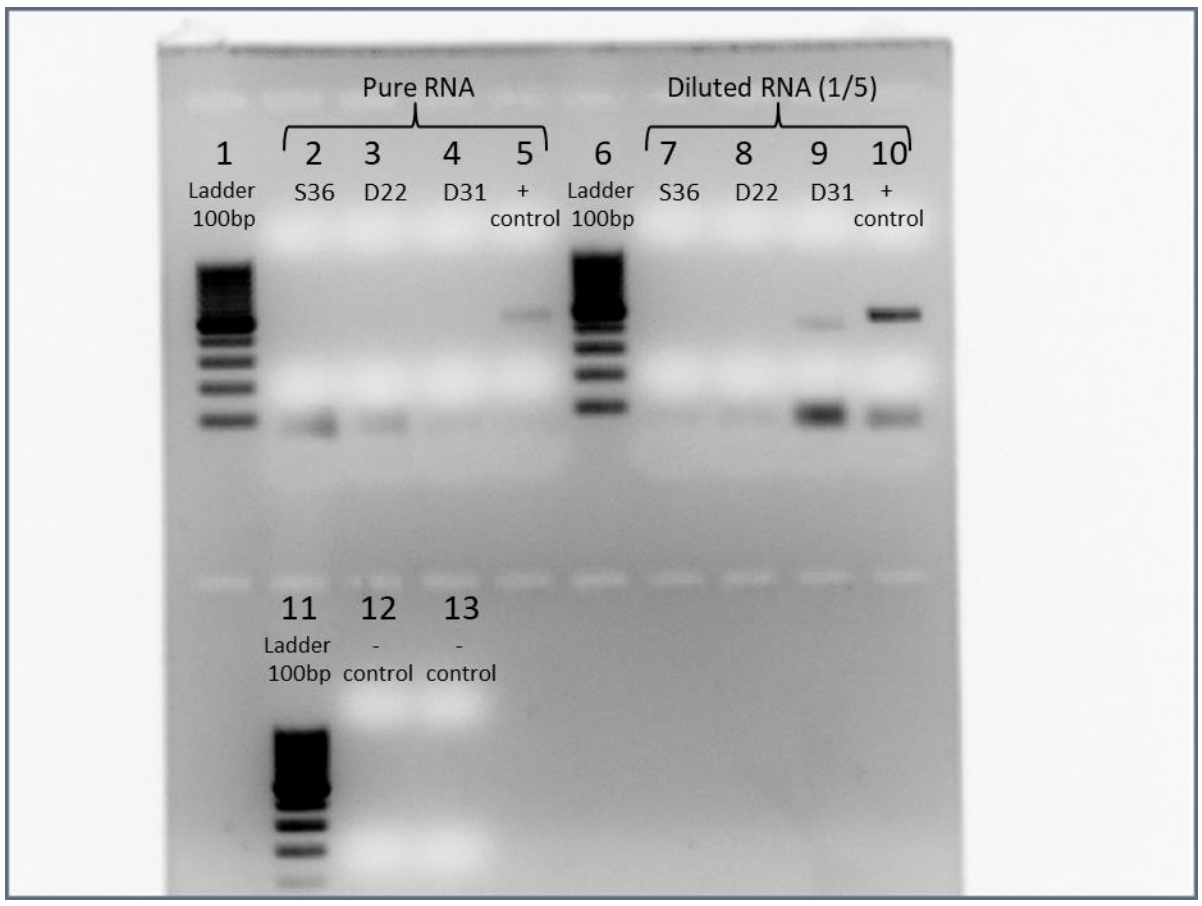

Figure 3. Visualization of Nested PCR products targeted the N gene of viruses from the Nairovirus genus on an agarose gel 2%. Line 1, 6 and 11: Ladder 100 bp. 1/ Nested PCR was run with pure cDNA issued from RT-PCR. Line 5: Positive controls, RNA from dugbe virus. Line 2: positive sample S36 (S site, France). Line 3: positive sample D22 (D site, the Netherlands). Line 4: positive sample D31 (D site, the Netherlands). Line 12: Negative control, water. 2/ Nested PCR was run with diluted cDNA (1/5 dilution) issued from RT-PCR. Line 10: Positive controls, RNA from dugbe virus. Line 7: positive sample S36 (S site, France). Line 8: positive sample D22 (D site, the Netherlands). Line 9: positive sample D31 (D site, the Netherlands). Line 13: Negative control, water.

Table 1. List of tick-borne viruses, tick species, targets, primers/probe sets, and positive controls.

[a] Plasmids are recombinant pBluescript IISK+ containing the target gene. # Primers and Probes (reverse complement) from Wolfel et al., 2007

Family	Genus	Species	Targeted gene	Name	Sequence	Length (bp)	Positive control
Asfarviridae	*Asfivirus*	African swine fever virus	Vp72	ASFV_F	CGATGATGATTACCTTTGCTTTG	84	Culture of ASFV Georgia strain
				ASFV_R	AAAATTCTCTTGCTCTGGATACG		
				ASFV_P	AAGCCACGGGAGGAATACCAACCCAG		
Orthomyxoviridae	*Thogotovirus*	Thogoto virus	M	Thogoto_F	GGTCCTCAAGAACGTCAGCA	113	Plasmid[a]
				Thogoto_R	CATGTAAGTACCAAGACTCATCG		
				Thogoto_P	AAAGTCGCCCTTCTCCGGGAAAGCAT		
		Dhori virus	PB1	Dhori_F	CAAGCTCTGGTGTGCCTGT	81	Plasmid[a]
				Dhori_R	CAGTTACTTCTGAGACAGCCT		
				Dhori_P	AGGAGGGGAAGAGAAGTTGGCCAAG		
Reoviridae	*Orbivirus*	Kemerovo virus	Vp3	Kemerovo_F	GTCAGACGGATTTTCGACCTC	71	Plasmid [a]
				Kemerovo_R	GCGAGCCAGATCCCGATGT		
				Kemerovo_P	ACGGGCCAACACTCGTTCATCACAG		
	Coltivirus	Colorado tick fever virus	Vp2	Colorado_F	TTCTTGCTTCTTCCCGGATCA	80	Culture of Florio VR-1233 strain
				Colorado_R	CGATTCGGTTTCCGGTAACAT		
				Colorado_P	CATGACCATATCCACGGGAAGCTATCA		
		Eyach virus (EYAV)	Vp2	Eyach_F	TGGCTGACAACATGACGGATA	98	Eyach virus grown in suckling mice brains
				Eyach_R	GGCCTCACGATACTTTCGATT		
				Eyach_P	ACGGGCTCGGTACTTCGGTTGAGAT		
Nairoviridae	*Orthonairovirus*	Crimean-Congo Hemorrhagic fever virus (CCHF)	N	CCHF_F#	CAAGGGGTACCAAGAAAATGAAGAAGGC	181	Plasmid [a]
				CCHF_R#	GCCACAGGGATTGTTCCAAAGCAGAC		
				CCHF_P1#	TGTCAACACAGCAGGGTGCATGTAGAT		
				CCHF_P2#	TGTAAGCACGGCAGGGTGCATGTAAAT		
				CCHF_P3#	ACTCCAATGAAGTGGGGGAAGAAGCT		
		Dugbe virus	N	Dugbe_F	GCACAAGGAGCACAAATAGAC	134	Culture of Dugbe virus
				Dugbe_R	TTTTTGCCTCCTCTAGCACTC		
				Dugbe_P	TGGCCCATCTCAAAGAGGAATTGAGAC		
		Nairobi sheep disease virus (NSDV)	G1	NSDV_G1_F	TCTAAGTGCTAGCCCTGATGT	112	Culture of NSDV
				NSDV_G1_R	GCCAACTGAGTGTTCTTCTTC		
				NSDV_G1_P	TTCTACAGGCCGTCCGTCAAGGAAGA		
			G1	NSDV_G1bis_F	ACTAAGTGCAAGCTCAGAAGC	112	Culture of NSDV
				NSDV_G1bis_R	ACCCACAGAATGTTCATCCTC		
				NSDV_G1bis_P	TCCTACTGTGTGTCCTTCAGGGGTTG		
Phenuiviridae	*Phlebovirus*	Uukuniemi virus (UUKV)	RNA-dependent RNA polymerase	Uuku_F	GTGGCAGCTTTTCTCTGGTTT	82	Culture of TC259 strain
				Uuku_R	GGGGAAACTGTCATGCCTAAT		
				Uuku_P	CCTTTTGCCAGTTTGGTCAGTTGCTCC		
Peribunyaviridae	*Orthobunyavirus*	Schmallenberg	N	SBV_F	CGTTGGATTGCTGATACATGC	102	Culture of SBV 1568 V3 strain
				SBV_R	GGCCCAGGTGCATCCCTT		
				SBV_P	AACCTCAGCAAGGGGCATGACAATCTG		
Flaviviridae	*Flavivirus*	Tick-borne encephalitis virus European subtype (TBE)	E	TBE_Euro_F	TCCTTGAGCTTGACAAGACAG	91	Culture of Absettarov, Hypr, Neudoerfl, Salem strains
				TBE_Euro_R	TGTTTCCATGGCAGAGCCAG		
				TBE_Euro_P	TGGAACACCTTCCAACGGCTTGGCA		
		Tick-borne encephalitis virus Far-Eastern subtype (TBE)	E	TBE_FarEast_F	TCAGAACACCTACCGACGG	121	Plasmid[a]
				TBE_FarEast_R	CTCCAAACTCAACCAGCCGT		
				TBE_FarEast_P	CTGGCAGGTCCACCGGGACTGGT		
		Tick-borne encephalitis virus Siberian subtype (TBE)	E	TBE_Sibe_F	TTGTTGTGCAGAGTCGCCAG	82	Plasmid[a]
				TBE_Sibe_R	TCGGAAGGTGTTCCAGAGTC		
				TBE_SIbe_P	TGGCGTTGACTTGGCTCAGACTGTCA		
		Louping ill virus (LIV)	E	Louping_F	GCTGTCAAGATGGATGTGTACAA	113	Culture of 369T2 strain
				Louping_R	CCACTCTTCAGGTGATACTTGT		
				Louping_P	CTTGGAGATCAGACTGGAGTGCTGCT		
		Langat virus	E	Langat_F	ATACCATAAAGGTGGAGCCAC	84	Culture of TP21 strain
				Langat_R	CTGTGAACGAGGCTGACTTC		
				Langat_P	ACACTGGAGAGTTTGTGGCAGCCAATG		
		Deer tick virus	5'NCR/C	Deertick_F	GACAGCTTAGGAGAACAAGAG	94	Culture of CT390, FDRSP-08, JHSP-08 strains
				Deertick_R	CGGTCACTTTCAGCTTTCGC		
				Deertick_P	CTGGGAGTGGTCATGGTGACTACTTC		
		Powassan virus	C	Powassan_F	TGGGGATTCTTTGGCACGC	75	Culture of LB, 64-7062 strains
				Powassan_R	GTGGTACCGTTTTCCAGAACA		
				Powassan_P	TTTTCAGCACTGGGGGTCTGGCCGT		
		West Nile virus	E	WN_F	CAGCGATCTCTCCACCAAAG	69	Culture of IS98, Kunjin, MP22 strains
				WN_R	GGGTCAGCACGTTTGTCATTG		
				WN_P	TGGCTTCTCCCATGGTCGGGCAC		
		Meaban virus	NS5	Meaban_F	TGAGAAGAGCGGTGGAGGA	87	Culture of Meaban virus
				Meaban_R	TTTCCTCCCTCAAGCTCGG		
				Meaban_P	CCAAGTCTTTCACGAGCCATCCGAG		
		Omsk Hemorrhagic fever virus	NS5	Omsk_F	AATGGGAGCATTCAGCTGGC	87	Plasmid[a]
				Omsk_R	GTCCGTCCTTCATCACCAAC		
				Omsk_P	TCATGGAAATGGTGCGAGCAGAAGGG		
		Kyasanur forest disease virus	M	Kyasanur_F	ACACGATGCACACACCTGC	72	Plasmid[a]
				Kyasanur_R	CACCAATGAAACTCTAGTCGTC		
				Kyasanur_P	AGAACCGGGACTTTGTCTCAGGGAC		
		Ixodes ricinus	CO1	Ix_ri_CO1_F	TGGGGCAGGAACTGGATGAA	180	Tick
				Ix_ri_CO1_R	CGTTCTAAAGATAGTCCTGGTG		
				Ix_ri_CO1_P	CAGTATACCCCCCACTTTCAGCAAATATTTCT		
		Ixodes persulcatus	CO1	Ix_per_CO1_F	CAGGGACAGGATGAACTGTTTA	166	Tick
				Ix_per_CO1_R	GATATTCCAGGGGAACGTATG		
				Ix_per_CO1_P	TCCTCCTCTATCATCTAACATCTCCCATTCA		

Table 2. Primers used to confirm the presence of viral RNA in ticks.

Species	Targeted gene	Primer name	Sequence (5' → 3')	Amplicon size (bp)	References
Tick-borne encephalitis virus European subtype	NS5 (RT-PCR)	FSM-1	GAG GCT GAA CAA CTG CAC GA	357	(Puchhammer-Stockl *et al.*, 1995)
		FSM-2	GAA CAC GTC CAT TCC TGA TCT		
	NS5 (nested PCR)	FSM-1i	ACG GAA CGT GAC AAG GCT AG	252	
		FSM-2i	GCT TGT TAC CAT CTT TGG AG		
Uukuniemi virus	RNA-dependent RNA polymerase (RT-PCR)	SegL_UukuV_i1+	ATG GAA GGG TTT GTC AGT CCC CC	559	(Mazelier *et al.*, 2016)
			AAG TTG CTG GAA GCC TTC AGA GTT GC		
	RNA-dependent RNA polymerase (nested PCR)		ATT CCA AAA CCC CAG AAG ATG	244	
		SegL_UukuV_1- SegL_UukuV_2+	TCC TCT TTG TTC TTA AGG TAA CC		
	G1/G2 (RT-PCR)	SegL_UukuV_2-	ACA CAA AGA CCT CCA ACT TAG CTA TCG ACA CAA AGA CCC TCC	1720	(Mazelier *et al.*, 2016)
	G1/G2 (nested PCR)	SegS_UukuV_1+ SegS_uukuV_1- SegS-UukuV_2+ SegS_UukuV_2-	ATG GCT ATG CCG GAG AAT TGG GTG CGC TCAGATCAATGATCTGAGGACAGTTGCAG CC	764	
Eyach virus	VP12 (RT-PCR)	COL-12S	GAT GCC CTG CAA CCG CGC TG	656	(Attoui *et al.*, 2002)
		COL-12R	GAC TGC AAT TAC CCG TCC CGG		
	VP12 (nested PCR)	Eyach-12Si	TAC TGC CTC TGC TTT TTT GAA	527	New design
		Eyach-12Ri	CGT CCC GGA AGA ATG ATG CTA		
	RNA-dependent RNA polymerase VP1 (RT-PCR a)	Eyach-2F	GCTAACGTACCCACAGTATG	460	(Attoui *et al.*, 2002)
		Eyach-2R	GGGTGTTCTCGGTGCACC		
	RNA-dependent RNA polymerase VP1 (RT-PCR b)	Eyach-1F	GAGGCCTGCCTACAAGAAGAC	279	
		Eyach-1R	CTTCAGCCACAATAACGCC		
CCHF virus	N (RT-PCR)	CCHF/F2	TGG ACA CCT TCA CAA ACT	536	(Rodriguez *et al.*, 1997)
		CCHF/R3	GAC AAA TTC CCT GCA CCA		
	N (nested PCR)	CCHF/F3	GAA TGT GCA TGG GTT AGC TC	260	
		CCHF/R2	GAC ATC ACA ATT TCA CCA GG		
Nairovirus genus	N (RT + preamplification + PCR)	PanNairo_S_F	TCT CAA AGA AAC ACG TGC CGC	400	(Lambert & Lanciotti, 2009)
		PanNairo_S_R	GTC CTT CCT CCA CTT GWG RGC AGC CTG CTG GTA		

Table 3. Prevalence estimation for each targeted virus for 3 sites in France, Denmark, Sweden, the Netherlands and one site in UK using the microfluidic tool (BioMark™ system).

Virus	Minimum and Maximum infection rate (95% confidence interval)												
	France			Denmark			Sweden			The Netherlands			UK
	Site M	Site A	Site S	Site BIS	Site KAL	Site AAB	HR	MH	RV	Aus	D	K	RP
ASFV	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Thogoto virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Dhori virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Kemerovo virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Colorado tick fever virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Eyach virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	**0.38% [0.122-0.884]**	< 0.21%[a]
CCHF-like virus	< 0.21%[a]	< 0.21%[a]	**0.07% [0.002-0.403]**	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	**0.15% [0.018-0.529]**	< 0.21%[a]	< 0.21%[a]
Dugbe virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Uukuniemi virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	**0.22% [0.045-0.649]**	**0.07% [0.002-0.403]**	**0.07% [0.002-0.403]**	< 0.21%[a]	< 0.21%[a]	**0.15% [0.018-0.529]**	< 0.21%[a]
Schmallenberg	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
TBEV European subtype	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	**0.30% [0.081-0.767]**	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
TBEV Far-Eastern subtype	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
TBEV Siberian subtype	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Louping ill virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Langat virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Deer tick virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Powassan virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
West Nile virus (WN)	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Meaban virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Omsk Hemorrhagic fever virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]
Kyasanur forest disease virus	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]	< 0.21%[a]

ASFV: African swine fever virus; TBEV: Tick-borne encephalitis virus; CCHFV: Crimean-Congo Hemorrhagic fever virus;

[a] All pools negative. If all 45 pools of 31 nymphs were negative, prevalence was recorded as < 0.21%, because the 95% probability of sampling n negative ticks from a population with prevalence p is given as (1-p)n.

Table 4. Homology between deposited sequences and reference sequences in GenBank.

Viral Species	Nb of samples tested	Nb of samples sent for sequencing	Nb of samples with an interpreted sequence	Deposited sequence	Length (bp)	Percentage of identity (%)	Reference sequence
TBEV European subtype	4	4	4	MH708169 Only one deposited because 100 % identity between the 4 sequences	252	98	Salem (FJ572210); KrM 93 (HM535611); Kumlinge (GU183380); Absettarov (KJ000002); Tobrman (KJ922515); FVG ML Raccolana (FJ159003)
Uukuniemi virus	7	6 for L segment 6 for S segment	5 for L segment 5 for S segment	MH708173-MH708177 for L segment MH708178-MH708182 for S segment	202-245 604-698	94-97 97 and 99	Uukuniemi virus L (D10759) Uukuniemi virus S (KM114248 and M33551 respectively)
Eyach virus	5 (K site) 1 (Overijssel province)	4 2 (RT-PCR a and b)	4 2	MH708170 Only one deposited because 100 % identity between the 5 sequences MH708171 and MH708172	510 460 and 278	95 99 and 97	EYAV-Gr VP12 gene partial cds (AF343061) EYAV-segment 1 complete sequence (AF282467 and AF343053)
CCHF virus	3	1	0	-	-	-	-