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Detection of pathogens in *Dermacentor reticulatus* in northwestern Europe: evaluation of a high-throughput array

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Abstract

Background: The geographic distribution of *Dermacentor reticulatus* is expanding in Europe. Surveillance of this tick species and its pathogens is desirable, as it

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transmits pathogens of public and veterinary importance. A high-throughput real-time PCR-based array was used to screen 1.741 *D. reticulatus* ticks from Belgium, Germany, The Netherlands, and Great Britain for the presence of 28 tick-borne bacteria and twelve protozoan parasites. The presence of pathogen DNA was confirmed by conventional PCR followed by sequencing.

Results: The array detected the presence of DNA from *Borrelia* spp. (7%), *B. afzelii* (0.1%), *B. garinii* (0.1%), *B. spielmanii* (0.1%), *B. miyamotoi* (0.2%), *Anaplasma marginale* (0.1%), *A. phagocytophilum* (0.1%), *Ehrlichia canis* (2%), *Rickettsia helvetica* (0.2%), spotted fever group *Rickettsia* (9.6%), *Francisella tularensis* or *Francisella*-like endosymbionts (95%), *Coxiella burnettii* (0.1%), *Babesia divergens* (0.2%), *B. canis* (0.9%) *B. vogeli* (5.6%), and *Theileria equi* (0.1%). Only the presence of *B. canis* and spotted fever group *Rickettsia* could be confirmed by conventional PCR and sequencing. The spotted fever *Rickettsia*-positive samples were all identified as *R. raoultii*.

Conclusions: We successfully detected and determined the prevalence of *B. canis* and *R. raoultii* in *D. reticulatus*. An high-throughput array that allows fast and comprehensive testing of tick-borne pathogens is advantageous for surveillance and future epidemiological studies. The importance of thorough validation of real-time PCR-based assays and careful interpretation is evident.

Keywords: Molecular biology, Microbiology

1. Introduction

Dermacentor reticulatus (Fabricius, 1794) is considered to be the second most important tick species in Europe, after *Ixodes ricinus*, in terms of its spread and impact on public and veterinary health [1, 2]. *Dermacentor reticulatus* is recorded in many European countries, but is relatively rare in the dry Mediterranean climate zone, and absent in the cold Scandinavian countries (https://ecdc.europa.eu/en/publications-data/dermacentor-reticulatus-current-known-distribution-january-2018). The occurrence of *D. reticulatus* is highly focal within its large distribution area [3], probably because of its ecological requirements [1]. Several studies indicated geographic expansion of *D. reticulatus* within Europe in the last several decades. These studies suggested that the geographical spread of *D.* reticulatus is facilitated by international tourism and trade, and that changes in climate, land use and environmental protection have resulted in more favorable habitats [4, 5, 6, 7, 8].

Dermacentor reticulatus transmits a set of pathogens to humans, which can cause serious disease if not diagnosed and treated appropriately in a timely manner. These pathogens are Omsk haemorrhagic fever virus, tick-borne encephalitis virus, *Rickettsia raoultii,* and *R. slovaca* [1], the latter two causing tick-borne

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lymphadenopathy (TIBOLA, [9]). *Dermacentor reticulatus* is also the vector of *Anaplasma marginale, Babesia canis, B. caballi*, and *Theileria equi*, which cause serious diseases and economic loss in domesticated animals [10, 11]. The list of pathogens detected in *D. reticulatus* using molecular techniques is much longer [1], and includes for example *Borrelia burgdorferi* s.l., *R. helvetica, A. phagocytophilum,* and *Coxiella burnetii*. It should be noted that molecular detection techniques have several advantages, but also weaknesses, including the inability to distinguish living from dead microorganisms and the risk exists for contamination or PCR artefacts from various sources. Whether *D. reticulatus* carries and transmits all these pathogens as infectious agents needs to be established in experimental or epidemiological studies.

Surveillance of tick-borne diseases ideally includes the monitoring of the geographic distribution of ticks, as well as the monitoring of tick-borne pathogens in ticks and vertebrate hosts ([12, 13]. For adequate monitoring of pathogens with relatively low infection rates, many ticks need to be tested. This becomes even more challenging when monitoring many pathogens. Recently, a high-throughput array was successfully developed and implemented for the molecular detection of 25 tick-borne bacteria and twelve parasites for *Ixodes ricinus* [14]. This array utilizes a microfluidic system (BioMark^{TM} dynamic array system, Fluidigm) that is capable of performing parallel real-time PCRs using either 96.96 chips or 48.48 chips resulting in either 9216 or 2304 individual reactions, respectively [15].

The aim of this study was to conduct and evaluate a monitoring of tick-borne human and animal pathogens in *D. reticulatus*, using a high-throughput array. Accordingly, the high-throughput array used for *I. ricinus* was modified, and used for the screening of 1.741 *D. reticulatus* ticks from Belgium, Germany, The Netherlands, and Great Britain. The presence of pathogen DNA was confirmed by conventional PCR followed by sequencing.

2. Materials and methods

2.1. Primers and probes design

Most primers and probes as well as the positive controls were already used and described in a previous study [14]. Pathogens, targeted gene fragments and primers/probe sets used in the microfluidic array approach are listed in Table 1. For each pathogen and tick, primers and probes were designed, two of them specifically for this study. Each design was validated with different type of reference DNA materials (Table 1) by real-time TaqMan PCR on a LightCycler® 480 (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.

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Table 1. List of primers, probes and positive controls used for the fluidigm array. Most primers and probes as well as the positive controls were already used and described in a previous study [14]. Length of the PCR product in base pairs (bp).

Pathogens	Target	Primers	Sequence	Length	Positive control
Borrelia burgdorferi	rpoB	Bo_bu_rpoB_F Bo_bu_rpoB_R Bo_bu_rpoB_P	GCTTACTCACAAAAGGCGTCTT GCACATCTCTTACTTCAAATCCT AATGCTCTTGGACCAGGAGGACTTTCA	83 bp	Culture of B31
Borrelia garinii	rpoB	Bo_ga_rpoB_F Bo_ga_rpoB_R Bo_ga_rpoB_P	TGGCCGAACTTACCCACAAAA ACATCTCTTACTTCAAATCCTGC TCTATCTCTTGAAAGTCCCCCTGGTCC	88 bp	Culture of NE11
Borrelia afzelii	flaB	Bo_af_fla_F Bo_af_fla_R Bo_af_fla_P	GGAGCAAATCAAGATGAAGCAAT TGAGCACCCTCTTGAACAGG TGCAGCCTGAGCAGCTTGAGCTCC	116 bp	Culture of VS641
Borrelia valaisiana	ospA	Bo_va_ospA_F Bo_va_ospA_R Bo_va_ospA_P	ACTCACAAATGACAGATGCTGAA GCTTGCTTAAAGTAACAGTACCT TCCGCCTACAAGATTTCCTGGAAGCTT	135 bp	Culture of VS116
Borrelia miyamotoi	glpQ	B_miy_glpQ_F B_miy_glpQ_R B_miy_glpQ_P	CACGACCCAGAAATTGACACA GTGTGAAGTCAGTGGCGTAAT TCGTCCGTTTTCTCTAGCTCGATTGGG	94 bp	Plasmid[a]
Borrelia spielmanii	fla	Bo_sp_fla_F Bo_sp_fla_R Bo_sp_fla_P	ATCTATTTTCTGGTGAGGGAGC TCCTTCTTGTTGAGCACCTTC TTGAACAGGCGCAGTCTGAGCAGCTT	71 bp	Plasmid
Borrelia lusitaniae	rpoB	Bo_lu_rpoB_F Bo_lu_rpoB_R Bo_lu_rpoB_P	CGAACTTACTCATAAAAGGCGTC TGGACGTCTCTTACTTCAAATCC TTAATGCTCTCGGGCCTGGGGGACT	87 bp	Culture of Poti-B1
Borrelia bissettii	rpoB	Bo_bi_rpoB_F Bo_bi_rpoB_R Bo_bi_rpoB_P	GCAACCAGTCAGCTTTCACAG CAAATCCTGCCCTATCCCTTG AAAGTCCTCCCGGCCCAAGAGCATTAA	118 bp	Plasmid[a]
Borrelia spp.	23SrRNA	Bo_sl_23S_F Bo_sl_23S_R Bo_sl_23S_P	GAGTCTTAAAAGGGCGATTTAGT CTTCAGCCTGGCCATAAATAG AGATGTGGTAGACCCGAAGCCGAGT	73 bp	Culture of B31
Anaplasma marginale	msp1b	An_ma_msp1_F An_ma_msp1_R An_ma_msp1_P	CAGGCTTCAAGCGTACAGTG GATATCTGTGCCTGGCCTTC ATGAAAGCCTGGAGATGTTAGACCGAG	85 bp	Experimentally infected cow
Anaplasma platys	groEL	An_pl_groEL_F An_pl_groEL_R An_pl_groEL_P	TTCTGCCGATCCTTGAAAACG CTTCTCCTTCTACATCCTCAG TTGCTAGATCCGGCAGGCCTCTGC	75 bp	Dog blood

(*continued on next page*)

Table 1. (*Continued*)

Pathogens	Target	Primers	Sequence	Length	Positive control
Anaplasma ovis	msp4	An_ov_msp4_F An_ov_msp4_R An_ov_msp4_P	TCATTCGACATGCGTGAGTCA TTTGCTGGCGCACTCACATC AGCAGAGAGACCTCGTATGTTAGAGGC	92 bp	Plasmid[a]
Anaplasma bovis	groEL	An_bov_groEL_F An_bov_groEL_R An_bov_groEL_P	GGGAGATAGTACACATCCTTG CTGATAGCTACAGTTAAGCCC AGGTGCTGTTGGATGTACTGCTGGACC	73 bp	Plasmid[a]
Anaplasma centrale	groEL	An_ce_groEL_F An_ce_groEL_R An_ce_groEL_P	AGCTGCCCTGCTATACACG GATGTTGATGCCCAATTGCTC CTTGCATCTCTAGACGAGGTAAAGGGG	79 bp	Plasmid[a]
Anaplasma phagocytophilum	msp2	An_ph_msp2_F An_ph_msp2_R An_ph_msp2_P	GCTATGGAAGGCAGTGTTGG GTCTTGAAGCGCTCGTAACC AATCTCAAGCTCAACCCTGGCACCAC	77 bp	Culture
Ehrlichia ruminantium	dsb	Eh_ru_dsb_F Eh_ru_dsb_R Eh_ru_dsb_P	CTCAGAGGGTAATAGATTTACTC GTATGCAATATCTTCAAGCTCAG ACTACAGGCCAAGCACAAGCAGAAAGA	107 bp	Culture of Gardel
Ehrlichia canis	dsb	Eh_ca_dsb_F Eh_ca_dsb_R Eh_ca_dsb_P	AATACTTGGTGAGTCTTCACTCA GTTGCTTGTAATGTAGTGCTGC AAGTTGCCCAAGCAGCACTAGCTGTAC	110 bp	Plasmid[a]
Ehrlichia chaffeensis	dsb	Eh_ch_dsb_F Eh_ch_dsb_R Eh_ch_dsb_P	TATTGCTAATTACCCTCAAAAAGTC GAGCTATCCTCAAGTTCAGATTT ATTGACCTCCTAACTAGAGGGCAAGCA	117 bp	*Amblyomma americanum*
Neoehrlichia mikurensis	groEL	Nm_groEL_F Nm_groEL_R Nm_groEL_P	AGAGACATCATTCGCATTTTGGA TTCCGGTGTACCATAAGGCTT AGATGCTGTTGGATGTACTGCTGGACC	96 bp	*Ixodes ricinus*
Rickettsia conorii	23S-5S ITS	Ri_co_ITS_F Ri_co_ITS_R Ri_co_ITS_P	CTCACAAAGTTATCAGGTTAAATAG CGATACTCAGCAAAATAATTCTCG CTGGATATCGTGGCAGGGCTACAGTAT	118 bp	Culture
Rickettsia slovaca	23S-5S ITS	Ri_sl_ITS_F Ri_sl_ITS_R Ri_sl_ITS_P	GTATCTACTCACAAAGTTATCAGG CTTAACTTTTACTACAATACTCAGC TAATTTTCGCTGGATATCGTGGCAGGG	138 bp	Culture
Rickettsia massiliae	23S-5S ITS	Ri_ma_ITS_F Ri_ma_ITS_R Ri_ma_ITS_P	GTTATTGCATCACTAATGTTATACTG GTTAATGTTGTTGCACGACTCAA TAGCCCCGCCACGATATCTAGCAAAAA	128 bp	Culture

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Table 1. (*Continued*)

Pathogens	Target	Primers	Sequence	Length	Positive control
Rickettsia helvetica	23S-5S ITS	Ri_he_ITS_F	AGAACCGTAGCGTACACTTAG	79 bp	Culture
		Ri_he_ITS_R	GAAAACCCTACTTCTAGGGGT		
		Ri_he_ITS_P	TACGTGAGGATTTGAGTACCGGATCGA		
Rickettsia aeschlimannii	ITS	Rick_aesch_ITS_F	CTCACAAAGTTATCAGGTTAAATAG	134 bp	Culture
		Rick_aesch_ITS_R	CTTAACTTTTACTACGATACTTAGCA		
		Rick_aesch_ITS_P	TAATTTTTGCTGGATATCGTGGCGGGG		
Spotted fever group	gltA	SFG_gltA_F	CCTTTTGTAGCTCTTCTCATCC	145 bp	
		SFG_gltA_R	GCGATGGTAGGTATCTTAGCAA		
		SFG_gltA_P	TGGCTATTATGCTTGCGGCTGTCGGT		
Bartonella henselae	pap31	Bar_he_pap_F	CCGCTGATCGCATTATGCCT	107 bp	Culture of Berlin 1
		Bar_he_pap_R	AGCGATTTCTGCATCATCTGCT		
		Bar_he_pap_P	ATGTTGCTGGTGGTGTTTCCTATGCAC		
Bartonella quintana	bqtR	Bar_qu_bqt_F	TCCATCACAAGATCTCCGCG	80 bp	Culture
		Bar_qu_bqt_R	CGTGCCAATGCTCGTAACCA		
		Bar_qu_bqt_P	TTTAAGAGAGGAGGTAGAAGAGGCTCC		
Francisella tularensis and *Francisella*-like endosymbionts	tul4	Fr_tu_tul4_F	ACCCACAAGGAAGTGTAAGATTA	76 bp	Culture of CIP 5612T
		Fr_tu_tul4_R	GTAATTGGGAAGCTTGTATCATG		
		Fr_tu_tul4_P	AATGGCAGGCTCCAGAAGGTTCTAAGT		
	fopA	Fr_tu_fopA_F	GGCAAATCTAGCAGGTCAAGC	91 bp	
		Fr_tu_fopA_R	CAACACTTGCTTGAACATTTCTAG		
		Fr_tu_fopA_P	AACAGGTGCTTGGGATGTGGGTGGTG		
Coxiella burnetii and *Coxiella*-like	icd	Co_bu_icd_F	AGGCCCGTCCGTTATTTTACG	74 bp	Culture
		Co_bu_icd_R	CGGAAAATCACCATATTCACCTT		
		Co_bu_icd_P	TTCAGGCGTTTTGACCGGGCTTGGC		
	IS1111	Co_bu_IS_F	TGGAGGAGCGAACCATTGGT	86 bp	
		Co_bu_IS_R	CATACGGTTTGACGTGCTGC		
		Co_bu_IS_P	ATCGGACGTTTATGGGGATGGGTATCC		
Babesia divergens	hsp70	Bab_di_hsp70_F	CTCATTGGTGACGCCGCTA	83 bp	Culture of RFS
		Bab_di_hsp70_R	CTCCTCCCGATAAGCCTCTT		
		Bab_di_hsp70_P	AGAACCAGGAGGCCCGTAACCCAGA		
Babesia caballi	RAP1	Ba_ca_rap1_F	GTTGTTCGGCTGGGGCATC	94 bp	Plasmid[a]
		Ba_ca_rap1_R	CAGGCGACTGACGCTGTGT		
		Ba_ca_rap1_P	TCTGTCCCGATGTCAAGGGGCAGGT		

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Table 1. (*Continued*)

Pathogens	Target	Primers	Sequence	Length	Positive control
Babesia canis (3 subspecies)	RNA 18S	Ba_ca_18S_F Ba_ca_18S_R Ba_ca_18S_P	TGGCCGTTCTTAGTTGGTGG AGAAGCAACCGGAAACTCAAATA ACCGGCACTAGTTAGCAGGTTAAGGTC	104 bp	Dog blood
Babesia canis vogeli	hsp70	Ba_vo_hsp70_F Ba_vo_hsp70_R Ba_vo_hsp70_P	TCACTGTGCCTGCGTACTTC TGATACGCATGACGTTGAGAC AACGACTCCCAGCGCCAGGCCAC	87 bp	Dog blood
Babesia venatorum (EU1)	RNA 18S	Ba_EU_18S_F Ba_EU_18S_R Ba_EU_18S_P	GCGCGCTACACTGATGCATT CAAAAATCAATCCCCGTCACG CATCGAGTTTAATCCTGTCCCGAAAGG	91 bp	Plasmid[a]
Babesia microti	CCTeta	Ba_mi_CCT_F Ba_mi_CCT_R Ba_mi_CCT_P	ACAATGGATTTTCCCCAGCAAAA GCGACATTTCGGCAACTTATATA TACTCTGGTGCAATGAGCGTATGGGTA	145 bp	Culture of R1
Babesia bovis	CCTeta	Ba_bo_CCT_F Ba_bo_CCT_R Ba_bo_CCT_P	GCCAAGTAGTGGTAGACTGTA GCTCCGTCATTGGTTATGGTA TAAAGACAACACTGGGTCCGCGTGG	100 bp	Culture of MO7
Babesia bigemina	RNA 18S	Ba_bi_18S_F Ba_bi_18S_R Ba_bi_18S_P	ATTCCGTTAACGAACGAGACC TTCCCCCACGCTTGAAGCA CAGGAGTCCCTCTAAGAAGCAAACGAG	99 bp	Plasmid[a]
Babesia major	CCTeta	Ba_ma_CCT_F Ba_ma_CCT_R Ba_ma_CCT_P	CACTGGTGCGCTGATCCAA TCCTCGAAGCATCCACATGTT AACACTGTCAACGGCATAAGCACCGAT	75 bp	Plasmid[a]
Babesia ovis	RNA 18S	Ba_ov_18S_F Ba_ov_18S_R Ba_ov_18S_P	TCTGTGATGCCCTTAGATGTC GCTGGTTACCCGCGCCTT TCGGAGCGGGGTCAACTCGATGCAT	92 bp	Plasmid[a]
Theileria equi	ema1	Th_eq_ema1_F Th_eq_ema1_R Th_eq_ema1_P	GGCTCCGGCAAGAAGCACA CTTGCCATCGACGACCTTGA CTTCAAGGCTCCAGGCAAGCGCGT	66 bp	Plasmid[a]
Theileria annulata	RNA 18S	Th_an_18S_F Th_an_18S_R Th_an_18S_P	GCGGTAATTCCAGCTCCAATA AAACTCCGTCCGAAAAAAGCC ACATGCACAGACCCCAGAGGGACAC	126 bp	Culture of D7
Ixodes ricinus	ITS2	Ix_ri_ITS2_F Ix_ri_ITS2_R Ix_ri_ITS2_P	CGAAACTCGATGGAGACCTG ATCTCCAACGCACCGACGT TTGTGGAAATCCCGTCGCACGTTGAAC	77 bp	Tick

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Table 1. (*Continued*)

Pathogens	Target	Primers	Sequence	Length	Positive control
Ixodes persulcatus	ITS2	Ix_pe_ITS2_F	TGCGTTGCGTCTTCTCTTGTT	111 bp	Tick
		Ix_pe_ITS2_R	TCGATAAAACCAGGTAGGAGGA		
		Ix_pe_ITS2_P	TTTCGGAGCAAGTACAGAGGGAGCAAA		
Dermacentor reticulatus	ITS2	De_re_ITS2_F	AACCCTTTTCCGCTCCGTG	83 bp	Tick
		De_re_ITS2_R	TTTTGCTAGAGCTCGACGTAC		
		De_re_ITS2_P	TACGAAGGCAAACAACGCAAACTGCGA		
Dermacentor marginatus	ITS2	De_ma_ITS2_F	GCACGTTGCGTTGTTTGCC	139 bp	Tick
		De_ma_ITS2_R	CCGCTCCGCGCAAGAATCT		
		De_ma_ITS2_P	TTCGGAGTACGTCGAGCTCTAGCAGA		
Escherichia coli	eae	eae-F	CATTGATCAGGATTTTTCTGGTGATA	102 bp	Culture of EDL933
		eae-R	CTCATGCGGAAATAGCCGTTA		
		eae-P	ATAGTCTCGCCAGTATTCGCCACCAATACC		

[a] Plasmids are recombinant pBluescript IISK+ containing the target gene.

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Thermal cycling conditions were as follows: 95 °C for 5 min, 45 cycles at 95 °C for 10s and 60 °C for 15s and one final cycle of cooling at 40 °C for 10s. Some pathogens were targeted by real time PCRs on two different sequences to improve detection (Table 1).

2.2. Study area and tick collection

The distribution of *D. reticulatus* in Great Britain was recently published by Medlock *et al.* (2017), with three main foci in Wales, Devon and Essex [16]. Samples from three separate locations in Wales (Morfa Harlech, Morfa Gwyllt and Borth) and one location in Essex (Old Hall marshes) were selected. Questing ticks were collected during spring using blanket dragging, with samples from Wales collected during 2010−2012 and from Essex in 2016. *Dermacentor reticulatus* ticks were collected by blanket dragging in Belgium at four locations: Beveren, De Panne, Moen and Straimont. Beveren and Moen were visited in 2010 [17]. Moen and Beveren were visited in 2011 on a few occasions. Ticks were collected in De Panne in 2012. Straimont was sampled on a few occasions in 2013. Ticks were collected using blanket dragging in Germany on 60 sampling sites in the federal state of Bavaria, Germany between 2010 and 2013 and *D. reticulatus* ticks were found at three of them. Sites were sampled at least once in spring or autumn. Ticks from the Netherlands were collected using blanket dragging from several locations in coastal areas, mostly situated in the southwestern part of the country. Typical habitats were moist open grassland grazed by cattle, in nature reserves along loughs. Surveillance took place in 2014, 2015 and 2016 and was most successful in the months of March and October.

2.3. DNA extraction and pre-amplification with a mixture of pathogen-specific primers

Ticks were identified to species level using a stereomicroscope and morphological keys [18]. *Dermacentor reticulatus* ticks were cut into pieces using disposable surgical knives and lysed overnight in lysis buffer (ATL buffer, Qiagen, Germany). The DNA extraction was performed using the Blood and Tissue kit (Qiagen, Germany). Ticks from Germany were washed twice in distilled water, air-dried and DNA was extracted individually using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for tissue. Ticks were disrupted in a TissueLyser (Qiagen) with 80µl phosphate buffered saline, pH7.4, and a 5mm stainless steel bead in 2ml Eppendorf tubes for 5 min at 20bpm. Incubation was carried out over night at 56 °C. For every 24 to 48 samples, a negative extraction control containing sterile water was included. Quality and quantity of the extracted DNA were checked with a photospectrometer (NanoDrop®ND-1000; PeqLab, Erlangen, Germany).The TaqMan PreAmp

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Master Mix (Applied Biosystems, France) was used for the pre-amplification of DNA lysates according to the manufacturer's instructions (TaqMan PreAmp Master Mix Kit Protocol). All forward and reverse primers, except those targeting tick species (Table 1), were pooled and mixed at a final concentration of 200 nM each. The reaction was performed in a final volume of 5 µl containing 2.5 µl TaqMan PreAmp Master Mix, 1.2 µl of pooled primers mix and 1.3 µl of DNA lysate, with one cycle at 95 °C for 10 min, 14 cycles at 95 °C for 15 sec and 4 min at 60 °C. At the end of the cycling program the reactions were diluted 1:10. Pre-amplified DNAs were stored at -20 °C until further processing.

2.4. High-throughput real-time PCR system

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) as described [14]. In short, amplifications were performed using 6-carboxyfluorescein (FAM)- and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene expression master mix (Applied Biosystems, France). The thermal profile comprised 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of a 2-step amplification profile consisting of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. Data were acquired on the BioMark™ Real-Time PCR System and analyzed using the Fluidigm Real-time PCR Analysis software to obtain cross point (Cp) values. Negative controls with water were included per chip. The detection of *D. reticulatus* DNA served as a confirmation of the tick species tested and as a positive control of the DNA extraction. A positive processing control, which is a DNA extract from the EDL933 strain of *Escherichia coli*, was added to each sample.

2.5. Confirmation by PCR and sequencing

Analysis of the qPCR was performed using the second derivative calculations for Cp (crossing point) values. Curves were assessed visually. A qPCR was considered positive when the Cp values were <40 and the amplification curves were sigmoid shaped. Alternatively, confirmation of the presence of pathogen DNA in samples was performed by conventional PCRs (Table 2), using specific primers, targeting different genes or regions than the ones used in the BioMark™ system. Amplicons were sequenced by dideoxy-dye terminal sequencing of both strands by Baseclear (Leiden, Netherlands). The sequences were stored and processed in Bionumerics (Version 7.1, Applied Math, Belgium) after subtraction of the primer sequences, and compared with known sequences from GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov).

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Table 2. PCR-based methods used to confirm the presence of pathogenic DNA in ticks. A qPCR was considered positive when the Cp values were <40 and the amplification curves were sigmoid shaped. Some confirmation tests only detect several (geno)species of a pathogens. A PCR was considered positive when it could be sequenced using Sanger sequencing and when the obtained sequence was at least 99% similar to known sequences from GenBank.

Pathogen	Target	PCR	Reference
Borrelia spp.	OspA	qPCR	[26]
	Flagelin B	qPCR	[26]
	23S-5S IGS	PCR	[27]
	GlpQ	PCR	[28]
	Flagelin B	PCR	[28]
	Bossp_16S-rRNA	PCR	[29]
	Bossp_IGS	Nested- PCR	[30]
	Bossp_p66	Nested- PCR	[31]
	MLST (8 targets)	Nested- PCR	[32]
E. canis	Msp2	qPCR	[33]
	GroEL	qPCR	[34]
	16S-rRNA	PCR	[35]
	GroEL	Nested-PCR	[36]
	16S -rRNA	PCR	[37]
Anaplasma spp.	Msp2	qPCR	[33]
	GroEL	qPCR	[34]
	16S-rRNA	PCR	[35]
	GroEL	Nested-PCR	[36]
Babesia & Theileria spp.	18S-rRNA	qPCR	[38]
	18S-rRNA	PCR	[39]
	BabG	PCR	[40]
F. tularensis	FopA	qPCR	[41]
	ISFtu	qPCR	[41]
Coxiella spp.	IS1111	qPCR	[42]
	Com	qPCR	[42]
SFG-*Rickettsia*	GltA	qPCR	[19]
	GltA	PCR	[43]
	16S-rRNA	PCR	[44]
	OmpA	PCR	[45]
	OmpB	PCR	[46]

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3. Results

3.1. Fluidigm-array

Most primer and probe combinations, 46 out of 48 (Table 1), were successfully tested and validated on *I. ricinus* [14]. Primers and probes targeting specifically *Anaplasma bovis* and *Rickettsia aeschlimannii* were designed for this study (Table 1). These primers and probes identified their corresponding positive control samples via Taqman® real-time PCRs on a LightCycler 480 apparatus, but did not react with any of the other positive control samples described in Table 1. Several of the targeted pathogens cannot be cultured, or are rare and consequently unavailable from field samples, therefore plasmids containing target sequences were used as positive controls. A total of 1.753 tick lysates were tested using the BioMark^{TM} system. Seven samples from the Netherlands were positive on the *I. ricinus* target and negative for *D. reticulatus.* The results from these samples were discarded from further analyses. One sample was positive for both the *I. ricinus* and the *D. reticulatus* target, probably due to a cross-contamination somewhere in the processing of the samples (Table 3). Five samples did not react with any of the tick targets, and were negative for all pathogens, whereas the *E. coli* target was positive. The results from these samples were also discarded from further analyses.

The remaining 1741 lysates were positive with the *D. reticulatus* target and analysed for the presence and absence of pathogen DNA. Among the targeted pathogens, 18 bacteria (*B. burgdorferi* s.s*, B. valaisiana, B. lusitaniae, B. bissetti, A. platys, A. ovis, A. centrale, A. bovis, E. chaffeensis, E. ruminantium, Neoehrlichia mikurensis, Rickettsia conorii, R. slovaca, R. massiliae, R. helvetica, R. aeschlimannii, Bartonella henselae,* and *B. quintana*) and eight protozoan parasites (*Babesia microti, B. bovis, B. caballi, B. venatorum, B. bigemina, B. major, B. ovis*, and *T. annulata*) were not detected in any of the 1741 *D. reticulatus* lysates. Of the 1741 *D. reticulatus*-positive lysates, samples were positive for *Borrelia spp.* (n = 120)*,* three targets of *B. burgdorferi* s.l. (n = 1), *B. miyamotoi* (n = 3), *A. marginale* (n = 1), *A. phagocytophilum* (n = 5), *E. canis* (n = 26), *R. helvetica* (n = 4), *SFG Rickettsia* (n = 167), *F. tularensis* or *F. tularensis*-like endosymbionts (n = 1655), *Coxiella burnetii* or *Coxiella-like bacteria* (n = 1), *Babesia canis* (n = 16), *B. divergens* (n = 3), *B. vogeli* (n = 87), and *Theileria equi* (n = 1) using the BioMarkTM (Table 3). In order to confirm the results obtained on the BioMark^{TM} system and to validate this new method on *D. reticulatus*, qPCR, classical PCR and sequencing were performed on extracted DNA for a subset of field samples.

3.2. Confirmation

The presence of *B. canis* was confirmed by a qPCR targeting the 18S-rRNA fragment in all 16 samples, and could be confirmed by conventional PCR followed by

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Table 3. Number of positive tick lysates from the four countries using the microfluidic tool (BioMark™ system). Pathogens detected with the microfluidic array are in **bold**. *One sample was positive for targets of the three different *B. burgdorferi* s.l. genospecies.

Pathogen	Belgium	Great Britain	Germany	The Netherlands
Samples tested	513	113	255	860
***Borrelia* spp.**	**32**	**8**	**16**	**64**
B. burgdorferi s.s	0	0	0	0
B. garinii	0	0	0	**1***
B. afzelii	0	0	0	**1***
B. valaisiana	0	0	0	0
B. lusitaniae	0	0	0	0
B. spielmanii	0	0	0	1*
B. bissetti	0	0	0	0
B. miyamotoi	0	0	0	**3**
Anaplasma marginale	0	0	0	**1**
A. platys	0	0	0	0
A. phagocytophilum	1	0	**1**	**3**
A. ovis	0	0	0	0
A. centrale	0	0	0	0
A. bovis	0	0	0	0
E. chaffeensis	0	0	0	0
E. ruminantium	0	0	0	0
E. canis	**5**	**3**	**10**	**8**
Neoehrlichia mikurensis	0	0	0	0
Rickettsia conorii	0	0	0	0
R. slovaca	0	0	0	0
R. massiliae	0	0	0	0
R. helvetica	**3**	0	0	**1**
R. aeschlimannii	0	0	0	0
SFG Rickettsia	**44**	**34**	**87**	**2**
Bartonella henselae	0	0	0	0
B. quintana	0	0	0	0
Francisella tularensis (tul4)	0	0	0	0
Francisella tularensis (fopA)	**458**	**112**	**251**	**834**
Coxiella burnetii (icd)	1	0	0	0
Coxiella burnetii (IS1111)	0	0	0	0
Babesia divergens	0	3	0	0
B. microti	0	0	0	0

(*continued on next page*)

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Table 3. (*Continued*)

Pathogen	Belgium	Great Britain	Germany	The Netherlands
Babesia canis	0	**16**	0	0
B. vogeli	0	0	**54**	**33**
B. bovis	9	0	0	0
B. caballi	0	0	0	0
B. venatorum	0	0	0	0
B. bigemina	0	0	0	0
B. major	0	0	0	0
B. ovis	0	0	0	0
Theileria equi	0	0	**1**	0
T. annulata	0	0	0	0
Ixodes ricinus	0	0	0	**1**
I. persulcatus	0	0	0	0
Dermacentor reticulatus	513	113	255	860
D. marginatus	0	0	0	0
Positive processing control	513	113	255	860

sequencing in 10 out of 16 samples. The ten obtained 18S-rRNA sequences were all identical and 100% similar to an 18S-rRNA sequence from the *B. canis* isolates Bc1, A1/A2 and several others retrieved from Genbank (accession numbers AY072926 and KX839230). From the 167 samples that reacted with the SFG *Rickettsia* on the array, 128 were confirmed by a qPCR targeting SFG *Rickettsia* [19] and 103 could be confirmed by a conventional PCR followed by sequencing. All these GltA sequence fragments were >99% identical and >99% similar to the IM16 isolate of *R. raoultii* (accession number KY474576).

The presence of *B. burgdorferi* s.l., which reacted with three targets in the high-throughput array, was confirmed by the OspA qPCR (Table 2), but could not be confirmed with any of the other confirmation (q)PCR tests for *B. burgdorferi* s.l. or *Borrelia* spp.. None of the 120 *Borrelia spp.*-positive and three *B. miyamotoi* samples could be amplified or confirmed with any of the 16 control *Borrelia* spp. and *B. burgdorferi* s.l. (q)PCRs (Table 4). The presence of *A. phagocytophilum, A. marginale*, *E. canis*, *R. helvetica, F. tularensis, C. burnetii*, *B. divergens*, *B. vogeli*, and *T. equi* could not be confirmed either.

4. Discussion

In this study, we evaluated a PCR-based method using multiple primers and probe sets to perform high-throughput monitoring of pathogens in an emerging tick species from Europe. An initial step of pre-amplification was necessary to increase the

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Table 4. Confirmed presence of tick-borne pathogen DNA in *D. reticulatus*. Samples which were positive in the microfluidic array were retested by other qPCR or PCR tests (Table 2) to confirm the presence of DNA of a tick-borne pathogen.

Pathogen	Fluidigm positive	Confirmed (from Table 2)	Countries
Borrelia spp.	120	No	
B. burgdorferi s.l.	1	1 (qPCR)	Germany
B. miyamotoi	3	No	
Anaplasma marginale	1	No	
A. phagocytophilum	5	No	
E. canis	26	No	
Rickettsia helvetica	4	No	
SFG *Rickettsia (R. raoultii)*	167	128 (qPCR), 103 (PCR/Seq)	All countries
F. tularensis and FLEs	1655	No	
Coxiella burnetii and *Coxiella*-like	1	No	
Babesia canis	16	16 (qPCR), 10 (PCR/Seq)	Great Britain
B. divergens	3	No	
B. vogeli	87	No	
Theileria equi	1	No	

sensitivity of the array, otherwise not all positive DNA controls could be detected. The array enabled important quality control steps concurrent with pathogen detection, namely the confirmation of the presence of tick DNA, the (anticipated) tick species and a positive processing control. These controls are often neglected/omitted in other tick screening studies [20, 21]. As a consequence, twelve samples were excluded from further analyses in this study. In one sample, the presence of *I. ricinus* and *D. reticulatus* DNA was detected. We assume that a contamination had taken place during the DNA extraction or PCR preparation.

Two commonly reported pathogens in *D. reticulatus, B. canis* and *R. raoultii* were detected by the array, which could also be confirmed by established qPCR and conventional PCR followed by sequencing. Not all *B. canis*- and *R. raoultii*-positive samples could be confirmed (Table 4), probably because of the relatively low DNA-load in the samples, as was evidenced by high Cp-values in these samples (not shown). The detection of *F. tularensis* using the *fopA-target* was compromised by the presence of *Francisella*-like endosymbionts in 95% of the *D. reticulatus* samples (Table 3, [22]). The other *F. tularensis* marker, *tul4,* remained negative. Therefore, we conclude that *F. tularensis* is absent or not-detectable in the investigated samples. Furthermore, the primers and probe sets for the sensitive and specific detection of *F. tularensis* need further optimization, so the current results obtained for these species should be interpreted with care.

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Three subspecies of *B. canis* could be detected by the primer/probe set targeting a small fragment of the 18SrRNA gene. Another primer/probe set targeting a fragment of the hsp70 gene was used for the specific detection of *B. canis vogeli*. Both of these qPCRs were specific when they were used on DNA reference samples and didn't cross-react with *I. ricinus* ticks. The presence of *B. canis* could be confirmed by conventional PCR and sequencing. However, the presence of *B. canis vogeli* in the *B. canis vogeli*-specific-positive samples from the array could not be confirmed. High-throughput screenings of different tick species (*D. marginatus, Rhipicephalus bursa,* and *Amblyomma variegatum*), also generated false-positive results, as they could never be confirmed by nested PCR (not shown). Therefore, a new primer/probe set should be designed for the detection of *B. canis vogeli*.

The presence of *E. canis* DNA in 28 samples and several negative controls could not be confirmed by alternative PCR-based methods. Probably, the signal arose from a previous laboratory contamination when a high concentration of the positive control, a plasmid, was accidentally used (not shown). As discussed previously, laboratory contaminations can be problematic when using DNA amplification techniques for the detection of pathogens [23]. This issue can be resolved by designing a new primers/probe set targeting another gene fragment of *E. canis.*

The array detected DNA of several tick-borne pathogens, namely *R. helvetica*, *A. phagocytophilum, B. burgdorferi* s.l., *C. burnetii* and *B. divergens.* These pathogens have been detected in *D. reticulatus* by means of molecular methods before [23], but their presence could not be confirmed by conventional PCRs in the present study. With molecular techniques alone, it is not possible to infer the presence of infectious agents in *D. reticulatus*, or to infer its vector competence for these agents. Further investigations on the vector competence of *D. reticulatus* are necessary before the results of these pathogens are meaningful for surveillance of vector-borne pathogens.

Both *A. marginale* and *T. equi* were detected by the array, each in one sample, but neither of them could be confirmed by a confirmatory PCR. One explanation might be that the array is more sensitive than the conventional PCRs, for example due to the pre-amplification step. Another possibility is that the primers/probe of *T. equi* is cross reacting with other samples. For this, new primer/probe sets are currently being designed. It was not possible to investigate this further, due to the limited number of positive samples ($n = 1$, each). Thus, these results should be interpreted with care. Further validation of the detection properties of the primer/probe combinations for *A. marginale* and *T. equi* should be performed in future studies.

This array has been developed for epidemiologic rather than diagnostic purposes. Therefore, detection limits and sensitivity have not been experimentally determined. Furthermore, the normal range of the pathogen concentration present in a naturally infected tick is extremely difficult, if not impossible, to determine. The detection

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limit of a pathogen in a defined area is also determined by the infection rate of a pathogen in the tick species. For example, other studies have already shown the presence of *B. canis* in The Netherlands, where *B. canis* was not detected in the 860 tick lysates [24, 25]. In other words, a sufficient number of ticks according to the expected prevalence should be screened to enable the detection of some pathogens.

5. Conclusion

This study clearly demonstrates the utility of a fast tool that allows comprehensive testing of high numbers of tick-borne pathogens in ticks, which can be easily customized to fit regional demands or to screen samples for new or emerging diseases. This study further demonstrates the importance of thorough validation of this novel approach and that careful interpretation of the results is necessary.

Further studies will have to confirm whether this approach heralds the necessary breakthrough in epidemiological surveillance of vector-borne pathogens, broadening the monitoring of human and animal diseases.

Declarations

Author contribution statement

Hein Sprong, Sara Moutailler: conceived and designed the experiments; Wrote the paper.

Manoj Fonville: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Cornelia Silaghi, Lisa Weis: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Arjan Stroo, Adolfo Ibañez-Justicia, Jolyon Medlock, Paul Heyman, Benjamin Cull, Kayleigh Hansford: Conceived and designed the experiments; Performed the experiments.

Christel Cochez: Contributed reagents, materials, analysis tools or data.

Arieke Docters van Leeuwen, Elodie Devillers: Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Additional data associated with this study is available on request.

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