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
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 burnetii* (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-ricetulatus-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...
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™ 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.
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     | GCTTACTCACAAAGGCGTCTTT                                                  | 83 bp  | Culture of B31            |
|                 |        | Bo_bu_rpoB_R     | GCACTCTCTTACAAAGGCGTCTTT                                                  |        |                           |
|                 |        | Bo_bu_rpoB_P     | AATGCTTGGACACAGGACCTTCA                                                   |        |                           |
| *Borrelia garinii* | rpoB    | Bo_ga_rpoB_F     | TGCCGAAACTTACCAAAAGGCGTCTTT                                                  | 88 bp  | Culture of NE11           |
|                 |        | Bo_ga_rpoB_R     | ACATCTCTCTTACACAGGACCTTCA                                                   |        |                           |
|                 |        | Bo_ga_rpoB_P     | TCTATCTCTTGACAGGGACCTTCA                                                   |        |                           |
| *Borrelia afzelii* | flaB    | Bo_af_flab_F     | GGAACAAATGCAAGAGACGCTGAA                                                   | 116 bp | Culture of VS641          |
|                 |        | Bo_af_flab_R     | GCTGCTAAAAGAAGACGACGTCCT                                                  |        |                           |
|                 |        | Bo_af_flab_P     | TTGCAAGCGCAAGCGACGACCTT                                                     |        |                           |
| *Borrelia valaisiana* | ospA    | Bo_va_ospA_F     | ACTCACAATGACAGACTGCTGAA                                                   | 135 bp | Culture of VS116          |
|                 |        | Bo_va_ospA_R     | GCTGCTAAAAGAAGACGACGTCCT                                                  |        |                           |
|                 |        | Bo_va_ospA_P     | TTCGCCAAGACGACGACGACGTCCT                                                   |        |                           |
| *Borrelia miyamotoi* | glpQ    | B_miy_glpQ_F     | CACGACCAGAAGATGACGACGACGAC                                                  | 94 bp  | Plasmid*                  |
|                 |        | B_miy_glpQ_R     | GCTGCTAAAAGAAGACGACGTCCT                                                  |        |                           |
|                 |        | B_miy_glpQ_P     | TTGCAAGCGCAAGCGACGACGTCCT                                                   |        |                           |
| *Borrelia spielmanii* | fla    | Bo_sp_flab_F     | ATCTATTTCCTGTGAGGGACG                                                      | 71 bp  | Plasmid                   |
|                 |        | Bo_sp_flab_R     | GCTGCTAAAAGAAGACGACGTCCT                                                  |        |                           |
|                 |        | Bo_sp_flab_P     | TTGCAAGCGCAAGCGACGACGTCCT                                                   |        |                           |
| *Borrelia lusitaniae* | rpoB    | Bo_lu_rpoB_F     | CAAGACTTACTCATAAAAGGCGTCT                                                  | 87 bp  | Culture of Poti-B1        |
|                 |        | Bo_lu_rpoB_R     | TGCAGTCTTCTCATTCAAATCC                                                    |        |                           |
|                 |        | Bo_lu_rpoB_P     | TAAATGCTTGAGGGACGACT                                                      |        |                           |
| *Borrelia bissetii* | rpoB    | Bo_bi_rpoB_F     | GCAACTACGACCTTCTTCACAG                                                    | 118 bp | Plasmid*                  |
|                 |        | Bo_bi_rpoB_R     | CAAATCCTGGCCTTACTTCTTCC                                                  |        |                           |
|                 |        | Bo_bi_rpoB_P     | GAAGTCTCCGGCGAGACTTAA                                                     |        |                           |
| *Borrelia spp.* | 23SrRNA | Bo_sl23S_F       | GAGTCTTAAAAGGCGGCAAGTTAG                                                  | 73 bp  | Culture of B31            |
|                 |        | Bo_sl23S_R       | CTTCAGCTGGCCATAATAG                                                       |        |                           |
|                 |        | Bo_sl23S_P       | AGATGCTTGGTACAGCGGAGCCAG                                                  |        |                           |
| *Anaplasma marginale* | msp1b  | An_ma_msp1_f     | CAGGCTCAACGGTGCACTG                                                       | 85 bp  | Experimentally infected cow |
|                 |        | An_ma_msp1_r     | GATATGCTGCTGGCCATTCC                                                      |        |                           |
|                 |        | An_ma_msp1_p     | ATGAAAGGCGACTGTTAGGAAGCGGAG                                                  |        |                           |
| *Anaplasma platys* | groEL  | An_pl_groEl_f    | TTTCGCGATCCTTGGAAGAC                                                      | 75 bp  | Dog blood                 |
|                 |        | An_pl_groEl_r    | CTTCCCTGATCCTGACGACG                                                      |        |                           |
|                 |        | An_pl_groEl_p    | TTGCTAGATCCTGGCCAGGCTT                                                     |        |                           |

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| Pathogens                  | Target | Primers                  | Sequence                                                                 | Length | Positive control       |
|---------------------------|--------|--------------------------|--------------------------------------------------------------------------|--------|------------------------|
| Anaplasma ovis            | msp4   | An_ov_msp4_F             | TCATCGACATGCCTAGTAATGCC                                                  | 92 bp  | Plasmid<sup>a</sup>    |
|                           |        | An_ov_msp4_R             | TTGTCGCGCACTCACTCA                                                     |        |                        |
|                           |        | An_ov_msp4_P             | AGCCAGAGACCTGTTAGTATGAGACC                                               |        |                        |
| Anaplasma bovis           | groEL  | An_bov_groEL_F           | GGGAGAGATAGTACACATCTCTGG                                                  | 73 bp  | Plasmid<sup>a</sup>    |
|                           |        | An_bov_groEL_R           | CTGATAGCTACAGTTAAGGCC                                                   |        |                        |
|                           |        | An_bov_groEL_P           | AGGTCGTTGTGTTAGTACTGCTGGACC                                              |        |                        |
| Anaplasma centrale        | groEL  | An_ce_groEL_F            | AGCTGCCCCTGTATACACAG                                                  | 79 bp  | Plasmid<sup>a</sup>    |
|                           |        | An_ce_groEL_R            | GATGTTGATGCCAAATTTGCTC                                                  |        |                        |
|                           |        | An_ce_groEL_P            | CTTGCATCTCCTAGACGAGTAAAGGG                                                |        |                        |
| Anaplasma phagocytophilum | msp2   | An_ph_msp2_F             | GCTATGGAAGGCCAGTTGG                                                     | 77 bp  | Culture                |
|                           |        | An_ph_msp2_R             | GTCTGAAGCGCTCGTTAACC                                                   |        |                        |
|                           |        | An_ph_msp2_P             | AATCTGACCTCAACCTGGCAACGACC                                             |        |                        |
| Ehrlichia ruminantium     | dsb    | Eh_ru_dsb_F              | CTCAGAGGTTAATAGTTTACTC                                                  | 107 bp | Culture of Gardel      |
|                           |        | Eh_ru_dsb_R              | GTATGCAATATCTCTAAAGCTC                                                  |        |                        |
|                           |        | Eh_ru_dsb_P              | ACTACAGGCCCAAGCAGAAGGACACAGAAAGA                                     |        |                        |
| Ehrlichia canis           | dsb    | Eh_ca_dsb_F              | AATACCTGTTGAGCTCCCTACTCA                                               | 110 bp | Plasmid<sup>a</sup>    |
|                           |        | Eh_ca_dsb_R              | GTTCTTGTTATGTTAGTCTGC                                                   |        |                        |
|                           |        | Eh_ca_dsb_P              | AAGTGGCAAGCAGCAGACTGCTGTAC                                               |        |                        |
| Ehrlichia chaffeensis     | dsb    | Eh_ch_dsb_F              | TATTGCTAAATACCTGCTTAAAAAGTC                                               | 117 bp | Amblyomma americanum   |
|                           |        | Eh_ch_dsb_R              | GAGCTATCTCCTGAATGCTGATTT                                                 |        |                        |
|                           |        | Eh_ch_dsb_P              | ATTGACCTCCTAACTGAGGAGGACA                                                |        |                        |
| Neoehrlichia mikurensis   | groEL  | Nm_groEL_F               | AGAGACATCTACCGATTGGGA                                                   | 96 bp  | Ixodes ricinus         |
|                           |        | Nm_groEL_R               | TCCCGGCGGTACCATGAGGCTT                                                  |        |                        |
|                           |        | Nm_groEL_P               | AGATGCTTGGATGTACTCGTGCAAGACC                                              |        |                        |
| Rickettsia conori         | 23S-5S ITS | Ri_co_ITS_F                  | CTCGAAAGTTATCAGGTTAATAG                                                  | 118 bp | Culture                |
|                           |        | Ri_co_ITS_R              | CGATACCTGCAAAAATAATTCTCG                                                 |        |                        |
|                           |        | Ri_co_ITS_P              | CTGGATATCGGCGAGGCTACAGAT                                                |        |                        |
| Rickettsia slovaca        | 23S-5S ITS | Ri_sl_ITS_F                  | GTATCTACTCAACAGTTATACAG                                                  | 138 bp | Culture                |
|                           |        | Ri_sl_ITS_R              | CTTAACTTTTATACTACATACACC                                                 |        |                        |
|                           |        | Ri_sl_ITS_P              | TAAATTTGCGATTCAGTGCCAGCG                                                  |        |                        |
| Rickettsia massilae       | 23S-5S ITS | Ri_ma_ITS_F                  | GTATTTGCACTACTAATGTTTACTG                                                 | 128 bp | Culture                |
|                           |        | Ri_ma_ITS_R              | GTTAATGTTGCGAGCAGTCAA                                                   |        |                        |
|                           |        | Ri_ma_ITS_P              | TAGCCCCGCAGTGATCATGCAAAA                                                  |        |                        |

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| Pathogens                    | Target | Primers          | Sequence                             | Length | Positive control   |
|------------------------------|--------|------------------|--------------------------------------|--------|--------------------|
|                             |        |                  |                                      |        |                    |
| Rickettsia helvetica         | 23S-5S ITS |                  |                                      |        |                    |
|                             |        | Ri_he_ITS_F      | AGAACCGTAGCGTACACTTAG                | 79 bp  | Culture            |
|                             |        | Ri_he_ITS_R      | GAAACCTCCTTCTAGGGGT                 |        |                    |
|                             |        | Ri_he_ITS_P      | TACGTGAGATTGATCGGATCCGGGGA          |        |                    |
| Rickettsia aeschlimannii     | ITS    |                  |                                      |        |                    |
|                             |        | Rick_aesch_ITS_F | CTCAACAGTTGTTACGTTAAATAG             | 134 bp | Culture            |
|                             |        | Rick_aesch_ITS_R | CTTAACGTTCTACGTACGGCA                |        |                    |
|                             |        | Rick_aesch_ITS_P | TAATTCTGTGGATATCCGGGTGTCG           |        |                    |
| Spotted fever group          | gltA   |                  |                                      |        |                    |
|                             |        | SFG_gltA_F       | CTTTTTGTAGCTTCTCTCATCC              | 145 bp |                    |
|                             |        | SFG_gltA_R       | CCGATGTTAGTTATTTTGGT                |        |                    |
|                             |        | SFG_gltA_P       | TGGCTATTATGCTTGGGCTTCGGGTG         |        |                    |
| Bartonella henselae          | pap31  | Bar_he_pap_F     | CGCGTGATCGTTATAGGGTT                | 107 bp | Culture of Berlin 1|
|                             |        | Bar_he_pap_R     | AGCGATTTGCTCATCTAAGT                |        |                    |
|                             |        | Bar_he_pap_P     | ATTTGTGCTGGTGTATCGGTAAGGG          |        |                    |
| Bartonella quintana          | bqtR   | Bar_qu_bqt_F     | TCCATCCAAGATATCGGCG                | 80 bp  | Culture            |
|                             |        | Bar_qu_bqt_R     | CGTGCCAATGCTGTAACCA                 |        |                    |
|                             |        | Bar_qu_bqt_P     | TTAAGAGAGGGAGGGAGGCAGGCTCC          |        |                    |
| Francisella tularensis and   | tul4   | Fr_tu_tul4_F     | ACCCGCACAGGAAGTTAAGATT              | 76 bp  | Culture of CIP 5612T|
| Francisella-like endosymbionts|       | Fr_tu_tul4_R     | GATATTGGAAGCTTGTATACG               |        |                    |
|                             |        | Fr_tu_tul4_P     | AATGGCGACCCAGAAGGTTCAGTGCT          |        |                    |
|                             | fopA   | Fr_tu_fopA_F     | GCGGATTACTAGGTCGAGAC                | 91 bp  |                    |
|                             |        | Fr_tu_fopA_R     | CAAACTGTGTTGAAACATTCTAG             |        |                    |
|                             |        | Fr_tu_fopA_P     | AGAGGTTGTTGGAAGTGTTGGGTG            |        |                    |
| Coxiella burnetii and        | icd    | CoBu_icd_F       | AGGCCCCGTGTTTATTTACG                | 74 bp  | Culture            |
| Coxiella-like                |        | CoBu_icd_R       | CCGAAATCACATTTCTACCTT               |        |                    |
|                             |        | CoBu_icd_P       | TTCAGGCTTATTTGGCGCGTCCGG            |        |                    |
|                             | IS1111 | CoBu_l5_F        | TGGAGGAGGGAACATTGTG                 | 86 bp  |                    |
|                             |        | CoBu_l5_R        | CATTACCGTTGAGTTGTCG                 |        |                    |
|                             |        | CoBu_l5_P        | ATCGGACGTTATATGGGTGAGGGTGATG        |        |                    |
| Babesia divergens            | hsp70  | Bab-di_hsp70_F   | CTTATTTGTCAGCCGCGTA                 | 83 bp  | Culture of RFS     |
|                             |        | Bab-di_hsp70_R   | CTTCCCGGATAAGCCCTCT                |        |                    |
|                             |        | Bab-di_hsp70_P   | AGAACCAGAAGGGCGCTAACCAGA           |        |                    |
| Babesia caballi              | RAP1   | Ba_ca_rap1_F     | GGTGGTGGTGAGGCGGCTC                 | 94 bp  | Plasmid            |
|                             |        | Ba_ca_rap1_R     | CAGGCGACTAGGCGTGTG                 |        |                    |
|                             |        | Ba_ca_rap1_P     | TCTGTCGCGATGTAAGGGGCGG             |        |                    |

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| Pathogens                  | Target | Primers          | Sequence                          | Length | Positive control |
|---------------------------|--------|------------------|-----------------------------------|--------|------------------|
| *Babesia canis* (3 subspecies) | RNA 18S | Ba_ca_18S_F      | TGGCCGTTCTTAGTGGGTG            | 104 bp | Dog blood        |
|                           |        | Ba_ca_18S_R      | AGAAGCAACCGAATACTCAAATA            |        |                  |
|                           |        | Ba_ca_18S_P      | ACCGCACTAGTTAGCGGGGTAAAGTCC       |        |                  |
| *Babesia canis vogeli*    | hsp70  | Ba_vo_hsp70_F    | TCACCTGTCCTGCTAATTC              | 87 bp  | Dog blood        |
|                           |        | Ba_vo_hsp70_R    | TGATAGCCATGCGTTGAGAC             |        |                  |
|                           |        | Ba_vo_hsp70_P    | ACGACTCCACGCGGCAAGCCC             |        |                  |
| *Babesia venatorum (EU1)* | RNA 18S | Ba_EU_18S_F      | CCCCGCCTGACCTAGTGGT            | 91 bp  | Plasmid^         |
|                           |        | Ba_EU_18S_R      | CAAAATCAATCCCCGGTACG             |        |                  |
|                           |        | Ba_EU_18S_P      | CATCGAATTCATGGCAGTTG             |        |                  |
| *Babesia microti*         | CCTeta | Ba_mi_CCT_F      | ACAATGGTTTTCCCGAGCAA            | 145 bp | Culture of R1    |
|                           |        | Ba_mi_CCT_R      | CGACATTTTCGGCAACCTATAT          |        |                  |
|                           |        | Ba_mi_CCT_P      | TACTCTGTTGACATGCTG              |        |                  |
| *Babesia bovis*           | CCTeta | Ba_bo_CCT_F      | GCAAGTAGTTGGTATGCA              | 100 bp | Culture of MO7   |
|                           |        | Ba_bo_CCT_R      | GTCCTGATCTGTAATGTA              |        |                  |
|                           |        | Ba_bo_CCT_P      | TAAAGCAACACTGGTGGTGC            |        |                  |
| *Babesia bigemina*        | RNA 18S | Ba_bi_18S_F      | ATTCGGTTAACAGACAGAC            | 99 bp  | Plasmid^         |
|                           |        | Ba_bi_18S_R      | TCCCCACGCTTGAGCA                |        |                  |
|                           |        | Ba_bi_18S_P      | CAGGACTCCCTTAAAGCAGAAACG         |        |                  |
| *Babesia major*           | CCTeta | Ba_ma_CCT_F      | CACTGGTGGTGTCCATCAA             | 75 bp  | Plasmid^         |
|                           |        | Ba_ma_CCT_R      | TCTCGAAGCATCCGATTT              |        |                  |
|                           |        | Ba_ma_CCT_P      | AACACTGTCACGACATGAC             |        |                  |
| *Babesia ovis*            | RNA 18S | Ba_ov_18S_F      | TCTGTGATGCTTTCATGTC             | 92 bp  | Plasmid^         |
|                           |        | Ba_ov_18S_R      | GCTGTTACCCGCGTC                |        |                  |
|                           |        | Ba_ov_18S_P      | TCGGAGCGGGTCACTCGATGC           |        |                  |
| *Theileria equi*          | ema1   | Th_eq_ema1_F     | GGCTCGCGAAGTAGGCA              | 66 bp  | Plasmid^         |
|                           |        | Th_eq_ema1_R     | TTGCGATATCAGACCTTGGA            |        |                  |
|                           |        | Th_eq_ema1_P     | TTCCAAAGCTCCAGGCAAGGCGT          |        |                  |
| *Theileria annulata*      | RNA 18S | Th_an_18S_F      | GCCGGTTAATCACCAGTTCAA           | 126 bp | Culture of D7    |
|                           |        | Th_an_18S_R      | AAACATCGCGGAAAAGGAC             |        |                  |
|                           |        | Th_an_18S_P      | ACATGCCACACCCAGAGGCAAC           |        |                  |
| *Ixodes ricinus*          | ITS2   | Ix_ri_ITS2_F     | CGAAACTCGATGAGACCC             | 77 bp  | Tick             |
|                           |        | Ix_ri_ITS2_R     | ATCTCACCACCGGACGT              |        |                  |
|                           |        | Ix_ri_ITS2_P     | TTTGAGAAATCCCGCTGACGT           |        |                  |

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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          | TCGATAAAAACCAGTAGGAGGA             |        |                  |
|                            |        | Ix_pe_ITS2_P          | TTTCGGAGCAAGTACAGAGGGAGCAAA        |        |                  |
| *Dermacentor reticulatus*  | ITS2   | De_re_ITS2_F          | AACCCCTTTCCGCTCCGTG               | 83 bp  | Tick             |
|                            |        | De_re_ITS2_R          | TTTTGCTAGAGCTCGACGTAC             |        |                  |
|                            |        | De_re_ITS2_P          | TACGAGGCAAACAACGCAAACTGCGA         |        |                  |
| *Dermacentor marginatus*   | ITS2   | De_ma_ITS2_F          | GCACGTTGCGTTTTGTGCC               | 139 bp | Tick             |
|                            |        | De_ma_ITS2_R          | CGCTCCGCGCAAGAATCT                |        |                  |
|                            |        | De_ma_ITS2_P          | TTCCGAAGTACGTGACCTGAGCA           |        |                  |
| *Escherichia coli*         | eae    | eae-F                 | CATTGATCAGGATTTTTCTGTGATA         | 102 bp | Culture of EDL933|
|                            |        | eae-R                 | CTCATGCAGAAATAGCCGTTA             |        |                  |
|                            |        | eae-P                 | ATAGTCTCGCCAGTATGCCAACAATACC      |        |                  |

*Plasmids are recombinant pBluescript II SK+ containing the target gene.*
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
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).
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]      |
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™ 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 BioMark™ (Table 3). In order to confirm the results obtained on the BioMark™ 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
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       |                 |
| B. garinii                      | 0       | 0             | 0       |                 |
| B. afzelii                      | 0       | 0             | 0       |                 |
| B. valaisiana                   | 0       | 0             | 0       |                 |
| B. lusitaniae                   | 0       | 0             | 0       |                 |
| B. spielmanii                   | 0       | 0             | 0       |                 |
| B. bissetti                     | 0       | 0             | 0       |                 |
| B. miyamotoi                    | 0       | 0             | 0       |                 |
| **Anaplasma marginale**         | 0       | 0             | 0       | 1               |
| A. platys                       | 0       | 0             | 0       |                 |
| A. phagocytophilum              | 1       | 0             | 0       |                 |
| A. ovis                         | 0       | 0             | 0       |                 |
| A. centrale                     | 0       | 0             | 0       |                 |
| A. bovis                        | 0       | 0             | 0       |                 |
| E. chaffeensis                  | 0       | 0             | 0       |                 |
| E. ruminantium                  | 0       | 0             | 0       |                 |
| E. canis                        | 5       | 3             | 10      | 8               |
| Neoehrlichia mikurensis         | 0       | 0             | 0       |                 |
| Rickettsia conorii              | 0       | 0             | 0       |                 |
| R. slovaca                      | 0       | 0             | 0       |                 |
| R. massiliae                    | 0       | 0             | 0       |                 |
| R. helvetica                    | 3       | 0             | 0       |                 |
| R. aeschlimannii                | 0       | 0             | 0       |                 |
| **SFG Rickettsia**              | 44      | 34            | 87      | 2               |
| Bartonella henselae             | 0       | 0             | 0       |                 |
| B. quintana                     | 0       | 0             | 0       |                 |
| Francisella tularensis (tul4)   | 0       | 0             | 0       |                 |
| **Francisella tularensis (fopA)** | 458   | 112           | 251     | 834             |
| Coxiella burnetii (icd)          | 1       | 0             | 0       |                 |
| Coxiella burnetii (IS1111)       | 0       | 0             | 0       |                 |
| Babesia divergens               | 0       | 3             | 0       |                 |
| B. microti                      | 0       | 0             | 0       |                 |

(continued on next page)
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 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 |

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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.

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                       |               |
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
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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