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Development of a PCR for *Borrelia burgdorferi* sensu lato, targeted on the *groEL* gene

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Abstract: *Borrelia burgdorferi* sensu lato (s.l.) is the etiological agent of Lyme disease, transmitted by ticks of the genus *Ixodes* Latreille. Diagnosis of Lyme disease in humans is often difficult and a detailed knowledge of the circulation of *B. burgdorferi* s.l. in tick hosts is therefore fundamental to support clinical procedures. Here we developed a molecular approach for the detection of *B. burgdorferi* s.l. in North Italian *Ixodes ricinus* (Linnaeus). The method is based on the amplification of a fragment of the *groEL* gene, which encodes a heat-shock protein highly conserved among *B. burgdorferi* s.l. species. The tool was applied in both qualitative and Real-time PCR approaches testing ticks collected in a North Italian area. The obtained results suggest that this new molecular tool could represent a sensitive and specific method for epidemiological studies aimed at defining the distribution of *B. burgdorferi* s.l. in *I. ricinus* and, consequently, the exposure risk for humans.

Keywords: Lyme diseases, *Ixodes ricinus*, qualitative PCR, detection, Real-time PCR.

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Lyme disease (LD) is a human tick-borne disease caused by bacteria belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, which currently includes 22 species widespread throughout the world (Mannelli et al. 2012, Mysterud et al. 2019). In Italy, the most common species causing LD are *Borrelia garinii*, *Borrelia afzelii* and *B. burgdorferi* sensu stricto (s.s.), with *Borrelia lusitaniae* and *Borrelia valaisiana* recorded as additional possible agents of the disease (Ciceroni and Ciarrocchi 1998, Pintore et al. 2015).

Currently hard ticks of the genus *Ixodes* Latreille represent the main vector of *Borrelia* spirochetes in the Northern hemisphere (Danielová et al. 2010, Scott et al. 2010, Rudenko et al. 2011). In particular, the main vector of LD in Europe is *Ixodes ricinus* (Linnaeus), which inhabits a wide range of habitats characterised by a humid climate and moderate temperatures, up to 1400 m a.s.l. (Ragagli et al. 2016). This determines a relatively high exposure risk for humans and makes an extensive knowledge on *B. burgdorferi* s.l. distribution pivotal for raising awareness in subjects exposed to *I. ricinus* bite and, consequently, to the risk of LD development.

The clinical diagnosis of LD in humans is often difficult because of the broad, and often generic, range of symptoms it can produce (Strle and Stanek 2009, Rizzoli et al. 2011). For this reason, knowledge of the circulation of *B. burgdorferi* s.l. in specific areas is fundamental to support LD diagnosis, especially in case of negative serological results or asymptomatic patients. Contrary to vertebrate hosts, detection, quantification and genotyping of *B. burgdorferi* s.l. in *I. ricinus* is not an issue, since *Borrelia* spirochetes are quite abundant in ticks.

Indeed, several DNA-based techniques targeting a variety of genes have been developed for the detection of bacteria belonging to *B. burgdorferi* s.l. species in tick hosts (e.g., Marconi and Garon 1992, Lee et al. 2003, Chu et al. 2008, Capelli et al. 2012). This is partly due to the complexity of the *B. burgdorferi* s.l. genome, which is characterised by the presence of genes carrying multiple mutations and various plasmids coding for proteins associated to different serotypes (Kòs et al. 2013).

Here we developed a molecular approach for the detection of *B. burgdorferi* s.l. in North Italian *I. ricinus* ticks. In

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Table 1. Sampling period, life stages, sex and number of tick specimens collected at 'La Fagiana' Natural Reserve.

Sampling period	Life stage/sex	No. of specimens
June 2018	nymph	39
	adult ♀	0
	adult ♂	0
April 2019	nymph	80
	adult ♀	11
	adult ♂	10

Table 2. Results of the molecular analyses on *Ixodes ricinus* (Linnaeus) samples obtained using *groEL* amplification approaches for *Borrelia burgdorferi* s.l. detection.

Life stage/sex (N)	% of positive samples (N)	
	Qualitative PCR *groEL*	Real Time PCR *groEL*
nymphs (39)	15.4% (6)	28.2% (11)
nymph pools (16)[b]	81.3% (13)	93.8% (15)
adult ♀ (11)	63.6% (7)	72.7% (8)
adult ♂ (10)	50.0% (5)	50.0% (5)
Total positive samples	40.8% (31)	51.31% (39)

detail, the proposed method is based on the amplification of a *groEL* gene fragment using both qualitative and Real-time PCR approaches. The *groEL* gene encodes a highly conserved 60-kDa heat-shock protein (GroEL) essential for the survival of cells in both physiological and stressful conditions (Lee et al. 2003). For the validation of this molecular tool, ticks sampled in a northern Italy area were tested.

In detail, sampling of ticks was carried out at 'La Fagiana' Natural Reserve (North Italy, 45°25'57.3"N; 8°50'10.2"E), an area of confirmed *B. burgdorferi* s.l. presence (Pistone et al. 2010). One hundred and forty tick specimens were collected during two sampling campaigns through dragging and flagging techniques from vegetation. Sampling period, life stages, sex and number of sampled specimens are detailed in Table 1.

Ticks were identified based on morphological features (Manilla 1998). Adult ticks from both periods and nymphs sampled in June 2018 were analysed as single specimens, whereas nymphs sampled in April 2019 were grouped in 16 pools of 5 nymphs each. All the specimens were stored in 70% ethanol at 4 °C until analysis. No larvae were collected in any of the two samplings.

The specimens were rehydrated and washed twice in 1X PBS for 20 min, cut in half with a sterile scalpel and subsequently homogenised in 1.5 ml tubes by crushing with a sterile pestle. Subsequently, DNA was extracted from single specimens or pool of nymphs using Isolate II Genome DNA (Bioline®, Memphis, Tennessee, USA) following the manufacturer's instructions. Proteinase K incubation was carried out overnight at 56 °C and DNA was eluted in one step with 50 µl of sterile water pre-heated at 72 °C. DNA was quantified by the Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Indianapolis, Indiana, USA) and stored at -20 °C.

A specific qualitative PCR for the amplification of a fragment of *calreticulin* (*cal*) gene of *I. ricinus* was performed to verify the extracted DNA quality and the absence of inhibitors, following the protocol described in Sassera et al. (2008).

The *groEL* gene sequences of the major representative of the *B. burgdorferi* s.l. complex circulating in Northern Italy (*B. garinii*, *B. burgdorferi* s.s., *B. afzelii*, *B. valaisiana*, *B. lusitaniae*) were aligned in BioEdit, version 7.0.5 (Hall 1999). A new set of primers was designed in a conserved region by means of EasyPrimer tool (https://skynet.unimi.it/index.php/tools/, Perini et al. 2020) and then manually adjusted: groEL-F: 5'-ACGATTTCTTATGTTGAGGG-3'; groEL-R; 5'-TCTCAAGAACTGGTAAAAG-3' (fragment size 160 bp). Primer sequences were analysed by Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) to avoid cross-reactions with other organisms.

Afterwards, the extracted DNA was tested to detect the *B. burgdorferi* s.l. presence in the area of study. Amplifications were performed in 10 µl of water containing at final concentration: 1× Reaction Buffer (Promega®, Madison, Washington, USA) 0.2 mM of each dNTP, 1 µM of each primer, 0.5 U of GoTaq G2 DNA polymerase (Promega), 1 µl of DNA sample. The amplification conditions were: 95 °C for 3 minutes; 40 cycles (95 °C for 20 seconds, 51 °C for 15 seconds and 72 °C for 20 seconds), 5 °C for 5 minutes. Amplicons were loaded and run in 2% agarose gel.

To verify the reliability of the proposed method, on the same samples we performed also a published nested PCR protocol amplifying a fragment of the 5S-23S rRNA intergenic spacer region of *B. burgdorferi* s.l. (Chu et al. 2008) following the protocol modified by Pistone et al. (2010).

The set of primers targeting the *groEL* gene was also used in Real-time PCR (CFX Connect Real-time PCR detection system; Biorad®, Hercules, Canada, USA). Amplification was performed in 20 µl of water containing at final concentration: 1× SsoAdvanced Universal SYBR Green Supermix (Biorad®), 0.25 µM of each primer, 1 µl of DNA sample. The thermal profile for Real-time PCR was as follows: 98 °C for 3 minutes; 40 cycles (95 °C for 10 seconds, 53 °C for 15 seconds and 72 °C for 15 seconds) and melt curve from 55 °C to 95 °C with increments of 0.5 °C per cycle. Each DNA was tested in duplicate. The obtained amplicons were subsequently load and run on 2% agarose gel.

The *groEL* qualitative and Real-time PCR products, as well as the 5S-23S rRNA products, were excised from agarose gel, purified using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega) and Sanger sequenced (Eurofins Genomics, Ebersberg, Germany). The obtained sequences were subjected to BLAST analyses.

All the collected tick specimens (nymphs, adult males and females) were identified as *I. ricinus*. The amplification of a *I. ricinus cal* gene fragment in each sample confirmed the good quality of the extracted DNA and the absence of inhibitors (data not shown).

In this study, a new set of primers amplifying a fragment of the *B. burgdorferi* s.l. *groEL* gene was designed. The *groEL* gene, compared to other molecular targets, such as 16S rRNA, is more heterogeneous among species and also useful for intraspecies differentiation (Park et al. 2004). Indeed, molecular approaches based on the amplification

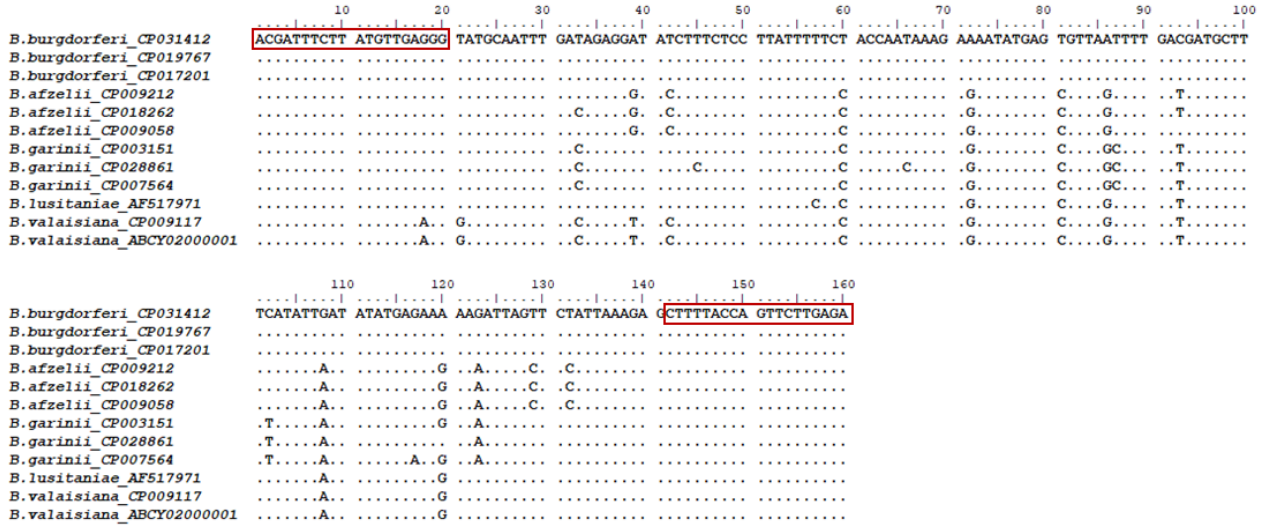

Fig. 1. Alignment of the *groEL* gene sequences of species of the *Borrelia burgdorferi* s.l. complex circulating in northern Italy. The primers designed in this work are highlighted in red frame.

of *groEL* gene are already published for the detection of *B. burgdorferi* s.l. and other bacteria such as *Anaplasma* spp. and *Rickettsia* spp. (Lee et al. 2003, Lew et al. 2003, Park et al. 2004, Campos-Calderón et al. 2016). The *groEL* gene sequences of LD *B. burgdorferi* s.l. species circulating in Northern Italy and available in GenBank were aligned (Fig. 1). The primers, designed on a conserved region, were analysed *in silico* to detect any potential cross-reaction with other bacterial species (such as common bacteria found in ticks) or eukaryoties organisms (e.g., vertebrate or tick DNA).

Results of qualitative and Real-time PCRs carried out on the 76 samples (i.e., 60 single specimens and 16 pools of 5 nymphs each), are reported in Table 2.

Thirty-one samples (41%) resulted positive to the newly designed qualitative PCR targeting *groEL* gene. Fifteen out of 31 positive samples were randomly sequenced and compared to sequences available in GenBank, with seven samples showing 100% identity with *B. lusitaniae* and eight samples 100% identity with *B. afzelii*.

Thirty-nine samples (51%) resulted positive to the Real-time PCR using the same primers. This approach allowed the detection of eight additional positive samples, all showing 100% identity with *B. lusitaniae*.

These additional Real-time PCR positive samples could be attributed to a low spirochete load that prevented the detection during electrophoresis when using the qualitative protocol. These samples, showed indeed a threshold cycle above 33, confirming the Real-time PCR as more sensitive than the qualitative one. Differences between polymerase enzymes, as well as between standalone polymerase enzymes and Real-time PCR mastermixes, could affect the PCR results. These differences could be due to polymerase-specific characteristics or to $MgCl_2$ adjustments (which are required for standalone polymerases; Witte et al. 2018). This can explain the additional positive samples detected using the Real-time PCR approach when compared to the qualitative one.

In order to verify the reliability of the proposed method, samples were also amplified using an already published nested protocol targeting a fragment of 5S-23S rRNA intergenic spacer region of *B. burgdorferi* s.l. (Chu et al. 2008) and previously applied for a tick survey in the same area of the present study (Pistone et al. 2010).

The obtained results showed the *groEL* Real-time PCR to be more sensitive and more specific than the nested PCR approach. Indeed, eight samples positive to the *groEL* amplification were negative when tested with the already published protocol. Conversely, the nested PCR protocol detected three additional positive samples compared to the *groEL* Real-time approach. The amplicons were thus sequenced to confirm the specificity of the amplification, and the obtained sequences were compared to those available in GenBank. No similarity was found, suggesting that, in certain conditions, the nested protocol may produce chimeric products. The protocol published by Chu and colleagues (2008) was firstly developed and tested on samples from Zhejiang province (South-East China), and also successfully applied on samples collected in Europe, including Italy (Zhan et al. 2009, Pistone et al. 2010, Veronesi et al. 2012). In our case, the production of non-specific amplicons may have been caused by ticks collected in the present study having a different microbial community or by the presence of vertebrate hosts' and bacterial DNA obtained during previous blood meals (Cogswell et al. 1996, Brettschneider et al. 1998). In any case, since both the *B. burgdorferi* s.l. amplicons and the non-specific products obtained with the 5S-23S rRNA nested PCR approach produced fragments of similar molecular size, a sequencing procedure would therefore be necessary to confirm the specificity of each amplification. This would result, in turn, in additional time-consuming procedures, as well as additional costs for PCR product purification and sequencing when performing epidemiological studies aimed at evaluating the presence of *B. burgdorferi* s.l. in ticks.

In contrast, results obtained with the proposed *groEL* PCR approach showed a high specificity of the amplification products. In addition, despite the short size of the *groEL* amplicon (160 bp), a high variability among *B. burgdorferi* s.l. genospecies was observed through in *silico* analyses (Fig. 1). Therefore, the proposed primers for the amplification of a fragment of *groEL* gene may allow: (1) the screening of samples for *B. burgdorferi* s.l. without additional sequencing procedures; (2) the discrimination of *B. burgdorferi* s.l. genospecies when the sequencing procedure is undertaken.

Moreover, the designed primers appear also suitable for the amplification of the *groEL* gene of several additional *B. burgdorferi* s.l. species causing LD and circulating in Europe (*B. bissettii*, *B. spielmanii*, *B. bavariensis*), as suggested by Supplementary Fig. 1 (alignment based on Franke et al. 2012). If this observation was experimentally confirmed, the proposed method could potentially be applied on molecular surveys performed in several European areas.

In conclusion, this approach, designed for the amplification of a *groEL* gene fragment, could represent a sensitive and specific method for epidemiological studies aimed at defining the distribution of *B. burgdorferi* s.l. in *I. ricinus* and, consequently, the exposure risk for humans. Rapid, sensitive and sensible methods are necessary to evaluate the risk of contracting LD linked to *B. burgdorferi* s.l. distribution in ticks, in order to undertake suitable control and prevention measures (Yang et al. 2012, Koś et al. 2013). Finally, further analyses are necessary to verify, at least in the area of the present study, whether the sensitivity of the proposed method is actually comparable to, or higher than, other existing protocols, or if it should be analysed combined with other gene targets/different methods, as suggested by other studies (Wodecka et al. 2010, Yang et al. 2012).

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