LoopTag FRET Probe System for Multiplex qPCR Detection of *Borrelia* Species

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Abstract

Laboratory diagnosis of Lyme borreliosis relies on some methods with known limitations. Molecular diagnostics using specific nucleic acid probes may overcome some of these limitations. We describe a novel reporter fluorescence real-time polymerase chain reaction (PCR) probe system, LoopTag, for the detection of *Borrelia* species. Advantages of the LoopTag system include the use of cheap conventional fluorescent dyes, ease of primer design, no restrictions on PCR product length, robustness, high specificity, applicability to multiplexed real-time PCR, melting curve analysis over a wide temperature range, high sensitivity, and easy adaptation to conventional PCR. Using the LoopTag probe system, we were able to detect all nine tested European species belonging to the *Borrelia burgdorferi* (sensu lato) complex and differentiate them from relapsing fever *Borrelia* species. As few as 10 copies of *Borrelia* were detectable in one PCR reaction. The novel multiplex probe real-time PCR system, designated LoopTag, is simple, robust, and incorporates melting curve analysis for the detection and differentiation of European species belonging to the *Borrelia burgdorferi* s.l. complex

Background

The diagnosis of Lyme borreliosis, a multi-system disorder caused by *Borrelia burgdorferi* complex species, relies on patient history, clinical symptoms, and serological tests like enzyme-bound immunosorbent assay (ELISA), which has inherent technical and biological limitations [1]. Polymerase chain reactions (PCR), particularly real-time PCR, offer a faster and more sensitive alternative to traditional culture-based methods for detecting Borrelia DNA. Quantitative PCR enables the determination of target DNA amounts, employing methods such as probe-based detection and melting temperature (Tm) analysis [2]. Various probe and primer systems, including Scorpions, Molecular Beacons, and Förster Resonance Energy Transfer (FRET), have been developed for signal generation in PCR [3]. FRET is advantageous for melting curve analysis as it allows differentiation of targets based on their melting points (Tm) [4]. A disadvantage of classical hybridisation probe systems is the need for two labelled probes to detect one target, which may reduce sensitivity due to the increased number of oligonucleotides involved [5]. This leads to reduced reaction complexity and higher consumables costs when designing multiplex PCRs. To overcome these limitations, we have developed a novel multiplexed probe real-time PCR system, that is applicable for the detection and differentiation of European species belonging to the *B. burgdorferi* s.l. complex.

Methods

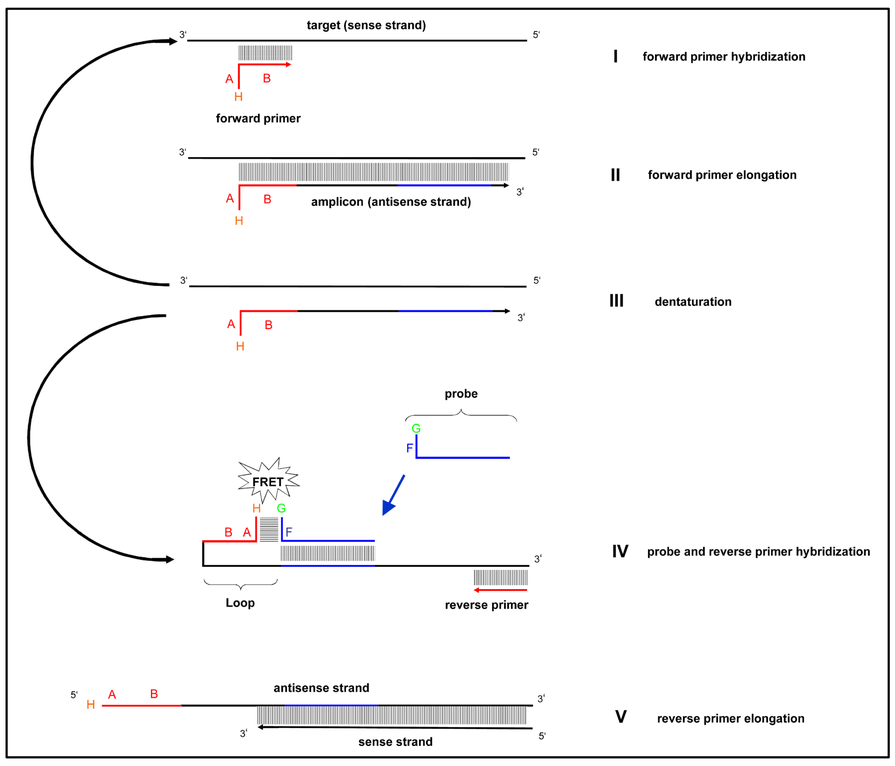

Fig. 1: Procedure of the LoopTag system during PCR. Please note the different reactions of the target, and the amplicon strand respectively. The forward primer of the *Borrelia* flagellin primer pair carries an 8 base pair long target-unspecific 5′-sequence (A), and a 20 base pair long target-specific sequence (B) which hybridizes to the target (stage I). The forward primer is elongated by the polymerase (stage II). After denaturation (stage III) the probe hybridizes to the antisense strand. The target-unspecific 5′-sequence of the antisense strand (A) forms a loop by hybridization to its complementary sequence, which is the target-unspecific part (F) of the probe. The loop brings together the fluorescence donor (G) and the fluorescence acceptor (H), both covalently attached to the probe and the forward primer, respectively. This results in a FRET signal proportional to the number of amplification products. The reverse primer of the *Borrelia* flagellin primer pair hybridizes to the antisense strand (stage IV) and is elongated by the polymerase (stage V). After stage V, the circle continues.

Discussion

- The main advantage of this system is that only one probe with a 3′-end fluorescent label is required for melting curve analysis (second label on primer) → reduced cost and number of oligonucleotides in multiplexing
- Compared to other probe systems based on loop formation (e.g. Molecular Beacons), the LoopTag system requires fewer probes/primers, the position of the detection probes can be selected flexibly and any standard PCR, regardless of amplicon length, could be adapted by adding the stem sequences to a primer and by designing the probe
- High sequence homology of the flagellin gene and standard deviations of melting temperatures can make it difficult to distinguish each species from another by several degrees Celsius → difficult to identify unknown samples by melting point alone
- Applications can be clinical research or routine clinical practice as a laboratory developed test, as all sequences in this study are disclosed
- Prospective application is the use of the LoopTag system in combination with planar array technologies → proof-of-concept study: LoopTag system for multiplex detection of PCR products on the surface of microbeads for real-time monitoring and surface melting curve analysis

Results

- Amplification of the flagellin gene of the different strains of the *B. burgdorferi* s.l. complex was detected (no amplification of related and non-*Borrelia* species)
- Different melting points of species-related PCR products allows differentiation of *Borrelia* Species based on melting curve analysis
- Pearson's product-moment correlation between the species-specific melting temperature and plateau height is significant, large, and positive ($r(7) = 0.94$, 95% CI (0.74, 0.99), $p < 0.001$)
- Establishment of an intern control based on an artificial DNA sequence for the detection and differentiation of *Borrelia* Species
- Comparison of the LoopTag system to a system using EvaGreen detection (as intercalating dye) revealed a similar efficiency and sensitivity

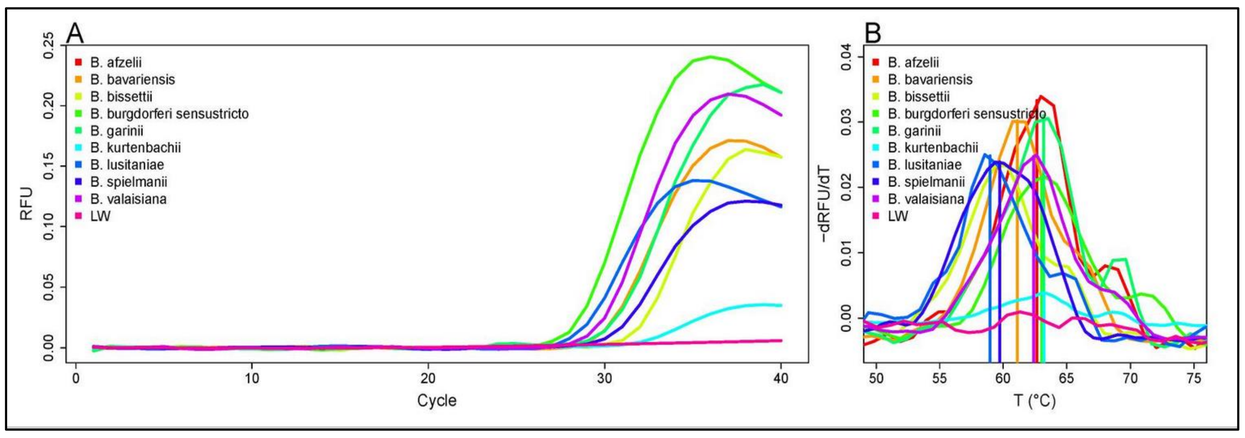

Fig. 2: Amplification of the flagellin gene. (**A**) Plots of the amplification curves for the detection of the flagellin gene of various *Borrelia*. (**B**) Melting peak analysis of the *Borrelia* species: *B. afzelii* PKo (62.4 ± 0.1 °C), *B. bavariensis* PBi (61.1 ± 0.4 °C), *B. bissettii* PGeb (60.2 ± 0.8 °C), *B. burgdorferi* s.s. B31 (63.7 ± 0.7 °C), *B. garinii* PLa (62.3 ± 0.3 °C), *B. kurtenbachii* 25015 (63.6 ± 0.3 °C), *B. lusitaniae* Poti B2 (59.7 ± 1.0 °C), *B. spielmanii* PSig2 (59.1 ± 0.5 °C), and *B. valaisiana* VS116 (62.4 ± 0.2 °C). A non-template water control (LW) exhibited no melting peak. Melting temperatures were calculated on the basis of three independent experiments.

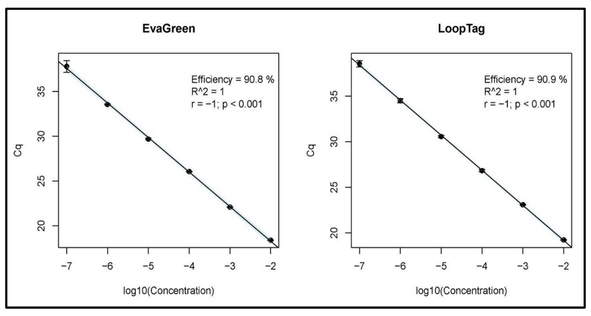

Fig. 3: Comparison of the LoopTag system to a system using the common intercalating dye EvaGreen for detection. We found no pertinent differences between the LoopTag and EvaGreen detection systems. The amplification efficiency was approximately 91%.

Conclusion

We have developed a novel probe system called LoopTag for the detection and differentiation of PCR products. The LoopTag system has been validated for the identification and differentiation of European species within the *B. burgdorferi* s.l. complex and for the discrimination of relapsing fever *Borrelia* species. Our results show high specificity and sensitivity up to 10 genome equivalents per PCR reaction. The system is simple and offers the ability to perform melting curve analysis.

Tab. 1: Minimal detectable amount of genome equivalents per species. DMAGE, Detectable minimal amount of genome equivalents per PCR reaction.
*, data are based on determinations of in vitro cultivated *Borrelia* species.
§, different strains of one species. Serotypes: PHei, TN, PRef, PLa, PWudII.

Borrelia	Species	DMAGE
	B. afzelii	≥10
	B. bavariensis	≥10
	B. bissettii	≥10
	B. burgdorferi s.s.	≥10
		PBr
		§
		: approx. 10
Borrelia burgdorferi s.l. complex	*B. garinii*	PHei: ≥10
		TN: approx. 700
		PRef: ≥10
		PLa: ≥10
		PWudII: ≥10
	B. kurtenbachii	100
	B. lusitaniae	≥10
	B. spielmanii	≥10
	B. valaisiana	≥10
Relapsing fever *Borrelia*	*B. anserina*	≥400.000 *
	B. duttonii	≥400.000 *
	B. miyamotoi	Not detectable
	B. parkerii	≥400.000 *
	B. recurrentis	Not detectable
	B. turicatae	≥200.000 *
Negative controls (other species)	*E. coli* (2 strains)	Not detectable
	Leptospira (2 strains)	Not detectable
	Treponema phagedenis (2 strains)	Not detectable

References

1. Deutschmann C., Roggenbuck D., Schierack P., Rödiger S. Autoantibody testing by enzyme-linked immunosorbent assay—A case in which the solid phase decides on success and failure. Heliyon. 2020;6:e03270. doi: 10.1016/j.heliyon.2020.e03270
2. Maurin M. Real-time PCR as a diagnostic tool for bacterial diseases. Expert Rev. Mol. Diagn. 2012;12:731–754. doi: 10.1586/erm.12.53.
3. Ranasinghe R.T., Brown T. Fluorescence based strategies for genetic analysis. Chem. Commun. (Camb. Engl.) 2005;44:5487–5502. doi: 10.1039/b509522k.
4. Marras S.A., Kramer F.R., Tyagi S. Multiplex detection of single-nucleotide variations using molecular beacons. Genet. Anal. Biomol. Eng. 1999;14:151–156. doi: 10.1016/S1050-3862(98)00018-7.
5. Schwaiger M., Péter O., Cassinotti P. Routine diagnosis of *Borrelia burgdorferi* (sensu lato) infections using a real-time PCR assay. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 2001;7:461–469. doi: 10.1046/j.1198-743x.2001.00282.x

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