***Borrelia* Multilocus Sequence Analysis based on housekeeping loci**

Lyme borreliosis (LB) is the most frequent vector-borne disease in humans in the temperate zone of the Northern Hemisphere. Clinical symptoms include arthritis, skin manifestations, such as acrodermatis chronica atrophicans (ACA) or erythema migrans (EM), and neurological disorders, such as facial palsy. The zoonotic disease is caused by several species belonging to the LB group of spirochaetes within the genus *Borrelia*. LB group spirochaetes have complex life cycles that involve ixodid (hard) ticks as vectors and a large number of vertebrate species as reservoir hosts, in particular small mammals and several bird species. Relapsing fever (RF) spirochaetes constitute another major group within the genus *Borrelia*. Except for *B. recurrentis*, which is transmitted by body lice, the majority of RF spirochaetes are transmitted by argasid (soft) ticks. Although relapsing fever was well known to occur in subtropical and tropical regions, recent work has identified a RF species, *B. miyamotoi*, that is transmitted by the same ixodid ticks that serve as vector for LB group spirochetes. Hence, this species occurs sympatrically with the LB group spirochetes in Europe, USA and Asia.

A MultiLocus Sequence Typing (MLST) scheme has been developed for *B. burgdorferi* by Gabriele Margos and Klaus Kurtenbach (University of Bath, United Kingdom), in collaboration with researchers from the U.S. (Yale University, NYMC, Centers for Disease Control, University of Utah), U.K. (University of Oxford, Imperial College London, University of Bath) and France (Institut Pasteur). This MLST scheme is based exclusively on chromosomal housekeeping genes. Due to primer design, this MLST scheme has been extended to MultiLocus Sequence Analysis (MLSA) comprising other species within the genus ***Borrelia***. The MLSA scheme has the power to characterize the spirochaetes at all the different phylogenetic levels required for evolutionary, epidemiological and fine-scale population/landscape genetic studies. Importantly, this scheme can be applied to infected ticks without the need to culture the spirochaetes, which can be fastidious and selective.

This feature renders the MLSA scheme a highly useful tool in large-scale epidemiological studies of these vector-borne bacteria.

The MLSA system has very recently been extended to be used with RF spirochetes. As the housekeeping genes considered here are more divers in RF spirochetes than in LB spirochetes, it was required to design degenerate PCR primers to amplify the genes of RF spirochetes. The primers given in Table 2 have been successfully used to amplify DNA of several RF species, including *B. miyamotoi*, *B. duttonii*, *B. persica* and *B. recurrentis* from cultured specimen. To obtain good quality sequences, it was required to design specific sequencing primers for individual species which are available for *B. miyamotoi*.

The development and validation of the scheme was funded mainly by The Wellcome Trust, London, U.K. (to K. Kurtenbach) and the NIH, U.S.A. (to D. Fish, Yale University, K. Kurtenbach, and I. Schwartz, NYMC). David M. Aanensen and Brian Spratt, Imperial College London, U.K. hosted the original Borrelia MLSA database.

Margos, G., A.G. Gatewood, D.M. Aanensen, K. Hanincová, D. Terekhova, S.A. Vollmer, M. Cornet, J. Piesman, M. Donaghy, A. Bormane, M.A. Hurn, E.J. Feil, D. Fish, S. Casjens, G.P. Wormser, I. Schwartz, and K. Kurtenbach. 2008. MLST of housekeeping genes captures geographic population structure and suggests a European origin of *Borrelia burgdorferi*. Proc. Natl. Acad. Sci. USA., published online 24/06/08

Table 1 Housekeeping genes and PCR primers for LB group spirochetes

gene (number*)	Primer 5' to 3'	Primer name	Product Length
***nifS* (BB0084)**	seminested		
Inner forward	same as outer forward		
Inner reverse	GTTGGAGCAAGCATTTTATG	nifR719	**719**
Outer forward	ATGGATTTCAAACAAATAAAAAG	nifF1	
Outer reverse	GATATTATTGAATTTCTTTTAAG	nifSR1049	
***clpA* (BB0369)**			
Inner forward	GACAAAGCTTTTGATATTTTAG	clpAF1255	
Inner reverse	CAAAAAAAACATCAAATTTTCTATCTC	clpAR2104	**849**
Outer forward	AAAGATAGATTTCTTCCAGAC	clpAF1237	
Outer reverse	GAATTTCATCTATTAAAAGCTTTC	clpAR2218	
***rplB* (BB0481)**	seminested		
Inner forward	CGCTATAAGACGACTTTATC	rplF40	**720**
Inner reverse	same as outer reverse		
Outer forward	TGGGTATTAAGACTTATAAGC	rplF2	
Outer reverse	GCTGTCCCCAAGGAGACA	rplR760	
***pyrG* (BB0575)**			
Inner forward	GATATGGAAAATATTTTATTTATTG	pyrF448	
Inner reverse	AAACCAAGACAAATTCCAAG	pyrR1154	**706**
Outer forward	GATTGCAAGTTCTGAGAATA	pyrF391	
Outer reverse	CAAACATTACGAGCAAATTC	pyrR1190	

***recG* (BB0581)**			
Inner forward	CTTTAATTGAAGCTGGATATC	recF917	
Inner reverse	CAAGTTGCATTTGGACAATC	recR1658	**741**
Outer forward	CCCTTGTTGCCTTGCTTTC	recF890	
Outer reverse	GAAAGTCCAAAACGCTCAG	recR1694	
***clpX* (BB0612)**			
Inner forward	AATGTGCCATTTGCAATAGC	clpXF403	
Inner reverse	TTAAGAAGACCCTCTAAAATAG	clpXR1124	**721**
Outer forward	GCTGCAGAGATGAATGTGCC	clpXF391	
Outer reverse	GATTGATTTCATATAACTCTTTTG	clpXR1273	
***pepX* (BB0627)**			
Inner forward	TTATTCCAAACCTTGCAATCC	pepXF449	
Inner reverse	TGTGCCTGAAGGAACATTTG	pepXR1115	**666**
Outer forward	ACAGAGACTTAAGCTTAGCAG	pepXF362	
Outer reverse	GTTCCAATGTCAATAGTTTC	pepXR1172	
***uvrA* (BB0837)**			
Inner forward	GCTTAAATTTTTAATTGATGTTGG	uvrF1434	
Inner reverse	CCTATTGGTTTTTGATTTATTTG	uvrR2111	**677**
Outer forward	GAAATTTTAAAGGAAATTAAAAGTAG	uvrF1408	
Outer reverse	CAAGGAACAAAAACATCTGG	uvrR2318	

PCR conditions for MLST housekeeping genes.

HotstarTaq Mastermix (Qiagen, Germany), 25 pmol of each outer primer, forward and reverse, and 2.5 μl of template DNA (purified DNA of isolates or tick lysates) were used for the first set of amplification cycles (25 ml final reaction volume). For PCR on tick-derived material, the $MgCl_2$ concentration was adjusted to 2.5 mM. For the second set of amplification cycles 50 pmol of each inner primer and 5 μl of product derived from the primary set of cycles were used (50 μl final reaction volume). This can be adjusted to smaller volumes, e.g. 30 μl reaction volume.

The PCR conditions for the housekeeping genes, except for *recG,* were as follows: for the first set of cycles, touchdown PCR was used with annealing temperatures starting from 55 °C and decreasing 1 °C each cycle. Specific conditions were 95 °C for 15 min, 94 °C for 30 sec, annealing temperature from 55 °C to 48 °C for 30 sec, and an extension step of 72 °C for 60 sec. An additional 20 cycles were run at 94 °C for 30 sec, annealing temperature of 48 °C, and extension at 72 °C for 60 sec. After a final extension step for 5 min at 72 °C, the samples were kept at 15 °C until further analysis. The conditions for the second set of 35 cycles were 95 °C for 7 min, 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 60 sec. After a final extension step for 5 min at 72 °C, the samples were kept at 15 °C.

For *recG*, the PCR conditons for the first set of cycles were 95 °C for 15 min, 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 60 sec, 30 cycles, and extension at 72 °C for 5 min. The conditions for the second set of cycles were identical. For a detailed description of the PCR for LB group spirochetes see also Wang et al. 2014; Current Protocols in Microbiology 34:12C.1-12C.31.

Relapsing fever group spirochetes

For relapsing fever spirochetes, degenerate primers were developed that permit amplification of a fragment compatible with *B. burgdorferi* MLST. A list of degenerate primers that work for several RF species can be obtained from the database curator (gabriele.margos@lgl.bayern.de). The degeneracy of the primers may render them unsuitable for sequencing; therefore, PCR products either have to be cloned into a plasmid vector and sequenced with vector specific primers or specific sequencing primers need to be designed. Below are given PCR and specifically designed sequencing primers for *Borrelia miyamotoi*, a relapsing fever group spirochete that occurs sympatrically with LB group spirochetes.

Table 2 PCR and sequencing primer for *Borrelia miyamotoi*, a relapsing fever group spirochete

PCR primer

gene (number*)	Primer 5' to 3'	Primer name	Product Length
***nifS* (BB0084)**			
forward	GAAAAAGTAAACTCCCTCAGAAAGG	BmnifF31	
reverse	CAATGATGCCTGCAATATTTGGTG	BmnifR892	861
***clpA* (BB0369)**			
forward	TTGATCTCTTAGATGATCTTGG	BmclpAF1268	

reverse	CAAACATAAACCTTTTCAGCCTTTAATA	BmclpAR2051	783
***rplB* (BB0481)**			
forward	ATTAAGACTTATARGCCAAAAAC	BmrplF18*	
reverse	GGCTGNCCCCAAGGWGAT	BmrplR761*	743
***pyrG* (BB0575)**			
forward	CTTYTAGTWATTGARATTGGTGGT	BmpyrF415	
reverse	CAGCATCAAYTATRCCACAAAC	BmpyrR1261	846
***recG* (BB0581)**			
forward	CTAGYATTCCTYTAATTGAGGC	BmrecF908*	
reverse	TTCRGTTAAAGGTTCCTTATAAAG	BmrecR1779*	871
***clpX* (BB0612)**			
forward	CTGTTGCYATTTGTTTTGAATGC(Y)TC	BmclpXF104	
reverse	TAAAGTTCTTTTGCCCAAGG	BmclpXR1277	1173
***pepX* (BB0627)**			
forward	AGAGAYTTAAGYTTAKCAGG	BmpepXF361	
reverse	GTTTCTCTTAAAGAYTGCATTCC	BmpepXR1202	841
***uvrA* (BB0837)**			
forward	GCTKAAATTTTTRATTGATGTTGGA	BmuvrF1435	
reverse	CARGGAACAAAAACATCRGGC	BmuvrR2306	871

*cross reactivity with *B. burgdorferi*

Sequencing primer

gene (number)	Sequencing primer 5' to 3'	Primer name
***nifS* (Bb0084)**		
forward	GAAAAAGTAAACTCCCTCAGAAAGG	BmnifF31
reverse	CAATGATGCCTGCAATATTTGGTG	Bmnif892R
***clpA* (BB0369)**		
forward	TTGATCTCTTAGATGATCTTGG	BmclpAF1268
reverse	CAAACATAAACCTTTTCAGCCTTTAATA	BmclpA2051R
***rplB* (BB0481)**		
forward	GACTTATAGGCCAAAAACTTC	BmrplF23
reverse	GATACAGGATGACGACCACC	Bmrpl759R
***pyrG* (BB0575)**		
forward	TTTAGTAATTGAGATTGGTGGTAC	BmpyrF417
reverse	TATTCCACAAACATTACGAGC	Bmpyr1252R
***recG* (BB0581)**		
forward	TAGCATTCCTTTAGTTGAGGC	BmrecF909
reverse	CTCAGCATGCTCAACTACC	Bmrec1671R
***clpX* (BB0612)**		
forward	TTATCTGTTGCTGTTTATAATC	BmclpF268
reverse	TTCAAACATAACATCTTTAAGTAATTCTTC	BmclpX1155R
***pepX* (BB0627)**		
forward	AGAGACTTAAATTTAGCAGGAGTTG	BmpepF361
reverse	TGCATTCCCCACATTGGAGTTC	Bmpep1187R
***uvrA* (BB0837)**		
forward	TTAAATTTTTAATTGATGTTGGACT	BmuvrF1437
reverse	TCTGTAAAAAACCCAACATAAGTTGC	Bmuvr2147R

The PCR conditions for the housekeeping genes **clpA** and **nifS** are as follows: for the first set of cycles, touchdown PCR was used with annealing temperatures starting from 58 °C and decreasing 1 °C each cycle. Specific conditions were 95 °C for 15 min, 94 °C for 30 sec, annealing temperature from 58 °C to 50 °C for 30 sec, and an extension step of 72 °C for 60 sec. An additional 35 cycles were run at 94 °C for 30 sec, annealing temperature of 50 °C, and extension at 72 °C for 60 sec. After a final extension step for 5 min at 72 °C, the samples were kept at 15 °C until further analysis. Due to high degeneracy of primers, *nifS* does not work for RF species other than *B. miyamotoi*. Therefore, for most RF species seven gene sequences can be obtained and incomplete allelic profiles can be submitted. This means that no ST number can be given but at least the seven loci can be used to generate phylogenies and allow strain comparison.

The PCR conditions for the remaining housekeeping genes were as follows: for the first set of cycles, touchdown PCR was used with annealing temperatures starting from 60 °C and decreasing 1 °C each cycle. Specific conditions were 95 °C for 15 min, 94 °C for 30 sec, annealing temperature from 60 °C to 52 °C for 30 sec, and an extension step of 72 °C for 60 sec. An additional 35 cycles were run at 94 °C for 30 sec, annealing temperature of 50 °C, and extension at 72 °C for 60 sec. After a final extension step for 5 min at 72 °C, the samples were kept at 15 °C until further analysis.