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Molecular Typing of *Borrelia burgdorferi*

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Abstract

Borrelia burgdorferi sensu lato is a group of spirochetes belonging to the genus *Borrelia* in the family of Spirochaetaceae. The spirochete is transmitted between reservoirs and hosts by ticks of the family Ixodidae. Infection with *B. burgdorferi* in humans causes Lyme disease or Lyme borreliosis. Currently, 20 Lyme disease-associated *Borrelia* species and more than 20 relapsing fever-associated *Borrelia* species have been described. Identification and differentiation of different *Borrelia* species and strains is largely dependent on analyses of their genetic characteristics. A variety of molecular techniques have been described for *Borrelia* isolate speciation, molecular epidemiology, and pathogenicity studies. In this unit, we focus on three basic protocols, PCR-RFLP-based typing of the *rrs-rrlA* and *rrfA-rrlB* ribosomal spacer, *ospC* typing, and MLST. These protocols can be employed alone or in combination for characterization of *B. burgdorferi* isolates or directly on uncultivated organisms in ticks, mammalian host reservoirs, and human clinical specimens.

Keywords

spirochetes; molecular typing; OspC; MLST; *Borrelia burgdorferi*; Lyme disease

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Internet Resources

<http://borrelia.mlst.net>

Borrelia burgdorferi MLST database,

<http://www.phyloviz.net/beta/Tutorial.html>

<http://www.phyloviz.net/wiki/tutorial/>

Phyloviz tutorial.

<http://eburst.mlst.net/v3/instructions/>

eBURST tutorial.

<http://www.megasoftware.net/tutorial.php>

MEGA tutorial.

<http://www.ub.edu/dnasp/DnaSPHelp.pdf>

DNASP tutorial.

<http://www.spatialepidemiology.net/>

Spatial epidemiology tutorial.

INTRODUCTION

Borrelia burgdorferi sensu lato is a group of spirochetes belonging to the genus *Borrelia* in the family of Spirochaetaceae (Johnson, 1984; Baranton et al., 1992; Wang and Schwartz, 2011). The spirochete is transmitted between reservoir hosts by ticks of the family Ixodidae, mainly ticks in the *Ixodes ricinus* complex that include *I. scapularis* and *I. pacificus* in the U.S. and *I. ricinus* and *I. persulcatus* in Eurasia. Infection with *B. burgdorferi* in humans may cause Lyme disease, or Lyme borreliosis, which is the most common vector-borne disease in North America and Europe (Stanek et al., 2011). Between 1992 and 2011, a total of 439,738 cases of Lyme disease were reported to the U.S. Centers for Disease Control and Prevention (CDC) (Orloski et al., 2000; Centers for Disease Control and Prevention, 2012). Recent CDC estimates suggest that the actual number of annual cases may exceed 300,000 (Kuehn, 2013). In Europe, there is great regional variation with respect to the incidence of Lyme borreliosis; the average number of cases per year ranges from <100 to >20,000 per year (go to <http://www.eucalb.com/> and select “epidemiology” from the left-hand menu). The clinical manifestations of human Lyme disease depend upon the stage of the infection and may affect the dermatological, neurological, cardiac, and musculoskeletal systems (Stanek et al., 2011).

Currently, more than 40 species have been described in the genus *Borrelia*. These include 20 *Borrelia* species within the *B. burgdorferi* sensu lato complex (Johnson, 1984; Margos et al., 2011; Stanek and Reiter, 2011) and more than 20 *Borrelia* species associated with relapsing fever. The genus *Borrelia* possesses certain genetic and phenotypic characteristics that are unique among prokaryotes. *Borrelia* cells are helical with dimensions of 0.2 to 0.5 µm by 10 to 30 µm, allowing them to be easily distinguished from other eubacteria based on the phenotypic features common for all spirochetes (Barbour and Hayes, 1986). *Borrelia* can also be differentiated from other pathogenic spirochetes such as treponemes and leptospirae on the basis of morphological traits, including the wavelength of the cell coils, the presence or absence of terminal hooks, the shape of the cell poles, and the number of periplasmic flagella (Holt, 1994). However, it is almost impossible to distinguish different species within the *Borrelia* genus, or to discriminate the Lyme borreliosis group of spirochetes from the relapsing fever borreliae, by their morphology. Therefore, the identification and differentiation of different *Borrelia* species and strains is largely dependent on analyses of their genetic characteristics.

The Lyme borreliosis group of spirochetes forms a bacterial species complex, *Borrelia burgdorferi* sensu lato, which now consists of 20 named and proposed genospecies (Margos et al., 2011; Ivanova et al., 2014). The bacteria are maintained in nature by complex zoonotic transmission cycles involving hard ticks of the genus *Ixodes* and a large number of small- and medium-sized vertebrate host species. Five of the named *Borrelia* species are regularly found in human patients. These causative agents of human Lyme borreliosis are *B. burgdorferi* sensu stricto in North America and Europe, *B. garinii*, *B. bavariensis*, *B. afzelii* in Europe and Asia, and *B. spielmanii* in Europe. For other species such as *B. lusitaniae*, *B. bissettii*, and *B. valaisiana*, the pathogenicity status is uncertain because they are rarely found in patients. The remaining 12 *Borrelia* species (not mentioned above) have not been

shown to be pathogenic to humans. Typing systems that accurately characterize species and strains within species are crucial for epidemiological, clinical, and evolutionary studies.

The genome of *B. burgdorferi* consists of an approximately 910-kb linear chromosome and a variable number of circular and linear plasmids that range in size from 9 to 62 kb (Fraser et al., 1997; Casjens et al., 2000, 2012). Several genetic features of the Lyme borreliae, including their highly conserved gene order and unique organization of the rRNA gene cluster, have been utilized for the development of molecular typing methods.

Typing of bacterial strains has great relevance for diagnostics and for clinical and epidemiological studies, as well as for population genetic or evolutionary research on bacterial pathogens. While many PCR-based approaches, especially for diagnostic purposes, target single genes (based on time and financial considerations), a technique that was termed multilocus sequence typing (MLST) was invented in the late 1990s and has been used with great effect for dissecting relationships of bacterial populations (Enright and Spratt, 1999; Spratt, 1999; Urwin and Maiden, 2003).

Current molecular techniques that are available for the classification and characterization of *B. burgdorferi* include DNA-DNA homology analysis, ribotyping, DNA sequencing of 16S rRNA and other conserved genes, species-specific PCR, PCR-based restriction fragment length polymorphism (RFLP) analysis, pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) fingerprinting, multilocus sequence typing/multilocus sequence analysis (MLST/MLSA), and whole genome sequencing (WGS). In this unit, we focus on three basic protocols, typing of the *rrs-rrlA* and *rrfA-rrlB* ribosomal spacer, *ospC* typing, and MLST. These approaches are widely used currently for *Borrelia* isolate speciation, molecular epidemiology, and studies of the association between different clinical presentations of Lyme borreliosis and distinct genotypes of the infecting *B. burgdorferi* strains. These protocols can be employed alone or in combination for molecular characterization of *B. burgdorferi* isolates or directly on uncultivated organisms in ticks, mammalian host reservoirs, and human clinical specimens.

CAUTION: *B. burgdorferi* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

BASIC PROTOCOL 1. TYPING OF *BORRELIA BURGDORFERI* BY PCR-BASED RFLP ANALYSIS OF THE *rrs-rrlA* RIBOSOMAL RNA SPACER LOCUS

All members *B. burgdorferi* sensu lato possess a unique rRNA gene organization that is distinct from that of other prokaryotes (Schwartz et al., 1992; Ojaimi et al., 1994). Two different rRNA-based PCR-RFLP typing methods have been developed for *B. burgdorferi*,

targeting either the *rrs-rrlA* spacer or the *rrfA-rrlB* spacer (see Alternate Protocol and Fig. 12C.5.1; Postic et al., 1994; Liveris et al., 1995; Rijpkema et al., 1995). Both methods employ PCR amplification followed by RFLP analysis by restriction endonuclease digestion of the PCR products and resolution of the fragments by gel electrophoresis. The approach is relatively simple, fast, and discriminative at the species and strain levels. Using nested PCR, the method can be applied directly to environmental or patient samples without the necessity of prior culture, thus avoiding the potential problem of clonal selection by in vitro cultivation (Liveris et al., 1996, 1999).

PCR amplification of the proximal 941 bp of the *rrs-rrlA* spacer, containing the region downstream of *rrs* and terminating at the conserved *ileT* (tRNA^{ile}) locus, followed by RFLP analysis using *MseI* or *TruI* restriction endonuclease digestion distinguishes *B. burgdorferi* sensu stricto strains into three ribosomal spacer types referred to as RST1, RST2, and RST3. Studies have shown that RST types correlate with pathogenic potential in humans and mice (Liveris et al., 1999; Wang et al., 2001, 2002; Wormser et al., 1999, 2008). Application of this typing method to uncultivated spirochetes from human tissues and field-collected ticks requires nested PCR amplification employing two sets of primers due to the low number of microorganisms in these samples.

The *rrs-rrlA* spacer PCR product can also be subjected to direct sequencing using primer P_B as sequencing primer (Bunikis et al., 2004). In one study, sequence polymorphism within the first 250 nucleotides of this PCR product resulted in delineation of 10 distinct intragenic sequence (IGS) types in a sample of 68 *B. burgdorferi* isolates. More recently, this approach allowed classification of 127 clinical isolates into 16 IGS types by sequencing of the first 250 nucleotides of the *rrs-rrlA* spacer (Hanincova et al., 2008). Overall, application of either approach depends on the level of desired resolution, laboratory resources, and technical expertise available.

To isolate DNA from tissue biopsy samples (e.g., erythema migrans skin lesions), anticoagulated whole blood, or *B. burgdorferi* cultures, a commercial nucleic acid extraction kit should be used. The DNeasy Blood and Tissue Kit (Qiagen) has proven to be reliable and consistent for isolation of DNA from these specimen types. DNA purification is accomplished in spin columns by following the manufacturer's spin column protocols with minor modifications (see below).

The kit buffer system allows direct cell lysis followed by selective binding of DNA to the DNeasy membrane in spin columns. Centrifugation removes contaminants and enzyme inhibitors such as proteins and divalent cations.

Materials

2-mm skin biopsy samples

BSK-II medium, incomplete (*UNIT 12C.1*)

DNeasy Blood and Tissue Kit (Qiagen) including:

Buffer ATL

600 mAU/ml proteinase K

Buffer AL

Buffer AW1 concentrate

Buffer AW2 concentrate

Minispin columns

2-ml collection tubes

96% and 70% ethanol

RNase-free H₂O (e.g., DEPC-treated; *APPENDIX 2A*)

Anticoagulated whole blood

Phosphate-buffered saline (PBS), pH 7.4

B. burgdorferi growing in culture (*UNIT 12C.1*)

Ixodes scapularis ticks (fresh stored in 70% ethanol)

15 mg/ml proteinase K (Roche)

Primer P_A: 5'-GGTATGTTTAGTGAGGG-3'; forward primer 1465–1481 of the *rrs* sequence

Primer P₉₅: 5'-GGTTAGAGCGCAGGTCTG-3'; reverse primer 941-924 of the spacer

Primer P_B: 5'-CGTACTGGAAAGTGCGGCTG-3'; forward primer 1505-1524 of the *rrs* sequence

Primer P₉₇: 5'-GATGTTCAACTCATCCTGGTCCC-3'; reverse primer to 908-886 of the spacer

10 mM (each) 4dNTP mix (*APPENDIX 2A*)

5 U/μl *Taq* DNA polymerase

10× *Taq* DNA polymerase buffer containing 1.5 mM MgCl₂

10 U/μl *Mse*I restriction endonuclease (New England Biolabs) and 10× NEBuffer 4 with 100 mg/ml BSA; or its isoschizomer *Tru*II restriction endonuclease (Fermentas) at 10 U/μl and 10× Fermentas buffer R containing 100 μg/ml BSA

6× DNA loading dye: 30% (v/v) glycerol/0.25% (w/v) bromphenol blue/0.25% (w/v) xylene cyanol

Spectrum Brand micro tissue grinders

37°, 56°, 65°C, and 72°C water baths or heat blocks

8 Quickstrip, 0.2-ml PCR tubes (Phenix Research Products)

Thermal cycler

Additional equipment for PCR (Kramer and Coen, 2001) and agarose gel, gel visualization, and gel documentation (Voytas, 2000)

NOTE: Particular care must be taken to prevent cross-contamination during DNA isolation. Perform all DNA extraction in a designated area in the laboratory, preferably in a dedicated extraction hood. Use barrier filter pipet tips throughout the procedures. Open all reagents as well as boxes of tubes and pipet tips only in the extraction hood. To facilitate detection of contamination among the samples if it occurs, add a “mock” extraction control (essentially an extraction without any DNA sample source) for each set of 10 sample extractions. In addition, a negative (i.e., no-DNA) control should be included for each set of 10 PCR reactions.

For skin biopsy specimens

- 1a** Grind 2-mm skin biopsy samples in 0.5 ml incomplete BSK-II medium, in a Spectrum Brand micro tissue grinder.
- 2a** Transfer 250 µl of the biopsy suspension to a 1.5-ml sterile microcentrifuge tube and microcentrifuge 3 min at 14,000 rpm, room temperature.
- 3a** Remove the supernatant and add 180 µl Buffer ATL followed by 20 µl of 600 mAU/ml proteinase K (both solutions provided in the DNeasy Blood and Tissue Kit).

IMPORTANT NOTE: Buffers ATL and AL may form precipitates upon kit storage. If necessary, warm to 56°C until the precipitates have fully dissolved. Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96%) as indicated on the bottle to obtain a working solution.

- 4a** Follow the Spin-Column Bench Protocol for Animal Tissues (steps 2 to 6 in the kit instruction manual) up to the elution step (step 7 in the kit manual).
- 5a** Elute the DNA from the spin column membrane with 100 µl of RNase-free water instead of the 200 µl AE buffer provided in the kit.

For EDTA-treated whole blood—CAUTION: Adhere to all blood-borne-pathogen protocols when handling blood! Also see *UNIT 1A.1*.

- 1b** Mix 100 µl of anticoagulated whole blood with 20 µl of 600 mAU proteinase K (from the DNeasy Blood and Tissue Kit) and adjust the volume to 220 µl with PBS in a 1.5-ml microcentrifuge tube.
- 2b** Follow the Spin-Column Bench Protocol for Animal Tissues (steps 2 to 6 in the kit instruction manual) up to the elution step (step 7 in the kit manual).
- 3b** Elute the DNA from the spin column membrane with 100 µl of RNase-free water instead of the 200 µl AE buffer provided in the kit.

Cultivated *B. burgdorferi* cells

- 1c** Prepare *B. burgdorferi* culture aliquots each containing about 5×10^6 cells in a 1.5-ml microcentrifuge tube.
- 2c** Centrifuge 5 min at 14,000 rpm, room temperature.

- 3c** Resuspend pellets in 200 µl of PBS containing 20 µl of 600 mAU proteinase K.
- 4c** Follow the Spin-Column Bench Protocol for Animal Tissues (steps 2 to 6 in the kit instruction manual) up to the elution step (step 7 in the kit manual).
- 5c** Elute the DNA from the spin column membrane with 100 µl of RNase-free water instead of the 200 µl AE buffer provided in the kit.

This protocol is to be used mainly for DNA isolation from *B. burgdorferi* reference cultures of known RFLP types that are used as positive controls (see step 25). Single-round PCR with primer set P_B-P₉₇ provides enough DNA product for RFLP analysis.

For *Ixodes scapularis* ticks

- 1d** Remove tick from 70% ethanol (if stored), place it on filter paper and let it dry for several minutes.
- 2d** Place dry tick in a 1.5-ml microcentrifuge tube with 180 µl of ATL buffer (from DNeasy kit) and cut it into at least four small pieces with an 18-G, 1½-in. needle (most importantly, cut the tick midgut).
- 3d** Add 25 to 30 µl of 15 mg/ml proteinase K (Roche).
- 4d** Mix thoroughly (use vortex mixer) and incubate at 56°C overnight.
- 5d** Add 200 µl Buffer AL (from DNeasy kit) and mix thoroughly.
- 6d** Incubate at 72°C for 10 min.
- 7d** Add 250 µl of 96% ethanol and mix rapidly.
- 8d** Pipet the ethanol mixture (including any precipitate) into a DNeasy Minispin column placed in a 2-ml collection tube (provided in kit).
- 9d** Microcentrifuge 1 min at 14,000 rpm, room temperature. Discard flowthrough with the collection tube.
- 10d** Place the DNeasy Mini spin column in a new 2-ml collection tube, add 500 µl Buffer AW1, and microcentrifuge 1 min at 14,000 rpm, room temperature. Discard the flowthrough and collection tube.
- 11d** Place the Minispin column in a new 2-ml collection tube, add 500 µl Buffer AW2 and microcentrifuge 1 min at 14,000 rpm, room temperature. Discard the flowthrough.
- 12d** Place Minispin column back into collection tube and microcentrifuge 3 min at 14,000 rpm, room temperature.
- 13d** Place Minispin column in a clean 1.5-ml microcentrifuge tube.
- 14d** Add 50 µl of hot (72°C) sterile, double-distilled water to the column, let stand for 1 min, then microcentrifuge 1 min at 14,000 rpm, room temperature.
- 15d** Repeat step 14d with another 50 µl of hot water to yield a total of 100 µl.

Purified DNA samples used for PCR should be stored at 4°C instead of −20°C, since frequent freezing and thawing degrades DNA integrity and could result in diminished PCR product yields.

Prepare primers for PCR amplification

General protocols for PCR are provided in Kramer and Coen (2001).

- 16** Dilute primers to 100 pmol/μl in sterile RNase-free water and store at −20°C in 50-μl aliquots labeled as “stock.” To make primer “working solutions” dilute the stock 1/20 in sterile RNase-free water to a final concentration of 5 pmol/μl.
- 17** Dilute 4dNTP mix 1/40 in sterile RNase-free water to final concentration of 250 μM (each dNTP).
- 18** Mix 2.5 μl of *Taq* DNA polymerase with 5.5 μl of 1× *Taq* polymerase buffer to prepare 8 μl of *Taq* DNA polymerase at 1.5 U/μl (sufficient for eight reactions).
- 19** Set up the following reaction in 8 Quickstrip, 0.2 ml PCR tubes with individual caps (final volume 45 μl/tube):

5μl 10× *Taq* DNA polymerase buffer with 1.5 mM MgCl₂

1μl 1/40 diluted dNTP mix

5μl 1/20 diluted stock primer P_A

5μl 1/20 diluted stock primer P₉₅

1μl 1.5 U/ml *Taq* DNA polymerase

28μl sterile RNase-free water.

For multiple samples, prepare a scaled-up version of the above PCR reaction designated as Master Mix-1 (MM-1) by multiplying the number of samples to be tested [samples and both positive and negative PCR controls as well as “mock” sample controls (see below)] by the above reaction mixture.

For nested PCR, prepare a second Master Mix designated as Master Mix-2 (MM-2) containing the same ingredients as in MM-1, but with primers P_B and P₉₇ and 32 μl of water per each reaction for a total volume of 49 μl per reaction.

Perform PCR amplification

- 20** Add 5 μl of DNA (from the “a,” “b,” “c,” or “d” steps, above) to 45 μl of MM-1.
- 21** Perform PCR using the following thermal cycling program:

1 cycle:	2 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)

	30 sec	52°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	2 min	72°C	(final extension).

- 22 For samples that require nested amplification due to low spirochete load, add 1 μ l of the first-round (P_A -P₉₅) PCR product to 49 μ l of MM-2 and perform a second amplification as described in step 21.

RFLP analysis

- 23 Add 10 μ l of the PCR amplification reaction to 10 μ l of a solution containing 2 U of either *Mse*I restriction endonuclease in 1 $\times$ NEBuffer 4 supplemented with 100 mg/ml BSA, or *Tru*II restriction endonuclease in Fermentas Buffer R containing 100 μ g/ml BSA, in 20 μ l total volume. Incubate *Mse*I digest for at least 1 hr at 37°C (may be extended to overnight if desired). If *Tru*II is used, incubate at 65°C instead of 37°C for the same time period.
- 24 Mix 10 μ l of the digestion reaction with 2 μ l of DNA loading dye and load onto a 2.4% agarose gel prepared in 1 $\times$ TBE buffer containing 0.5 μ g/ml ethidium bromide (Voytas, 2000).

For proper identification of RFLP typing results, each gel must include all three RST-type reference strains (positive controls) in addition to both negative PCR and “mock” sample controls. The inclusion of all three reference strains also allows for easy identification of samples containing multiple B. burgdorferi strains. Expected RFLP profiles of these strains are shown in Figure 12C.5.2.

- 25 Resolve the digestion products by electrophoresis at a constant current of 120 mA. Visualize DNA fragments by UV illumination (Fig. 12C.5.2).

These procedures are described in Voytas (2000).

NOTE: Nested PCR is particularly vulnerable to external DNA contamination. Please see Critical Parameters and Troubleshooting section for more information.

ALTERNATE PROTOCOL. PCR-RFLP TYPING BASED ON THE *rrfA-rrlB* SPACER

PCR amplification of the *rrfA-rrlB* spacer yields species-specific PCR products between 225 to 266 bp. RFLP analysis using *Mse*I or *Tru*II restriction endonuclease allows differentiation of members of *B. burgdorferi* sensu lato into 8 distinct species (Postic et al., 1994). However, due to the small size of the amplicon, resolution of the PCR restriction digests requires 16% acrylamide–0.8% bisacrylamide gels instead of agarose gels as in *rrs-rrlA* spacer typing. The originally published protocol by Postic et al. (1994) does not employ nested PCR to amplify the target, and thus application of this typing method to uncultivated spirochetes from human tissues and field-collected ticks is less sensitive than employing nested PCR (Lin et al., 2001). A variation of *rrfA-rrlB* typing uses nested PCR, and has been used on uncultured clinical and tick samples (Rijpkema et al., 1995; Lunemann et al., 2001).

However, detection of sequence variations is not RFLP based, but rather employs a probe-based reverse line blotting (RLB) approach that is more complex and technically demanding.

Additional Materials (also see Basic Protocol 1)

Primer P₁: 5'-CTGCGAGTTCGCGGGAGA-3'; forward primer 77–95 of the *rrfA* sequence

Primer P₂: 5'-TCCTAGGCATTACCATTA-3'; reverse primer 20–37 of the *rrlB* sequence

16% acrylamide-bisacrylamide gel in 1× TBE buffer without stacking gel (APPENDIX 3M; omit SDS)

0.5 µg/ml ethidium bromide in 1× TBE buffer

1. Carry out Basic Protocol 1 through step 19, except substitute primers P₁ and P₂ for primers P_A and P₉₅.
2. Perform PCR as in Basic Protocol 1, step 21.
3. Add 10 µl of the PCR amplification reaction to 10 µl of a solution containing 2 U of either *Mse*I restriction endonuclease in 1× NEBuffer 4 supplemented with 100 µg/ml BSA, or *Tru*II restriction endonuclease in Fermentas Buffer R containing 100 µg/ml BSA, in 20 µl total volume. Incubate *Mse*I digest for at least 1 hr at 37°C (may be extended to or overnight if desired). If *Tru*II is used, incubate at 65°C instead of 37°C for the same time period.
4. Mix 10 µl of the digestion reaction with 2 µl of DNA loading dye and load onto a 16% acrylamide-0.8% bisacrylamide gel in 1× TBE buffer without a stacking gel.
Omit SDS from this gel.
5. Electrophorese for 3 hr at 100 V in 1× TBE buffer.
6. Soak gel in 1× TBE containing 0.5 µg/ml ethidium bromide for 10 min, followed by 10 min soaking in water to remove background ethidium bromide.
7. Visualize DNA fragments by UV illumination.

BASIC PROTOCOL 2. GENOTYPING OF *B. BURGDORFERI* BY OUTER SURFACE PROTEIN C (OspC) SEQUENCING

The polymorphic outer surface protein C (OspC) gene of *B. burgdorferi* is located on circular plasmid 26 (Fraser et al., 1997). Although the exact function of OspC remains unknown, it is essential for *B. burgdorferi* to establish a productive initial infection in mammals (Stewart et al., 2006; Tilly et al., 2006), and different allelic variants of this protein are associated with a differential capacity to cause disseminated infection in mammals (Seinost et al., 1999; Wormser et al., 1999, 2008; Wang et al., 2001, 2002). OspC typing has also been employed to investigate *B. burgdorferi* genetic diversity in

environmental samples (e.g., ticks) (I.N. Wang et al., 1999; Qiu et al., 2002; Brisson and Dykhuizen, 2004; Barbour and Travinsky, 2010).

B. burgdorferi samples are genotyped by amplifying a 617-bp region of the *OspC* gene with primers OC6 (+) and OC623 (–) (Table 12C.5.1; Qiu et al., 2002; Seinost et al., 1999; I.N. Wang et al., 1999). DNA extracted from clinical isolates (obtained from the culture of blood or erythema migrans lesions collected from Lyme patients), infected *Ixodes scapularis* nymphs, or tissues of infected mice can be used for *OspC* typing (Wormser et al., 1999, 2008; Wang et al., 2001, 2002; Qiu et al., 2002).

Materials

Specimen (one of the following):

5-ml culture of *B. burgdorferi*

1 to 60 g of mouse tissue (ear, heart, bladder, joint)

I. scapularis nymphs

DNA isolation kit: DNeasy Tissue Kit (Qiagen) for total genomic DNA from ticks *or* Gentra Puregene DNA isolation kit (Qiagen) for *B. burgdorferi* or mouse tissues

1 mg/ml collagenase A (Sigma) in PBS, pH 7.4 (see *APPENDIX 2A* for PBS)

0.2 mg/ml proteinase K (see recipe)

10× PCR buffer with 2.5 mM MgCl₂ (Fermentas)

5 U/μl *Taq* DNA polymerase (Fermentas)

10 mM (each) 4dNTP mix (*APPENDIX 2A*)

Primers OC6(+) and OC623 (–) (Table 12C.5.1)

Positive control: *B. burgdorferi* genomic DNA which has been previously tested for the presence of *ospC*

Column-based PCR purification kit (e.g., Qiagen, cat. no. 28106)

Centrifuge

55°C water bath or heat block

18-G, 1½ needles (BD PrecisionGlide)

Thermal cycler

Multiple sequence alignment program (e.g., DNASTAR)

Additional equipment for PCR (Kramer and Coen, 2001) and agarose gel), gel visualization, and gel documentation (Voytas, 2000)

DNA isolation

- 1a** For *B. burgdorferi* cultures: Centrifuge the cultures 10 min at 3300 × g, room temperature. Using a commercially available DNA isolation kit, follow

manufacturer's protocol (Qiagen) to proceed with total genomic DNA extraction from the *B. burgdorferi* cells.

- 1b** *For mouse tissue:* Digest 1 to 60 g of tissue with 20× (v/w; e.g., 1.0 ml added for 50 g tissue sample) of collagenase A in PBS at 37°C for a minimum of 4 hr. Add equal volume of 0.2 mg/ml proteinase K solution and incubate overnight at 55°C. Using a commercially available DNA isolation kit, extract DNA, following the manufacturer's protocol (Qiagen), from 100 to 200 µl of the digested tissue sample.
- 1c** *For I. scapularis:* Place individual ticks in separate sterile microcentrifuge tubes and dissect with an 18-G needle, concentrating especially on bisecting the midgut. Proceed with DNA extraction as per manufacturer's instruction (Qiagen DNeasy Tissue Kit).

Sequence analysis of *ospC*

- 2** Prepare a 50-µl reaction mixture containing:
 - 5 to 10 ng of *B. burgdorferi* genomic DNA from step 1a, b, or c
 - 5µl 10× PCR buffer
 - 200 µM of each dNTP (add from 10 mM stock)
 - 2 µM each of primers OC6(+) and OC623 (–) (Table 12C.5.1)
 - 2.5 U of *Taq* DNA polymerase.
- 3** Include a positive control (*B. burgdorferi* genomic DNA which has been previously tested for the presence of *ospC*) and a negative control (no *B. burgdorferi* DNA) in each set of reactions in order to rule out nonspecific amplification or contamination.
- 4** Perform PCR using the following thermal cycling program:

1 cycle:	1 min	96°C	(initial denaturation)
45 cycles:	30 sec	94°C	(denaturation)
	30 sec	54°C	(annealing)
	1 min	72°C	(extension).

- 5** Examine the amplified product for purity by electrophoresis on a 1% agarose gel (Voytas, 2000).
- 6** Purify the product using a commercially available column-based purification kit (e.g., Qiagen).
- 7** Perform DNA sequencing of the purified PCR product in both directions, using the PCR primers.
- 8** To assign *ospC* major groups to the samples tested, compare the test DNA sequence to existing sequences of *ospC* major groups available in Genbank

(Table 12C.5.2) with the use of a multiple sequence alignment program (e.g., DNASTAR). *ospC* major groups are designated using the criteria that *ospC* alleles are less than 2% different within a group and greater than 8% divergent between groups (I.N. Wang et al., 1999).

BASIC PROTOCOL 3. MULTILOCUS SEQUENCE TYPING (MLST) OF *B. BURGDORFERI*

Multilocus sequence typing or multilocus sequence analysis (MLSA, referring to the application of MLST at the genus level) is a highly sensitive method for characterization of bacterial strains (Aanensen and Spratt, 2005; Bishop et al., 2009). The technique described here has recently been developed for the Lyme borreliosis group of spirochetes (the *B. burgdorferi* sensu lato species complex) and permits characterization of *Borrelia* strains at different phylogenetic levels required for evolutionary, epidemiological, and population/landscape genetic studies (Hoen et al., 2009; Margos et al., 2009). The technique requires amplification and sequence comparison of several housekeeping genes. For *Borrelia*, eight housekeeping loci, which are scattered across the main linear chromosome (to avoid local bias), have been chosen (see Table 12C.5.3 below). As the method considers single point mutation differences, amplicons need to be sequenced in forward and reverse directions for comparison. Good-quality sequences should be compared to the data available in the MLST database, and among each other, to obtain allele numbers. It is important that these sequences be of the same fragment length as those available through the MLST Web site (<http://borrelia.mlst.net>). For each strain, the allele numbers of the loci make up a chain of eight integers that corresponds to the allelic profile of the strain. Finally, the allelic profile determines the sequence type. Novel alleles or novel sequence types must to be submitted to the MLST database (via the curator) to obtain appropriate allele or ST numbers. For analysis of MLST/MLSA data, either concatenated sequences for all loci (giving one string of DNA for each strain) or allelic profiles can be used in downstream methods such as phylogenetic analysis, eBURST/goeBURST, or population genetics methods (Margos et al., 2011). Figure 12C.5.3 gives an overview of the steps involved in MLST analysis.

The following protocol is the result of some improvements to determine the best conditions for a successful and significant use of the MLST method. Depending on the origin and concentration of the DNA, there are two variants of PCR that can be used to amplify the product: single and (semi-)nested PCR. Single-step PCR can be used for cultured material when more than 0.5 ng/μl of clean DNA is available. For tick material (where *Borrelia* DNA may be scarce and some background can be expected), or from patients containing a lower concentration, a (semi-)nested PCR is advisable (see alternate steps within the protocol).

Since PCR, and in particular nested PCR, are highly sensitive methods, special care must be taken to avoid laboratory contamination of samples. Further information is presented in the “troubleshooting” section.

Materials

Nuclease-free (e.g., DEPC-treated; *APPENDIX 2A*) double-distilled H₂O

Hot-Start *Taq* DNA Polymerase (e.g., HotStarTaq DNA Polymerase, Qiagen)

10× PCR buffer (provided with the *Taq* polymerase)

20 mM (each) 4dNTP mix (*APPENDIX 2A*)

Primers for PCR amplification of the MLST genes (Table 12C.5.4)

HotStar Taq DNA Polymerase Master Mix (Qiagen; already contains *Taq* polymerase, 2× PCR buffer, dNTPs, and 3 mM MgCl₂)

Purified *Borrelia* DNA isolated from ticks or patients (see Basic Protocol 1)

ExoSAP (Affymetrix) or a PCR purification kit (Roche, Qiagen, Life Technologies) for PCR clean up

0.2-ml thin walled PCR tubes

Microvolume spectrophotometer: NanoDrop (e.g., Thermo Scientific, PEQLAB)

96-well PCR plates (e.g., Twin.tec 96 well plate, Eppendorf)

Thermal cycler

Sequence analysis software (e.g., Lasergene SeqMan from DNASTAR; MEGA; or DNASP)

PHYLOViZ 1.0 software

goeBURST 1.2.1 software

eBURST software

MEGA software

Additional equipment for PCR (Kramer and Coen, 2001) and agarose gel), gel visualization, and gel documentation (Voytas, 2000)

NOTE: For the *Borrelia* MLST/MLSA scheme, the genes listed Table 12C.5.3 are employed (Margos et al., 2008).

Single PCR

1a *To perform PCR with a reaction mix prepared in the lab:* Prepare a reaction master mix (total reaction volume of 30 µl, consisting of the following ingredients and pipet it into labeled PCR tubes. Per reaction:

3µl of 10× PCR buffer (provided with the *Taq* polymerase; buffer concentration and composition may vary between companies)

16µl of nuclease-free double distilled water

1µl dNTPs (20 mM stock solution of mixed A, T, G, C)

3µl forward primer (5 pmol/µl)

3µl reverse primer (5 pmol/µl)

1µl *Taq* polymerase (5 U/µl).

- 1b** To perform PCR using a commercially available PCR master mix: Pipet the following components into labeled PCR tubes. Per reaction:

15 μ l 2 $\times$ HotStarTaq DNA Polymerase Master Mix

6 μ l nuclease-free, double-distilled water

3 μ l forward primer (5 pmol/ μ l)

3 μ l reverse primer (5 pmol/ μ l).

For tick DNA extracts, increase the MgCl₂ concentration to 2 mM by adding 0.3 μ l of 50 mM MgCl₂ stock solution per reaction instead of water (i.e., 15.7 μ l water for a 30- μ l volume in step 1a; 5.7 μ l water for a 30- μ l volume in step 1b).

For either method, prepare a single reaction mix for each primer set in a larger tube. Multiply the volumes by the number of total samples (add two additional due to inaccuracy and loss during pipetting), mix it well and dispense 27 μ l per well/tube into labeled PCR tubes or plates.

- 2** Add 3 μ l purified DNA template per well/tube (diluted to approximately 0.5 ng/ μ l) in a separate room to avoid contamination.

PCR amplification

- 3** Centrifuge briefly (5 sec at maximum speed) to make sure nothing adheres to the lid.
- 4** Include negative PCR controls (containing all PCR components except DNA; instead of template DNA use the same amount of distilled water) as a quality control.
- 5** Optionally include a positive control (containing DNA of a definitely positive strain) which is helpful to verify the outcome of the reaction.
- 6** Because of the different melting temperatures of the housekeeping genes different amplification protocols must be employed. The genes *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *rplB*, and *uvrA* are amplified in the first step by touch-down PCR (Table 12C.5.5) that differs only in terms of the annealing temperature (Table 12C.5.6). For touch-down PCR, the initial annealing temperature is higher than the melting temperature of the primer. It is decreased by 1°C per cycle (in our case for example, 58°C, 57°C, 56°C, and so on) until the desired annealing temperature is reached. For *recG* a stable annealing temperature of 55°C is used (Table 12C.5.7).
- 7** Amplify the DNA using the PCR protocols in Tables 12C.5.5 to 12C.5.7.
- If nonspecific bands are produced during the PCR reaction using the lowest annealing temperature, try the higher temperature (58° to 50°C), except for *clpA*. If there are still nonspecific bands, it is possible to increase the annealing temperature to 60° to 52°C.

(Semi-) nested PCR (optional)

For samples that have a low DNA concentration (e.g., tick DNA extracts), a (semi-) nested PCR can be used.

- 8 For this, the single PCR protocol described above should be conducted followed by the (semi-) nested PCR step, which means the steps above are repeated a second time using the “inner primer” (Table 12C.5.4). These bind to the amplified fragment at a different position from the outer primer, so that a slightly shorter but more specific sequence is generated. The contamination risk is higher with this method; see Troubleshooting section for details.
- 9 As an alternative, instead of repeating the touch-down PCR, you can also use the simplified protocol described in Table 12C.5.8 for the second round of PCR (nested step) for *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *rplB*, and *uvrA* (for *recG* use an annealing temperature of 55°C again).
- 10 Perform agarose gel electrophoresis (Voytas, 2000) to ensure that there is (i) a good yield of PCR product, (ii) a single product band, and (iii) the amplicons are of the predicted size.

DNA sequencing

- 11 Each sample with a positive electrophoresis result can be cleaned up/purified and sequenced or be stored at –20°C. To clean up the PCR product an enzyme-based product like ExoSAP-IT System (Affymetrix) is recommended (please follow the manufacturer’s instructions).
- 12 Whether nested or single PCR was employed, use the inner primers for sequencing of amplicons in both forward and reverse directions. This provides a double control ensuring that you will be able to clearly identify all bases (Fig. 12C.5.4).
- 13 If a bad, scrambled sequence read is obtained (Fig. 12C.5.5), see Troubleshooting in the Commentary of this unit.

Make sure that any ambiguities are resolved. If there are clearly two peaks at the same position in forward and reverse sequence in an otherwise good-quality sequence trace, you may have a mixed specimen (Fig. 12C.5.6).

Sequence analysis

- 14 To analyze the sequence data, manually compare forward and reverse sequences for each individual sample. This can be done conveniently using DNASTAR Lasergene software (or equivalent) because it allows simultaneous comparison of forward and reverse strands. The corrected sequences should be saved as FASTA files. To further process your sequencing data, you can use sequence analysis software e.g., MEGA or DNASP (both freely available), to align and compare your sequences or to do statistical analysis, for example estimating

codon usage, nucleotide composition, or nucleotide pair frequencies. There are several things to consider for this sequence analysis:

- a. If some bases cannot be identified as unambiguous because of a bad sequence read, repeat the DNA sequencing step (or potentially even the PCR reaction).
- b. Be careful with mixed sequences. If there are two base peaks in one position, you must consider that you have a mixed sequence of two or more strains. Often, in such a case, there are also further positions with similar events in the same sequence. If you have a mixed infection, it is possible that it will not be apparent in all loci, but if present even in one locus, the entire sample should be omitted from further analyses.
- c. Make sure the sequence is the correct length (for the exact fragment length, download and use gene sequences from the MLST.net Web site as a reference).
- d. More extensive analyses of the data are only possible if sequences for all eight housekeeping genes are successfully determined.

The corrected sequences can be compared to those in the MLST database (<http://www.mlst.net/>) to identify the allele numbers (as described in the steps below).

Step-by-step use of the mlst.net database

- 15 Go to the mlst.net Web site (<http://www.mlst.net>; also see Fig. 12C.5.7).
- 16 Choose DATABASES and select “B. burgdorferi” or go directly to <http://borrelia.mlst.net>.
- 17 At this screen, different options are shown. For example, you can download alleles and sequence types or get some organism-specific information. Do NOT use the function “concatenate sequences,” as this will shorten the concatenated sequences.
- 18 To analyze the sequence data click on Locus Query → single locus. Make sure to choose the appropriate “allele you wish to query” and copy the corresponding sequence into the space available (Fig. 12C.5.8). Click on “Simple results” to obtain the allele number.

If a message appears that the allele is not found in the database, for example:

```
< PWat was not found in the database, however it
shows 99% similarity to Allele28 >
```

the sequence may represent a new allele. In that case, check where the differences in the sequence are located and go back to the original DNA sequencing trace file to make sure that the sequence is correct.

Also see Troubleshooting in the Commentary and follow the steps described there.

- 19 You can also choose Locus Query → Multiple locus and copy all eight housekeeping gene sequences into the appropriate spaces.
- 20 As an alternative, all sequences for one gene can be loaded at once. Choose Batch Query → Batch locus and make sure the data is in FASTA or MEGA format. Choose the appropriate gene and copy the data into the space. Click on “Submit”.
- 21 The allele number will then be shown by the database (Fig. 12C.5.9).
- 22 If a new allele type has been found, contact the curator of the database and submit the data to obtain the proper new allele number.
- 23 Once all eight allele numbers for the strain have been determined, choose Profile Query → Allelic, enter the allele numbers for each locus into the appropriate space, and click “Query entire data base.” If you have a known ST, a message should appear reading Your sequence type is X, and a list of this ST currently in the database should appear. If your ST is not yet in the database, the message reads: Your sequence type is not found, the closest matches are shown below. If the sequence type is not known, contact the curator.
- 24 Alternatively, choose Batch Query → Batch Allelic to enter all the allelic profiles for all strains (in a Tab-delimited format) and identify all sequence types simultaneously. Click on Submit.
- 25 To obtain information about strain, sequence type, species, country, or allelic profile, go to Profile query → database query, choose the desired information by clicking on “id,” and choose from the pull-down menu. You also have different choices using the pull-down menu at the “=” sign to modify your search. For example, if one combines: “ST = 1” and clicks Submit, a list with all strains that are ST 1 will be presented, and by clicking on the id number of strains within this list, full details can be visualized.

When using strain names, source names, etc., in the database query function, they must be typed exactly as in the database.

Data analysis

- 26 Many further analyses of the MLST data, which are all based on sequence types, allelic profiles, or concatenated sequences, are possible. These include analysis of the relationship between STs, phylogenetic trees, or geographic distribution of STs. The most frequently used programs for MLST data analysis are eBURST, goeBURST, and PHYLOViZ algorithms (Feil et al., 2004; Francisco et al., 2009, 2012), MEGA (Tamura et al., 2011), and spatial epidemiology. To facilitate handling of these software systems use the tutorials at the URLs listed in Internet Resources.

COMMENTARY

Background Information

Taxonomy of *B. burgdorferi*—The spirochetes represent a phylogenetically ancient and distinct group among the prokaryotes. They are currently categorized into a single order, *Spirochaetales*, in the class *Spirochaetia* of the phylum *Spirochaetes* phy. nov. In the second edition of *Bergey's Manual of Systematic Bacteriology*, the order *Spirochaetales* is reclassified into four families: *Spirochaetaceae* (family I), *Brachyspiraceae* (family II), *Brevine-mataceae* (family III), and *Leptospiraceae* (family IV) (Krieg et al., 2010). The family *Spirochaetaceae* consists of four genera: *Spirochaeta* (Genus I), *Borrelia* (Genus II), *Cristispira* (Genus III), and *Treponema* (Genus IV) (Krieg et al., 2010).

To date, more than 40 arthropod vector-borne *Borrelia* species have been described. These *Borrelia* species are classified into two major groups based on their ecological and epidemiological characteristics and disease association. The first group contains 19 *B. burgdorferi* sensu lato or Lyme *Borrelia* species that have been validated or proposed since 1984 (Krieg et al., 2010). The second group includes 25 validated or proposed relapsing fever *Borrelia* species transmitted by *Argasidae* soft ticks, with a few exceptions (i.e., louse-borne *B. recurrentis* and hard tick-borne *Borrelia miyamotoi*, *Borrelia turcica*, and *Borrelia lonestari*).

Fourteen *Borrelia* species belonging to the Lyme *Borrelia* group have been recognized by the International Committee on Systematics of Prokaryotes (Margos et al., 2011; Stanek and Reiter, 2011; Wang and Schwartz, 2011). These are *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia japonica*, *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia tanukii*, *Borrelia turdi*, *Borrelia sinica*, *Borrelia spielmanii*, *Borrelia americana*, *Borrelia carolinensis*, *Borrelia bavariensis*, and *Borrelia kurtenbachii*. Five additional *Borrelia* species in the Lyme *Borrelia* group have been proposed, but their taxonomic status has not been definitively validated. These are *Borrelia andersonii*, *Borrelia bissettii*, *Borrelia californiensis*, *Borrelia yangtze*, and *Borrelia finlandensis*. Among these, only *B. burgdorferi* sensu stricto, *B. garinii*, *B. bavariensis* (formerly part of *B. garinii*), and *B. afzelii* are well known to cause human diseases. Rare human cases associated with infections of *B. valaisiana*, *B. lusitaniae*, and *B. bissettii* have been reported; the pathogenicity of these species in humans remains to be elucidated (Collares-Pereira et al., 2004; Diza et al., 2004; Rudenko et al., 2009a).

In 2004, a novel *Borrelia* species, *Borrelia turcica*, was described, which showed less than 20% DNA homology with *B. burgdorferi* and *Borrelia hermsii* and formed a mono-phyletic cluster separated from both Lyme and relapsing fever-related *Borrelia* species on a phylogenetic tree based on 16S rRNA gene sequences (Guner et al., 2004).

Phenotypic typing methods—Conventional bacterial phenotypic typing methods such as biotyping, antibiotic susceptibility profiling, and bacteriophage typing cannot be applied to the genus *Borrelia* due to the extreme fastidiousness of the organisms and the inability to form confluent lawns on solid media. Serotyping based on two outer surface proteins, outer surface protein A (OspA) and outer surface protein C (OspC), represents the most

commonly used phenotypic method for *B. burgdorferi* [see review (G. Wang et al., 1999)]. In addition, multi-locus enzyme electrophoresis (MLEE) typing, which involves comparison of the mobility of metabolic enzymes on gel electrophoresis, has been employed successfully in differentiation of *B. burgdorferi* species (Balmelli and Piffaretti, 1996). Both serotyping and MLEE are rarely used currently due to the intensive labor involved and low discriminatory power.

Molecular typing methods—In addition to ribosomal DNA spacer, *ospC*, and MLST typing as described in this unit, several other molecular typing methods, including DNA-DNA homology analysis, ribotyping, DNA sequencing of 16S rRNA or other conserved genes (i.e., *fla*, *ospA*, *hbb*), species-specific PCR, PFGE, RAPD fingerprinting, variable-number tandem repeat (VNTR), and WGS analysis, have been developed and used for the identification and classification of *Borrelia* species.

DNA homology (DNA-DNA hybridization) analysis has been used as a reference method for species delineation of *Borrelia* (Baranton et al., 1992; Kawabata et al., 1993). As recommended by the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics, the phylogenetic definition of a species generally would include strains with approximately 70% or more DNA-DNA relatedness and with a T_m of 5°C or less (Wayne et al., 1987). Usually, *Borrelia* isolates within a species have 70% to 100% DNA homology, while isolates from different species exhibit only 30% to 65% DNA homology.

Grouping of bacteria by ribotyping is based on the profiles obtained after restriction enzyme digestion of chromosomal DNA and hybridization with a probe derived from a highly conserved ribosomal RNA. This approach has been used mainly for identification of the *B. burgdorferi*, *B. garinii*, and *B. afzelii* that are pathogenic in humans (Baranton et al., 1992; van Dam et al., 1993; Postic et al., 1996). Similarly, discrimination between different pathogenic *B. burgdorferi* species has been achieved by PFGE (Busch et al., 1996) and RAPD fingerprinting, or arbitrarily primed PCR analysis (Welsh et al., 1992; Wang et al., 1998). Analysis of the plasmid profiles of *B. burgdorferi* isolates also provides a potential for strain and species identification (Xu and Johnson, 1995).

Several MLSA scheme have been introduced for the Lyme borreliosis group of spirochetes (Bunikis et al., 2004; Richter et al., 2006; Attie et al., 2007; Margos et al., 2008; Rudenko et al., 2009b; Crowder et al., 2010), but the one described here (Margos et al., 2008) adheres to the principles of MLST systems as originally developed and defined by Spratt and Maiden (Maiden et al., 1998). It uses eight chromosomally located housekeeping genes that are scattered across the main linear chromosome of *Borrelia*. The system described in this unit has been used for taxonomic purposes (Margos et al., 2013, 2014) and to study populations of Lyme borreliosis spirochetes in Europe, North America, and Asia, both from questing ticks and human patients (Hoen et al., 2009; Takano et al., 2011; Vollmer et al., 2011, 2013; Margos et al., 2012; Hanincova et al., 2013; Mukhacheva and Kovalev, 2013). It is also being used to investigate population expansions of *B. burgdorferi* into Canada (Ogden et al., 2011, 2013).

With the advances in whole-genome sequencing technology and reduced cost on the next-generation of WGS platforms, it is anticipated that more *Borrelia* genome sequences will be available and that genome-wide SNP analysis will soon become a new tool for interspecies and intraspecies comparison of different *Borrelia* species and strains.

Applications of molecular typing techniques—Molecular typing techniques have been widely employed for *Borrelia* species delineation, population genetics and epidemiology, and pathogenicity studies of different *B. burgdorferi* species, genotypes, and strains. Traditionally, delineation of a new *Borrelia* species relied on DNA homology analysis between a newly identified species with representative strains from related, well-established species. The taxonomic status of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, etc., is validated on the basis of DNA homology analysis. DNA sequencing analysis of some highly conserved genes like *rrs* and *fla* were also used to describe novel *Borrelia* species such as *B. valaisiana* (Wang et al., 1997), *B. andersonii* (Marconi et al., 1995), and *B. tanukii* (Fukunaga et al., 1996). In recent years, MLST/MLSA (Richter et al., 2006; Postic et al., 2007; Margos et al., 2009; Rudenko et al., 2009b, 2011) and WGS (Casjens et al., 2011) have been employed to characterize *Borrelia* strains and to delineate new *Borrelia* species.

Analysis by multilocus enzyme electrophoresis had revealed a clonal population structure of *B. burgdorferi* based on the linkage disequilibrium of allele distributions (Boerlin et al., 1992). This conclusion had also been drawn from RAPD fingerprinting and comparison of the sequences of some highly conserved chromosomal genes such as *fla* and *p93* (Welsh et al., 1992; Dykhuizen et al., 1993), or the *rrs-rrlA* intergenic spacer and plasmid-borne *ospC* (Hanincova et al., 2008).

Despite *B. burgdorferi* sensu stricto retaining a clonal population structure at some spatial scales, genetic diversity of *B. burgdorferi* has been well documented among different species and strains, as evidenced by the delineation of 20 different species in the *B. burgdorferi* sensu lato complex (Margos et al., 2011; Stanek and Reiter, 2011; Ivanova et al., 2014). The exact mechanisms for generation of this genetic diversity remain unknown. As revealed by numerous studies using different molecular typing techniques, selection of distinct genotypes by reservoir hosts and ticks, or adaptive evolution, may be mechanisms for generating genetic diversity (Vollmer et al., 2013). Lateral gene transfer and recombination between *Borrelia* isolates belonging to the same species and between different species (although this may occur mainly in plasmid encoded loci) in hosts and vectors with mixed infections may also play a role in the evolution and maintenance of the genetic heterogeneity of *B. burgdorferi* in nature (Margos et al., 2012; Hanincova et al., 2013).

Clinical manifestations of human Lyme disease depend on the stage of the infection, and may affect dermatological, neurological, cardiac, and musculoskeletal systems. They may also vary depending on the *Borrelia* species causing the disease. Although all species cause erythema migrans (EM) as the early manifestation, *B. burgdorferi* sensu stricto may more frequently cause arthritis, and *B. afzelii* causes a chronic skin condition (acrodermatitis chronica atrophicans, ACA) as a late manifestation; *B. garinii* is more frequently associated

with early neurological manifestations (e.g., radiculoneuritis; van Dam et al., 1993; G. Wang et al., 1999). Further, differences in pathogenicity and dissemination of distinct *B. burgdorferi* subtypes have been suggested by molecular epidemiology studies. For example, the RST1 *B. burgdorferi* (Wang et al., 2002) and certain *ospC* genotypes (i.e., types A, B, H, I, K) have been associated with hematogenous dissemination in laboratory mice and in patients with Lyme disease (Seinost et al., 1999; Dykhuizen et al., 2008; Wormser et al., 2008). Recent studies also suggest that MLST sequence types have predictive value in terms of *B. burgdorferi* pathogenicity (Hanincova et al., 2013).

Critical Parameters

Typing method and discriminatory power—Taxonomic resolution and discriminatory power vary among different molecular typing methods. In general, the whole genome–based approaches such as PFGE, RAPD, MLST, and WGS have the highest resolution. They can be used for differentiation of *B. burgdorferi* strains between and within species. Of the three protocols described in this unit, MLST and ribosomal spacer-based PCR-RFLP typing have been employed to characterize *B. burgdorferi* strains from different sources and geographic locations at the species level (Vitorino et al., 2008; Hoen et al., 2009; Margos et al., 2009, 2010, 2011; Ogden et al., 2011, 2013; Vollmer et al., 2011, 2013), whereas *ospC* typing is more frequently used to differentiate strains within the same species and for disease association studies (Seinost et al., 1999; Wormser et al., 2008). Depending on the study purpose (i.e., required discriminatory power) and available resources, appropriate selection of one or more molecular typing methods is critical to yield species, subtype, and strain-specific information.

Optimization of sample preparation and PCR conditions—All three protocols described in this unit are capable of characterizing *B. burgdorferi* in various tick vectors, mammalian host reservoirs, and human clinical samples. Due to the low bacterial load in some biological materials, such as blood and synovial fluid from patients, a sample preparation protocol must be optimized to ensure a sufficient yield of microbial DNA.

Mixed infection by more than one species or subtype of *B. burgdorferi* has been reported in many sample types (Liveris et al., 1999; Gern et al., 2010). Purity of the *B. burgdorferi* strain DNA has a direct effect on the final interpretation of typing results. While some molecular typing methods (i.e., *OspC* typing, PFGE, RAPD) require the use of pure cultured spirochetes, most PCR-RFLP based methods can be used for molecular typing of *B. burgdorferi* directly in a variety of field-collected and clinical specimens (Liveris et al., 1999; Rauter et al., 2002; Xu et al., 2013). If desired, it may be possible to purify the individual isolates in a mixture by limited-dilution cloning and analyzing the resultant purified isolates (Hanincova et al., 2013).

Selection of distinct genotypes by reservoir hosts and ticks, or adaptive evolution, may have contributed to the observed genetic diversity of *B. burgdorferi* populations (Theisen et al., 1995; Kurtenbach et al., 1998). In addition, selection of particular genotypes or strains of *B. burgdorferi* during in vitro cultivation has been demonstrated (Xu et al., 2013). In vitro selection of *B. burgdorferi* by culture has been suggested by comparative analysis of *B.*

burgdorferi as measured directly in patient tissues and cultured isolates (Liveris et al., 1999; Brisson et al., 2011). When cultured isolates are used for molecular typing studies, it is necessary to recognize this potential “culture bias” in data interpretation.

Nested PCR, due to increased manipulations involved in the setup of two sequential PCR reactions and the high total number of amplification cycles, is particularly vulnerable to external DNA contamination. In order to avoid this problem, strategic planning, cleanliness, and caution must be implemented by laboratory personnel. All DNA samples should be prepared in a dedicated extraction hood, equipped with a UV lamp, and the hood should be irradiated for 20 min prior to each round of DNA extractions. If such a hood is unavailable, samples should be processed in a different room from the one where DNA gels are electrophoresed and PCR tubes are usually opened. Work areas on laboratory benches should be thoroughly cleaned with a 10% Clorox solution before use. Dedicated micropipettors and barrier filter tips should be used for all procedures. Ideally, PCR reaction mixtures should be prepared in a different biological hood from that used for DNA extraction. UV irradiation of this hood prior to initiation of work is fundamental for prevention of PCR contamination. It is critical that PCR tubes used for nested PCR be sealed with individual caps, not strip caps. For MLST, PCR should be done with one or two genes first before setting up a larger experiment, so one can see which samples provide good data and which samples produce problems with non-specific background or fail to generate a PCR product. One must be sure to set up sufficient and appropriate negative controls; it is very important to account for contamination even at a low level.

DNA sequencing—Use only DNA sequencing traces that permit unambiguous identification of bases. The use of low-quality traces that do not permit clear identification of bases will lead to errors in the sequences. If in doubt, repeat the sequencing reaction or even the PCR reaction, if necessary. If this does not resolve the problem, do not include the sample in further analyses. Often, when problems arise (e.g., with mixed samples, low quality of DNA, background noise), they occur in several genes, and those samples will be difficult to decipher. For MLST, mixed samples cannot be considered in the analysis (unless they are purified by limiting dilution cloning) because it is virtually impossible to determine the correct sequences.

Troubleshooting

Species delineation by MSLT and WGS—In MLST, *Borrelia* species identification and delineation are based on the concatenated sequences from the eight genomic loci. Although several MLST/MLSA schemes have been introduced for *Borrelia*, the one described in this unit adheres to the principles of MLST systems as originally intended by Spratt and Maiden (Urwin and Maiden, 2003). The species threshold level using this MLST system was determined to be 0.017. It was adjusted to the loci employed in this system, and is consistent with the species threshold suggested by Postic et al. (2007).

The recent polymorphism analysis of a novel Lyme *Borrelia* genome (isolate SV1) placed it on a branch outside the 14 sequenced *B. burgdorferi* genomes. The chromosome of SV1 was 1.75% different from its most closely related *B. burgdorferi* sensu stricto isolates, and was

proposed as a new species, *Borrelia finlandensis* sp. nov. However, it is closely related and corresponds to strains NE49 and Z41293 that were used by Margos et al. (2009) and described by Postic et al. (2007) as being ‘borderline’ *B. burgdorferi* sensu stricto. For several of the *Borrelia* species delineated by MLSA, ecological data were taken into consideration, e.g., host associations between *B. garinii* and *B. bavariensis* or *B. spielmanii* (Margos et al., 2009; Richter et al., 2006), and this has been suggested to be an important consideration when describing new bacterial species in general (Cohan, 2002).

PCR—If no PCR product is obtained, products are not the correct size, or products are of the correct size but too weak, repeat the PCR reactions with more template DNA or try a nested PCR. If there is nonspecific background (i.e., several bands) or bands of unexpected size, increasing the annealing temperature may resolve the problem.

DNA sequencing—If bad DNA sequencing results (see, e.g., Fig. 12C.5.5) are obtained, the sequencing must be repeated in order to obtain good-quality reads. Occasionally, redoing the procedure from the PCR step is advisable. If two base peaks are obtained in only one strand, it is most likely due to some background in the sequencing reaction. If one strand is very clear, in general that read can be trusted. An example of this is shown in Figure 12C.5.10. There is some background in the lower sequence (indicated with blue arrows), while the upper sequence is very clear. Therefore, this is considered an issue with the lower sequence reaction only, but not an indication of mixed sequences.

MLST database (mlst.net) analysis—If any of the following messages are received, follow the suggested solutions.

```
PWat was not found in the
database, however it shows 99%
similarity to Allele XX
```

This message may indicate the presence of a new allele. To verify this possibility, click on Locus Query → Single locus (paste the sequence in the available space) → “View DNA mismatches” to obtain a figure that shows the DNA mismatches between your sample and the closest related allele. Review the sequencing trace files again and ensure that the difference is real by carefully reviewing the noted mismatch position in your sequence. If a new allele or sequence type is found, contact the curator to obtain new, correct allele and sequence type numbers.

```
This is not a DNA sequence!
or Your sequence is of non-
standard length or SEARCH RE-
SULTS: This is not a DNA se-
quence! Your sequence must
contain only A,C,T,G or the
padding character --
```

Make sure that the sequence does not contain any characters other than A, T, G, C. If there are -, N or other characters in the data, this error message will be generated.

Your sequence is of non-standard length, standard clpA alleles are 579 bases in length, your query is 578 bases long please check- you have selected the correct locus - currently clpA The start and end points of your sequence are correctly trimmed.
If you are sure all is correct Click here to compare to other non-standard alleles

Make sure that the sequence is of the correct length and direction (i.e., 5' to 3').

SEARCH RESULTS: Sequence not found, closest similarity is 33%, probably wrong sequence.

This message probably indicates that the sequence is in the wrong orientation. Reverse the sequence direction and re-submit. If this does not resolve the problem, eliminate the sample from further analysis.

Time Considerations

Protocols in this unit involve multiple steps, including sample and DNA preparation, PCR amplification, and subsequent RFLP and DNA sequencing analysis. Depending on the types of samples, they may take 1 to 2 days to complete; however, this also strongly depends on the total number of samples, since much time is required for sample preparation, pipetting, data analysis, etc.

Sample and DNA preparation from cultured isolates can require as little as 1 to 3 hr. By contrast, DNA preparation from animal and human tissues typically entails an overnight digestion with proteinase K to increase the yield of DNA.

The PCR amplification step should require 2 to 3 hr for most protocols. RFLP analysis of the PCR product involves restriction enzyme digestion and gel electrophoresis, requiring a minimum of 1½ to 2 hr. *ospC* typing and MLST are DNA sequencing-based methods; typically, the sequencing is performed at a core facility or by a commercial service. This adds additional time to the technique, and time considerations vary with every specific situation. Furthermore, both of the latter methods require DNA sequence analysis that can

add 1 to 2 days to the method. Thus, of the three approaches described here, rRNA spacer PCR-RFLP analysis yields the most rapid results, but provides the lowest level of discrimination. On the other hand, MLST requires the greatest time investment, but yields the greatest strain discrimination, in addition to phylo-genetic information, if desired. *OspC* typing is intermediate in terms of time considerations, but cannot be used for phylogenetic analysis due to the high sequence diversity and possible recombination between *ospC* alleles.

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Figure 12C.5.1.

Typing of *B. burgdorferi* sensu lato isolates by *rrs-rrlA* or *rrfA-rrlB* intergenic spacer PCR-RFLP analysis.

Figure 12C.5.2.

rRNA spacer-based PCR-RFLP analysis of *B. burgdorferi* sensu stricto strains. RST1, strain B31; RST2, strain 297; RST3, strain N40. Migration positions of DNA size markers (M) are shown in left margin.

Figure 12C.5.3.

Multilocus Sequence Typing. Targeted PCR is used to amplify several genes distributed throughout the genome. Internal fragments of similar length for each gene are used. For each individual gene, fragments of identical length are aligned and compared to sequences in a virtual strain collection center, an MLST database, and to each other, permitting determination of an allelic profile for each strain. The allelic profile determines the sequence type that can be used to infer relationships of descent within bacterial species based on models of clonal expansion and diversification. Concatenated sequences of all genes can be used for phylogenetic inferences. The cumulative nature of an MLST database makes it an attractive instrument to understand intra- and interspecific relationships of bacteria. Reproduced with permission from IGE.

Figure 12C.5.4.
Example for a high-quality sequence trace file.

Figure 12C.5.5.
Examples of bad-quality, unreadable sequence trace files.

Figure 12C.5.6.

Mixed sequence trace indicated with an arrow (forward, upper sequence; reverse, lower sequence).

Figure 12C.5.7.
B. burgdorferi MLST database screen shot.

Figure 12C.5.8.

Example screen shot for Locus Query → Single locus → choose gene → copy sequence in available space.

Figure 12C.5.9.

Result of batch allele determination in the MLST database.

Figure 12C.5.10.

Example of sample in which only the forward or reverse sequence reads provide clear, unambiguous sequence.

Table 12C.5.1Primers for *ospC* PCR Amplification

Primer	Position in <i>ospC</i>	Sequence (5'-3')	T_m
OC6 (+)	6	AAA GAA TAC ATT AAG TGC GAT ATT	54°C
OC623 (-)	623	TTA AGG TTT TTT TTG GAC TTT CTG C	54°C

Table 12C.5.2GenBank Accession Numbers for *OspC* Major Groups

<i>ospC</i> type	GenBank accession numbers
<i>ospC</i> major group A to major group K	EU482041 to EU482051
<i>ospC</i> major group L	EU375832
<i>ospC</i> major group M	EU482052
<i>ospC</i> major group N	EU482053
<i>ospC</i> major group O	FJ997281
<i>ospC</i> major group T	EU482054
<i>ospC</i> major group U	EU482055
<i>ospC</i> major group X	HM047876
<i>ospC</i> major group Y	HM047877

Table 12C.5.3

MLST Genes

Gene no. ^a	Locus	Gene product/description
BB0369	<i>clpA</i>	Clp protease subunit A
BB0612	<i>clpX</i>	Clp protease subunit X
BB0084	<i>nifS</i>	Aminotransferase
BB0627	<i>pepX</i>	Dipeptidylaminopeptidase
BB0575	<i>pyrG</i>	CTP synthase
BB0581	<i>recG</i>	DNA recombinase
BB0481	<i>rplB</i>	50 S ribosomal protein
BB0837	<i>uvrA</i>	Exonuclease ABC, SU A

^a According to the numbering of the *B. burgdorferi* B31 sequence (GenBank accession number GI 6626249).

Table 12C.5.4

Primer Sequences, Size of Amplicon, and Fragment Used for MLST^a

Gene	5'-3' primer sequence	Amplicon size (bp)	Size of fragment used for MLST (bp)	Primer length (bp)	Primer start position in B31 genome	T _m (°C)
<i>niS</i>			564			
<i>IF^b</i>	same as outer forward					
<i>IR^b</i>	GTTGGAGCAAGCATTTTATG	719		20	719	54.3
<i>IF/OF^b</i>	ATGGATTTCAAACAAATAAAAAAG			23	1	52.2
<i>OR^b</i>	GATATTATTGAAATTCCTTTTAAAG	1027		23	1027	50.4
<i>clpA</i>			579			
<i>IF</i>	GACAAAAGCTTTTGATATTTTAG			24	1255	55
<i>IR</i>	CAAAAAAACAATCAAAATTTTCTATCTC	823		27	2078	57.6
<i>OF</i>	AAAGATAGATTCTTCCAGAC			21	1237	53.5
<i>OR</i>	GAAATTCATCTATTAAAAAGCTTTC	981		24	2218	48.3
<i>pyrG</i>			603			
<i>IF</i>	GATATGGAAAAATATTTTATTATTG			25	448	52.7
<i>IR</i>	AAACCAAGACAAATTTCCAAG	687		20	1135	52.3
<i>OF</i>	GATTGCAAGTTCTGAGAATA			20	391	52.3
<i>OR</i>	CAACACATTACGAGCAAAATTC	799		20	1190	52.3
<i>recG</i>			651			
<i>IF</i>	CTTTAATTGAAAGCTGGATATC			21	917	53.5
<i>IR</i>	CAAGTTGCAITTTGGACAATC	722		20	1639	54.3
<i>OF</i>	CCCTTGTTCCTTGCCTTC			19	890	58.4
<i>OR</i>	GAAAGTCCAAAACGCTCAG	804		19	1694	55.2
<i>clpX</i>			624			
<i>IF</i>	AATGTGCCATTTGCAATAGC			20	403	54.3
<i>IR</i>	TTAAGAAAGACCCCTCTAAAATAG	721		22	1124	54.7
<i>OF</i>	GCTGCAGAGATGAATGTGCC			20	391	50
<i>OR</i>	GATTGATTTTCATATATAACTCTTTTG	859		24	1250	55
<i>pepX</i>			570			
<i>IF</i>	TTATTCCAAACCTTGCAATCC			20	449	55.5
<i>IR</i>	TGTGCCTGAAGGAACATTTG	648		20	1097	56

Gene	5'-3' primer sequence	Amplicon size (bp)	Size of fragment used for MLST (bp)	Primer length (bp)	Primer start position in B31 genome	T _m (°C)
OF	same as inner forward					
OR	GTTCCAATGTCAAATAGTTTC	791		20	1153	52,3
<i>uvrA</i>			570			
IF	GCTTAAATTTTAAATTGATGTTGG			24	1434	55
IR	CCTATTGGTTTTTTGATTTATTG	677		23	2111	53,9
OF	GAAATTTTAAAGGAAATTAAGTAG			26	1408	55,2
OR	CAAGGAACAAAAACATCTGG	891		20	2299	54,3
<i>rplB</i>			624			
IF	CGCTATAAGACGACTTTATC			19	40	54,3
IR	same as outer reverse	703				
OF	TGGGTATTAAAGACTTATAAGC			21	2	
OR	GCTGTCCCCAAGGAGACA	741		18	743	

^aThese primers (often supplied as 100 pmol/μl stocks) should be diluted to a concentration of approximately 5 pmol/μl.

^bIF = inner forward; IR = inner reverse; OF = outer forward; OR = outer reverse.

Table 12C.5.5**Amplification Protocol for Touch-Down PCR**

a.	<i>Taq</i> activation/initialization	95°C	15 min ^a	
b.	Denaturation	94°C	15 sec	9 cycles
c.	Annealing	Individual, see Table 6	30 sec	
d.	Extension	72°C	60 sec	
e.	Denaturation	94°C	15 sec	30 cycles
f.	Annealing	Endpoint temperature	30 sec	
g.	Extension	72°C	60 sec	
h.	Final Extension	72°C	5 min	
i.	Hold	12°C	-	

^aThis time is required for Qiagen HotStarTaq; other *Taq* polymerases may require different lengths of time for activation, which will be given by the manufacturer.

Table 12C.5.6**Primer Annealing Touch-Down PCR**

<i>clpA, clpX, nifS, pepX, pyrG, rplB, uvrA</i>	55°-48°C	30 sec/cycles
Or as an alternative (except <i>clpA</i>): <i>clpX, nifS, pepX, pyrG, rplB, uvrA</i>	58°-50°C or 60°-52°C	30 sec/cycles

Table 12C.5.7Amplification Protocol for *recG*

a.	<i>Taq</i> activation/initialization	95°C	15 min	
b.	Denaturation	94°C	15 sec	40 cycles
c.	Annealing	55°C	30 sec	
d.	Extension	72°C	60 sec	
e.	Final Extension	72°C	5 min	
f.	Hold	12°C	—	

Table 12C.5.8

Simplified (Semi-) nested PCR Protocol

a.	<i>Taq</i> activation/soak	95°C	15 min	
b.	Denaturation	94°C	15 sec	35 cycles
c.	Annealing	50°C	30 sec	
d.	Extension	72°C	60 sec	
e.	Final extension	72°C	5 min	
f.	Hold	12°C	—	