Inoculation of mice

Note: Groups of at least 6 mice should be used in order to determine statistical significance between groups.

1. Grow low passage, infectious *B. burgdorferi* clones from freezer stock to mid-log phase.

2. Determine the density of the culture using a Petroff-Hausser counting chamber.

3. Obtain enough mice (C3H/HeN, Harlan Sprague-Dawley) to have 6 mice per strain of *Borrelia*. Prebleed approximately 100 µl of blood from each mouse. Centrifuge blood at 9000 rpm for 7 minutes at 4ºC. Pipet off the serum (upper, yellowish layer) and transfer it to a clean microcentrifuge tube. Store prebleed sera at -20ºC.

4. **For intraperitoneal/subcutaneous inoculation** each mouse will receive an inoculum of $5x10^3$ spirochetes. Dilute each culture in BSK-H to a working concentration of $2x10^4$ spirochetes/ml. Needle inoculate mice, using a 25G 5/8 needle, with 250 µl (200 µl intraperitoneal, 50 µl subcutaneous). This results in a final dose of $5x10^3$ spirochetes per mouse.

5. **For intradermal inoculation** each mouse will receive an inoculum of $5x10^3$ spirochetes. Shave the backs of the mice, dilute the inocula to a working concentration of $5x10^4$ spirochetes/ml and inject 50 µl under the skin in two different locations on the backs of the mice (100 µl total), using a 26G 3/8 intradermal bevel needle. This results in a final dose of $5x10^3$ spirochetes per mouse.

NOTE: We typically use an inoculum of $5x10^3$ spirochetes. However, the amount of the inocula can be altered appropriate to the experiment.

6. It is extremely important to analyze the *Borrelia* cultures used for the inocula to determine the actual number of spirochetes delivered to the mice as well as the plasmid content of the cultures for every experiment. Dilute inoculum cultures to a concentration of $1x10^3$ spirochetes/ml and plate 100 µl in solid BSK medium twice per culture. Once the colonies appear on the plates perform colony forming unit counts. Given that 100 µl of a $1x10^3$ spirochetes/ml culture were plated you expect to find 100 colonies per plate. If this is not the case recalculate the amount of the inocula based on the number of colonies observed. This is the actual dose given to the mice. The plasmid content of the inocula is determined by two methods. (1) Screen 10-20 colonies per inoculum by PCR (see protocol for Bb colony PCR) for the presence of the virulence plasmids lp25, lp28-1, lp36 and shuttle vector, if applicable. This provides a measure of the percent of the individuals in the population that harbor all of these plasmids, which are essential for infectivity and gives you a measure of what fraction of the population is infectious. (2) Make genomic DNA from the remainder of the inocula and screen the bulk population for total plasmid content using the Akins primer set (Elias, 2002) in order to verify that all of the inocula contain the expected panel of plasmids.

7. Bleed (collect at least 100 µl of blood) and sacrifice mice 3 weeks post inoculation (this time can be varied appropriate to the experiment). Isolate mouse sera as described in #3 above.

8. Analyze mouse serum for seroconversion of antibodies against *Borrelia* proteins. Prepare 12.5% SDS-PAGE gels with enough wells to run three lanes per mouse serum to be analyzed. Protein samples include 1. total protein lysate from *E. coli* (negative control), 2. total protein lysate from *E. coli* expressing recombinant *Borrelia* antigen P39 (a *Borrelia* protein expressed during mammalian infection), 3. total protein lysate from wild type, low-passage *Borrelia* (10 µl of 10^6 spirochetes/µl). Transfer proteins to a nitrocellulose membrane. Stain membranes with Ponceau Red in order to visualize the protein in the different lanes and using a razor blade cut the membranes into strips containing the three protein samples described above (three gel lanes). Rinse membranes with deionized water to remove the Ponso Red and block membranes for 1 hour at room temperature with 5% skim milk in TBS-Tween. Dilute mouse sera to 1:200 in 1 ml of TBS-Tween. Label each membrane strip with a mouse number and incubate each strip with the diluted serum from the corresponding mouse in a seal-a-meal bag for 1 hour at room temperature. Remove the membranes from the seal-a-meal bags and wash the membranes in bulk 2x 5 minutes with TBS-Tween. Incubate membranes in bulk with antimouse secondary antibody for 1 hour at room temperature. Wash the membranes in bulk 2x 10 minutes with TBS-Tween. Develop western blot and determine whether or not each individual mouse is infected by seroreactivity to the *Borrelia* proteins.

9. Harvest mouse tissues ear, bladder and joint from sacrificed mice for reisolation of spirochetes from infected tissues. Harvest the second ear, heart and second joint tissues and store at -80°C for subsequent isolation of spirochete DNA from infected tissues. Surface sterilize ear and joint tissues and place the tissues in individual tubes containing 10 ml of BSKII + *Borrelia* antibiotics (see *Borrelia* recipes). Place the bladder tissues (without surface sterilization) in individual tubes containing 10 ml of BSKII + *Borrelia* antibiotics (see *Borrelia* recipes). Allow cultures to grow at 35ºC for approximately one week. Analyze cultures for the presence of spirochetes (reisolation positive) under a dark field microscope.

10. Alternatively, mouse infection can be assessed by serology (as described in #8) and reisolation of spirochetes from a small piece of ear tissue sampled using an ear punch. Surface sterilize the ear punch and place in 10 ml of BSKII + *Borrelia* antibiotics and look for spirochete growth (as described in #9). This method does not require that the mice be sacrificed.

11. Compare the number of infected mice in one group to another using an appropriate method of statistical analysis.

Determination of infectious dose (ID_{50})

Follow protocol for inoculation of mice. However, inject groups of at least 6 mice with serial dilutions of inocula (*i.e.*, 10-fold, 5-fold, 2-fold) that are estimated to flank the number of spirochetes required to infect 50% of the mice in a single group. At least 4 different doses should be used for accurate calculation of the ID_{50}. Determine the number of infected mice and calculate the ID_{50} according to the method of Reed and Muench (1938), see example below.

Dose	Number of mice uninfected	Number of mice infected	Total		Percent infected[c]
			Uninfected[a]	Infected[b]	
10^4	6	0	22	0	0
10^5	5	1	16	1	6
10^6	6	0	11	1	8
10^7	3	3	5	4	**44**
10^8	2	4	2	8	**80**

[a]The sum of all of the uninfected mice at that dose and higher.
[b]The sum of all of the infected mice at that dose and lower.
[c]The total infected divided by the sum of the total uninfected and total infected multiplied by 100.

Using the numbers in the table above perform the following calculations:

$$\frac{\text{50-44 (the percent infected below 50\%)}}{\text{80 (the percent infected above 50\%)-44 (the percent infected below 50\%)}}$$

$$=.17$$

$\log 10^7$ (dose at which less than 50% of the mice become infected)

$$=7$$
$$.17+7=7.17$$

inverse log7.17
$ID_{50}=1.5x10^7$