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Identification of *Borrelia burgdorferi ospC* genotypes in canine tissue following tick infestation: Implications for Lyme disease vaccine and diagnostic assay design

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ABSTRACT

In endemic regions, Lyme disease is a potential health threat to dogs. Canine Lyme disease manifests with arthritis-induced lameness, anorexia, fever, lethargy, lymphadenopathy and, in some cases, fatal glomerulonephritis. A recent study revealed that the regional mean for the percentage of seropositive dogs in the north-east of the USA is 11.6%. The outer surface protein C (OspC) of Lyme disease spirochetes is an important virulence factor required for the establishment of infection in mammals. It is a leading candidate in human and canine Lyme disease vaccine development efforts. Over 30 distinct *ospC* phyletic types have been defined. It has been hypothesized that *ospC* genotype may influence mammalian host range. In this study, *Ixodes scapularis* ticks collected from the field in Rhode Island were assessed for infection with *B. burgdorferi*. Ticks were fed on purpose bred beagles to repletion and infection of the dogs was assessed through serology and PCR. Tissue biopsies ($n = 2$) were collected from each dog 49 days post-tick infestation (dpi) and the *ospC* genotype of the infecting strains determined by direct PCR of DNA extracted from tissue or by PCR after cultivation of spirochetes from biopsy samples. The dominant *ospC* types associated with *B. burgdorferi* canine infections differed from those associated with human infection, indicating a relationship between *ospC* sequence and preferred host range. Knowledge of the most common *ospC* genotypes associated specifically with infection of dogs will facilitate the rational design of OspC-based canine Lyme disease vaccines and diagnostic assays.

Introduction

Borrelia burgdorferi, the primary causative agent of Lyme disease in North America, is transmitted to mammals through the bite of *Ixodes* spp. ticks (Burgdorfer et al., 1982; Benach et al., 1983). In 2009, nearly 38,000 cases of confirmed and probable human Lyme disease were reported in the USA.[1] In Europe, additional *Borrelia* spp., including *B. garinii*, and *B. afzelii*, have been identified and demonstrated to cause Lyme disease (Baranton et al., 1992; Marconi and Garon, 1992). While the incidence of Lyme disease is thought to be higher in Europe, estimates of case numbers are not known accurately due to a lack of uniform reporting criteria. Lyme disease is a potential health threat to dogs in both North America and Europe (Krupka and Straubinger, 2010; Little et al., 2010). In dogs, Lyme disease may manifest with arthritis-induced lameness, anorexia, fever, lethargy, lymphadenopathy and, in some cases, fatal glomerulonephritis (Little et al., 2010). A recent study revealed that the percentage of seropositive dogs in the Northeast states of the USA ranges from 7.1% in New York to 19.8% in Massachusetts, with a regional mean of 11.6% (Bowman et al., 2009). In the Midwest of the USA, this value ranges from <1% in several states to 10.2% in Wisconsin, with a regional mean of 4.0% (Bowman et al., 2009).

Strategies to prevent infection in dogs have focused on acaricides and vaccination. Acaricides are effective at decreasing tick burdens, but do not provide complete protection and, depending on delivery approach, may require frequent (monthly) applications. While several Lyme disease vaccines have been licensed for use in dogs, the broad protective efficacy and ability to induce long term protective immunity is low (Littman et al., 2006; Earnhart and Marconi, 2008; Marconi and Earnhart, 2010). In a recent study, a multi-strain *B. burgdorferi* bacterin vaccine provided protection against tick challenge for 1 year (LaFleur et al., 2010). However, *Borrelia* spp. based bacterin vaccines are expensive to produce and it is difficult to maintain consistent quality. There is a clear

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and pressing need for development of subunit vaccines for canine Lyme disease.

Outer surface protein C (OspC) is a 22 kDa surface-exposed lipoprotein (Fuchs et al., 1992) encoded by a stable and universal 26 kb circular plasmid (Marconi et al., 1993a,b; Sadziene et al., 1993). OspC is an essential virulence factor that has been postulated to participate in interactions with tick or host-derived ligands at the tick–host interface that are required for the establishment of infection (Hovius et al., 2008; Earnhart et al., 2010; Onder et al., 2012). OspC elicits robust and protective antibody responses, making it an attractive candidate for vaccine development (Earnhart and Marconi, 2008; Marconi and Earnhart, 2010).

A challenge encountered in efforts to develop OspC as a vaccine is its inherent genetic diversity (Theisen et al., 1993, 1995; Wilske et al., 1993, 1996; Lagal et al., 2006; Earnhart and Marconi, 2007c). Over 30 distinct *ospC* phyletic types have been identified (Wang et al., 1999; Seinost et al., 1999a; Brisson and Dykhuizen, 2004; Earnhart and Marconi, 2007c). Immune responses elicited by OspC protective epitopes are 'phyletic type' specific (Earnhart et al., 2005; Buckles et al., 2006; Earnhart and Marconi, 2007b). Hence, a broadly protective OspC based vaccine must include protective epitopes derived from *ospC* variants most commonly associated with mammalian infection (Earnhart and Marconi, 2007a). The use of cocktails consisting of multiple recombinant OspC proteins has not proven effective, possibly due to misdirection of immune responses to immunodominant epitopes that do not elicit protective antibody responses (Earnhart and Marconi, 2008; Marconi and Earnhart, 2010). Chimeric recombinant proteins offer a conceptually promising approach for the development of broadly protective vaccines (Earnhart and Marconi, 2007a). An octavalent recombinant chimeric protein that consists of linear epitopes derived from the loop 5 and α helix 5 domains of OspC elicited bactericidal antibody against strains producing OspC type proteins represented in the vaccine construct (Earnhart et al., 2007; Earnhart and Marconi, 2007b,c). This finding indicates that a chimeric approach to Lyme disease vaccine development is possible.

To facilitate the rational design of a cost effective, broadly protective, OspC based vaccine intended for use in dogs, it is essential to identify the *ospC* genotypes most commonly associated with strains that successfully infect dogs. Towards this goal, field caught *Ixodes scapularis* ticks collected from Rhode Island were fed on laboratory raised dogs, tissue biopsies were collected and the *ospC* genotypes of strains present in tissue and tissue-derived cultures were determined. The results demonstrate that unique *ospC* types rarely recovered from human Lyme disease patients predominate in canine infections. This observation indicates that a chimeric OspC based Lyme disease vaccine intended for use in dogs must consist of different component epitopes than a vaccine intended for use in humans. The data have significant implications for the design of a broadly protective OspC based vaccine for use in dogs.

Materials and methods

Tick collection and analysis

Adult *I. scapularis* ticks were collected in southern Rhode Island in the spring of 2008 by flagging. The percentage of ticks infected with *B. burgdorferi* was determined by direct fluorescent microscopy using standard methods and labeled anti-*B. burgdorferi* antibody (Nicholson et al., 1996).

Infestation of dogs with field caught ticks

All procedures were conducted in compliance with regulations of the Animal Welfare Act. The study protocol (#KZ-1201e-2005-08pxm) was reviewed and approved (16 May 2008) by Pfizer's Animal Care and Use Committee prior to the start of the study. Fifteen purpose-bred beagles of both sexes (7 males, 8 females; 9–10 weeks of age; Marshall Bioresources) were assigned identification numbers and divided into four groups, designated T01 ($n = 4$), T02 ($n = 4$), T03 ($n = 4$) and T04 ($n = 3$). The dogs were fitted with Elizabethan collars and housed in one-over-one condominium style cages. The dogs were acclimatized to the collars by having them wear the collars for increasing amounts of time over several days.

Thirteen days prior to tick infestation, serum was collected from each dog. Dogs in groups T01, T02, T03 and T04 were infested with 0, 25, 50 or 75 adult *I. scapularis* ticks, respectively, using secured infestation chambers placed on each side of the mid-thorax. The chambers were adhered using Elastikon (Johnson and Johnson). To ensure that the dogs did not interfere with the chambers, each was fitted with a lightweight jacket. Ticks were fed to repletion, removed and serum samples (5 mL) collected on days −13, 21, 35, 49, 63, 77 and 92. Skin biopsies (2 mm) were collected at 49 dpi. Seroconversion was assessed with the 4DX SNAP test (IDEXX) and C6 titers determined by IDEXX using a semi-quantitative ELISA (Lyme Quant C6 Test).

To cultivate spirochetes from infected dogs, approximately half of each 2 mm skin biopsy was placed in 6 mL BSK-H media (Sigma) supplemented with 6% rabbit serum (Sigma) and *Borrelia* antibiotic cocktail (phosphomycin, amphotericin B and rifampicin; Sigma) and the cultures maintained at 37 °C under 5% CO_2. Clonal populations were obtained from the cultures by sub-surface plating (Sung et al., 2001). Colonies were excised from the plates and placed in BSK-H media for cultivation. The uncloned isolates recovered from the tissue biopsies were designated as DRI, indicating that they originated from dogs (D) that were infected using ticks collected in Rhode Island (RI). DRI is followed by a portion of the identifier number that was assigned to each dog. Lower case letters that follow the number indicate a specific clone derived from the uncloned isolate by subsurfacing plating as detailed above.

DNA extraction and PCR

DNA was extracted from the remaining half of each skin biopsy using the Qiagen DNeasy Kit and from cultures of *B. burgdorferi* clonal populations as described previously (McDowell et al., 2001). The *ospC* and *flaB* genes were amplified by PCR using DNA (100 ng) extracted from tissues and from the supernatant of boiled *B. burgdorferi* cell lysates. PCR was performed using standard conditions, GoTaq polymerase (Promega) and the following primers; *ospC*-F1 5′-GACGACGACAAGATTGAATACATTAAGTGCAATATTAATGAC-3′ and *ospC*-R1 5′-GAGGAGAAGCCCGGTTTACAAATTAATCTTATAATATTGATCTT AATTAAGG-3′. The primers used to amplify *flaB* have been described previously (Zhang and Marconi, 2005). The amplicons were assessed by agarose gel electrophoresis and ethidium bromide staining.

The PCR products obtained from amplification of DNA extracted from tissues were excised from the gels using the Qiagen Gel Extraction Kit, cloned into the pET46EK/LIC vector (Novagen) and the plasmids were propagated in *Escherichia coli* NovaBlue cells (Novagen). The resulting *E. coli* colonies were screened for *ospC* by PCR using cell lysates obtained by boiling *E. coli* colonies in water. Portions of the *ospC* PCR positive colonies were transferred into Luria–Bertani broth media (2 mL) and grown overnight. Cells were harvested by centrifugation and plasmids were extracted using the Qiagen MiniPrep kit. PCR amplicons obtained by amplification of *ospC* from *B. burgdorferi* colonies were directly sequenced after purification and were not cloned into a plasmid.

Phylogenetic analysis

DNA sequencing was performed by Eurofins MWG Operon. Phylogenetic analyses were conducted as described previously (Earnhart and Marconi, 2007c). Neighbor joining trees were generated using ClustalX 2.0.10 software in the multiple alignment mode with the default settings and a Gonnet matrix, and were visualized using N–J Plot version 2.2. Previously determined sequences of known *ospC* type were included in the analysis in order to assign type designations to the sequences determined in this study.

Results

Analysis of the prevalence of B. burgdorferi in ticks collected from Rhode Island

Using a standardized direct fluorescent microscopy method (Nicholson et al., 1996), 52% of the *I. scapularis* ticks field-collected in southern Rhode Island during the Spring of 2008 were infected with *B. burgdorferi*. Tick infection rates (TIRs) for Spring-collected adult female *I. scapularis* collected at the same set of sites in southern Rhode Island ranged from 50% to 70% from 2004 to 2009 (average 55.4%). Relatively high TIRs in nymphal (~25%) and adult (55%) *I. scapularis* and abundance levels of both developmental stages are consistent with the high incidence of human Lyme disease cases in Rhode Island (~17 per 100,000 people statewide from 2006 to