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Quantitative analysis of *Borrelia burgdorferi* gene expression in naturally (tick) infected mouse strains

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Abstract Adaptation of *Borrelia burgdorferi* in the vector and vertebrate host is mediated by mechanisms that regulate differential expression of outer surface lipoproteins (Osps). In this study, real time PCR was applied to quantify tissue-specific expression of four linear plasmid (lp54)-encoded (*ospA, zs7.a36*, *zs7.a66 zs7.a68*) and one circular plasmid (cp26)-encoded (*ospC*) gene from *B. burgdorferi* sensu stricto, in a natural setting of tick-infected immunodeficient (C.B-17 SCID) and immunocompetent (BALB/c and AKR/OlaHsd) mice for up to 120 days post-infection (p.i.). Early during infection (day 30 p.i.) high numbers of spirochetes were found in the heart and joint, but not the ear and spleen tissues of disease-susceptible SCID mice. In disease-susceptible AKR mice spirochetes colonized the ear and joint tissues, but were undetectable in tissues of disease-resistant BALB/c mice. Later in infection (day 120 p.i.), spirochetes had expanded (~1,000-fold) in all SCID tissues tested but were undetectable in AKR and BALB/c mice. Of the five genes analyzed, only *zs7.a36* transcripts were detected in various tissues of all infected mouse strains, though at differing levels, whereas *ospC* transcripts were only found in tissue specimens of SCID mice. Furthermore, gene expression of *ospC* and *zs7.a36* appears to be differentially regulated in distinct organs of individual mice. In contrast, transcripts for *ospA*, *zs7.a66*, and *zs7.a68* were not detected in any of the mouse strains, independent of their immune status and/or the severity of their infection/inflammatory responses. Late during infection (day 120 p.i.), transcription of *zs7.a36* and *ospC* was down-regulated in the tissues of SCID mice despite expansion of spirochetes. This type of quantitative analysis may be helpful to further disclose principles of pathogenesis of Lyme borreliosis and to design strategies for its therapeutic treatment.

Keywords *Borrelia burgdorferi* · Tick infection · Gene expression · Tissue tropism

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Introduction

Lyme disease in humans is caused by three genospecies of the *Borrelia burgdorferi* sensu lato (*B.b*.s.l.) complex; *B.b*. sensu stricto (s.s.), *B. afzelii*, and *B. garinii*. Co-evolution of this tick-borne spirochete and mice, its main natural reservoir host [3], have led to a steady state between pathogen and mammals that is characterized, in most cases, by persistence of *B. burgdorferi* and lack of disease symptoms in the carrier [57]. There is increasing evidence that the pathogen has evolved various strategies to evade innate and adaptive immunity, including binding of host-derived factors, such as plasminogen [21] and complement inhibitors [31], differential up- and down-regulation of outer surface proteins (Osp) [2, 15, 38,57] and antigenic variation [65]. In fact, recent data suggest that specific antibodies to OspC may select for surface-antigen non-expressors thereby contributing to spirochetal persistence in mice [36]. However, many *osp* genes, which are expressed in spirochetes seeding unfed ticks, e.g. *ospA* and *zs7.a68*, *lp6.6* seem to be inactive in mammals, independent of the immune status of the host [16, 34, 41, 56, 61,67]. Other spirochetal genes, e.g. *ospC*, *bbk32*, *bbk50*, and *zs7.a36*, are likely to be expressed by spirochetes during the entire enzootic cycle, including the arthropod vector and the mammalian host [19, 56, 61,67]. Yet another group of *B. burgdorferi* genes, e.g. *eppA, pG, p21, bbK2.10*, and *p35,* seem to be selectively

expressed in the mammalian environment, at least temporarily [1, 2, 10, 14, 15, 59,60].

To date there is little information available on whether the various in vivo expressed spirochetal surface antigens are suitable candidates for immunotherapy. The feasibility of targeting and eliminating spirochetes during chronic infection in mice is indicated by our recent findings that passive transfer of OspC-specific Ab leads to resolution of chronic *B. burgdorferi* s.s. infection, elicited by either experimental or tick challenge in immunodeficient and -competent mice [66,67]. However, such an approach may be invalidated by the selection of variants that do not express OspC under different conditions [36]. Moreover, the recent finding that upon feeding, homogeneous populations of *B. burgdorferi* within the tick gut give rise to transmittable progenies with extensive antigenic heterogeneity adds a further dimension to the problem of immune surveillance [44]. It is thus clear that only detailed knowledge of the regulation of *B. burgdorferi* gene expression in situ will help to elucidate those structures, which are integral to spirochetal persistence and thus of relevance for the development of therapeutic regimens.

To date, all studies but one [64], in which *B. burgdorferi* s.s. was quantified in mouse tissue samples, were pursued with needle-inoculated recipients [42, 45,62], and only in one case was *B. burgdorferi* gene expression examined in skin specimens of naturally (tick) challenged C3H mice for up to 2 weeks [28]. In light of the notion that needle exposure by no means reflects the situation of tick infection [23, 25,54] and the highly improved real-time PCR technique to quantify *B. burgdorferi* in situ [24, 27, 43, 46, 48, 62, 63,64], we have now performed a detailed quantitative analysis of the spirochete burden and population dynamics in different tissues of mice following natural (tick) infection by *B. burgdorferi* s.s. (ZS7) for a period of 4 months post-infection (p.i.). For this purpose we have selected one cp26-encoded gene, *ospC* [40], and four previously described lp54-encoded genes, i.e. *ospA*, *zs7.a36, zs7.a66,* and *zs7.a68* [20,61]—the respective antigens of which are suggested to be either putative vaccine candidates [61,66] and/or relevant for complement resistence [32]—and three strains of mice with distinct immune status and susceptibility to *B. burgdorferi* s.s.-induced disease/infection [50,53].

Materials and methods

Mice, spirochetes, ticks and infection

Adult female mice of strains C.B-17 SCID (H-2^d, SCID), AKR/OlaHsd (H-2^k, AKR), and BALB/c (H-2^d) were bred under specific pathogen-free conditions at the Max-Planck-Institut für Immunbiologie, Freiburg, Germany. Animals between 6 and 8 weeks of age were used throughout the experiments. For tick-mediated infection of mice, 10–12 *Ixodes ricinus* nymphs, previously fed on ZS7 *B. burgdorferi* (s.s.) infected mice (L. Gern, Neuchâtel, Switzerland) [22], were let feed on each mouse until saturation (4–6 days). The first day of feeding was considered day one of the infection. Mice were monitored for clinical symptoms (arthritis), blood was taken on days 30 or 60 for serological analysis and organs were removed on days 30 or 120 from the respective infected mice, including from uninfected controls, for DNA/RNA isolation, as described.

Serology

ELISA was used to quantify *B. burgdorferi*-specific antibodies in infected mice. Plates were coated with *B. burgdorferi* ZS7 sonicate [50] at 10 μg/ml. A standard curve was generated with mAb LA2 (IgG2b) [33] at 1 mg/ml and the second Ab goat anti-mouse Ig-AP (SBA-Birmingham, Ala.) at 1:2,000. Mouse sera were tested at dilutions of 1:50 and incubated 2 h at room temperature.

DNA isolation from murine tissue

For genomic DNA isolation, tissue pieces (30–50 mg, equivalent to 1/2 heart, 1/3 spleen, whole ear or joint) were added to 1,000 μl 0.1% (1 mg/ml) collagenase A (Roche) in PBS, and incubated 4–6 h at 37°C, with shaking. An equal volume of 0.2 mg/ml proteinase K (Gibco BRL) was added in the following buffer: 200 mM NaCl, 20 mM TrisHCl pH8, 50 mM EDTA pH8, plus 1% SDS. Samples were incubated overnight at 55°C, with occasional shaking. DNA was recovered with 1.7 μl glycogen (Boehringer Mannheim, 20 μg/μl stock solution) and an equal amount of isopropanol and let precipitate at −80°C. All samples were washed twice with 80% EtOH. Pellets were resuspended in 180–360 μl molecular biology-grade water (Eppendorf). The DNA concentration of each sample was determined by photometric analysis. This protocol generated higher yields of *B. burgdorferi*-specific DNA than compared to phenol/chloroform extractions (data not shown).

RNA isolation from murine tissue

Thirty to 50 mg of murine tissues were added to 1.5 ml cold RNAzol (WAK-Chemie) and RNA was isolated according to the manufacturer's recommendations. RNA was treated with DNAse I (Roche) for 30 min at 37°C and cleaned according to the Phase Lock Gel Heavy (Eppendorf) tube protocol. Concentrations were measured photometrically.

Real-time PCR

Real-time PCR using a LightCycler was carried out as described by Roche Molecular Biochemicals with 18 µl SYBR Green I Master Mix and 2 µl, at various DNA concentrations, of sample. Average DNA yields from the tissues of infected mice were: heart, 5.5×10^5 ng; spleen, 1.8×10^6 ng; ear, 2.3×10^5 ng; joint, 4.5×10^5 ng. The reaction conditions with primers for *zs7.a66* and *zs7.a68* were as follows: initial denaturation at 95°C for 10 min then 45 cycles at 95°C for 15 s, 55°C 5 s, 72°C 10 s; melting curve at 95°C 0 s, 65°C 15 s, 95°C 0 s at 0.1C/s; cooling at 40°C 30 s. The annealing conditions were altered to 56°C for 8 s for *zs7.a36* and hypoxanthine-guanine phosphoribosyltransferase (*hprt*), and to 50°C for 8 s for *ospC*. The elongation time for the latter primers was increased to 15 s. For OspA, the annealing conditions were 55°C for 8 s with a 25 s elongation time; for flagellin, the annealing step was also at 55°C for 10 s, and the elongation at 72°C for 11 s. The following plasmids were used as standards: pGEX-ZSA 36#1 containing *zs7.a36* inserted into pGEX-2T (Promega) (*Bam*H1/*Eco*R1); pGEX-ZSA66(2)#20 with *zs7.a66* inserted into pGEX 2T (*Bam*H1/*Eco*R1); pGEX-ZSA68(2)#7 with *zs7.a68* inserted into pGEX-2T (*Bam*H1/*Eco*R1); pUEX-Spiro with *ospC* (without leader) inserted into pGEX-2T (*Bam*H1/*Eco*R1). pUEX-Spiro#9 containing *flagellin* inserted into pUEX1(Amersham) (*Bam*H1); pBOS-OspA#3 containing *ospA* inserted into pEF-BOS (*Bst*X1). Purification was carried out using the Qiagen plasmid purification kit according to the high-copy plasmid protocol.

For control experiments to test real-time PCR sensitivity (see Table 1): serial dilutions of cultured spirochetes (from 10^5–100) were mixed with 30–50 mg of mouse tissue (heart, spleen, ear, or joint) and processed for DNA as described. Total DNA yields per whole tissue (average whole tissue weights: heart, 75 mg; spleen, 100 mg; ear, 30 mg; joint, 50mg) were as follows: heart, 2.4×10^5 ng; spleen, 1.9×10^6 ng; ear, 1.4×10^5 ng; joint, 3×10^4 ng. Tissue DNA was then diluted to a range of 10–100 ng/µl for real-time PCR detection of *B. burgdorferi*. Copy sensitivity limits correspond to number of theoretical *B. burgdorferi* present (after dilution) per PCR reaction or ng tissue DNA.

Table 1 Sensitivity of primer pairs for detection of *Borrelia burgdorferi* genes in plasmid DNA. The copy number is given as the lowest reliable gene copy number per PCR (2 µl sample)

B. burgdorferi gene	$MgCl_2$ (mM)	Fragment size (bp)	Plasmid standard	Copy number
Zs7.a36	3	260	pGEX-ZSA36#1	30
Zs7.a66	3.5	348	pGEX-ZSA66#20	300
Zs7.a68	3.5	180	pGEX-ZSA68#7	3
OspC	3	300	pGEX-ZSC#1	17
OspA	4	578	pbos OspA#3	30
Fla	3	240	pUEX Spiro#9	2

Primers

Primers of the following sequences were obtained from BIG Biotech GmbH: for *zs7.a36,* reverse 5′ aaagagatttctttaactcccg 3′, forward 5′ aagattgttgctcagtgggg 3′ for a 260 bp fragment. For *zs7.a66,* 5′ gtgtgttagagttaagctgtgg 3′, 5′ ttcaagccgttacaaccgtacc 3′ for a 348 bp fragment. For *zs7.a68,* 5′ catcaataagatcgtaaggacc 3′, 5′ tctggagaccttagca cttctg 3′ for a 180 bp fragment. For *ospC,* 5′ ctgatgagtctgttaaagggcc 3′, 5′ gcttcagagcatttcttagctg 3′ for a 300 bp fragment. For *ospA*, 5′ tgtaagcaaaatgttagc 3′, 5′ aagttcaactgaaacttccc 3′ for a 578 bp fragment. For *flagellin*, 5′ gggtacagaattaatcgagc 3′, 5′ tgtatagaacctctgtctgc 3′ for a 240 bp fragment. For *hprt,* HPRT 1 5′ gctggtgaaaaggacctct 3′, HPRT 2 5′ cacaggactagaacacctgc 3′ for a 250 bp fragment.

Quantitative real-time PCR assay

The real-time RT-PCR assay used the double-stranded DNA-specific dye SYBR Green I. First-strand complementary DNA synthesis was performed on 3–30 µg of DNAse I-treated total RNA with random hexamers and AMV RT, using a first strand RT kit (Roche). Amplification of the *hprt* gene on cDNA from mouse mRNA was used as a control for total RNA extraction and for the standardization of the results of target gene transcriptional activity. The quantification of each *B. burgdorferi* gene expression was performed on 20 ng of cDNA preparations. At least two aliquots from each mouse/tissue were tested in duplicate or triplicate in at least two separate PCR reactions. Errors are given between separate cDNA dilutions tested on different days. The corresponding RNA samples not previously subjected to reverse transcription were also amplified to measure the amount of contaminating chromosomal DNA. For all genes tested, no contamination by genomic DNA was detectable after DNAse I treatment of RNA, indicating specific detection of *B. burgdorferi* cDNA (data not shown). For the determination of *B. burgdorferi* gene expression in mouse tissues, the results were standardized against *hprt* expression. Trace detection is indicated as <0.1 copies/100HPRT.

Quantification of gene copy numbers

Quantitative analysis was performed using the LC software (Roche Diagnostics). The generation of quantitative data was based on the different PCR kinetics of samples with different levels of target genes. We used a relative quantification in which the expression levels of *B. burgdorferi* target genes were compared to the data from a standard curve. Calculations were based on following data: 1 µg of 1.000 bp ds DNA $=1.52$ pmol. To convert µg to pmol (for ds DNA) the following formula was used: $\mu g \times 10^6$ pg/1 µg $\times$ pmol/660 pg $\times$ $1/N$ = pmol, where N is the number of nucleotides and

Table 2 Sensitivity of real-time PCR. Detection limits for *B. burgdorferi* gene *zs7.a68*. Each PCR reaction contained 2 µl of sample at 10–100 ng/µl mouse tissue DNA. *N/A* Not applicable

Test material	Tissue	Copy sensitivity for quantification/PCR	Copy sensitivity/ng tissue DNA
pGEXZA68 DNA	–	≥3	N/A
ZS7 *B. burgdorferi*spirochetes	–	≥10	N/A
B. burgdorferi ($1–10^3$/PCR)	Heart	≥160	≥1
Plus unprepared tissue	Spleen	Not detected	Not detected
–	Ear	≥148	≥1
–	Joint	≥680	≥7

330 pmol/pg is the average MW of a nucleotide. The amount of chromosome equivalent per microliter was calculated considering the length of the *B. burgdorferi* chromosome and plasmids, 1.5 Mb [20], and assuming one copy per genome for the four genes analyzed. Copy numbers quantified are averages from tests of at least three concentrations of each DNA sample, tested in at least two separate PCR reactions. No spirochetal DNA could be amplified in tissues from uninfected control mice.

B. burgdorferi culture and harvesting

Low passage ZS7 *B. burgdorferi* (third) were grown in BSK-H (Sigma) medium and incubated at 32°C at 5% CO_2. For all control experiments, *Borrelia* were grown for 96 h to the exponential stage, visualized by dark field microscopy, counted with a Neubauer-improved counting chamber, harvested by centrifugation at 10,500 *g*, 20°C, for 20 min, and resuspended in cold PBS.

Results

Sensitivity and specificity of real-time PCR for detection of *B. burgdorferi* DNA

To define optimal conditions for the quantification of *B. burgdorferi* DNA by real time PCR, a set of primer pairs specific for lp54-encoded *ospA*, *zs7.a36, zs7.a66, zs7.a68,* and cp26-encoded *ospC* from strain ZS7 were tested on plasmids harboring the respective genes by using various experimental protocols adapted from previous studies [7, 39, 47, 48,62]. Highest sensitivity was obtained for *zs7.a68* with a detection limit of about three copies/PCR (Table 1, Fig. 1). Plotting the C_t values relative to the number of individual plasmids resulted in a linear correlation with a coefficient (r) value of >0.98 for all genes (Fig. 1B, shown only for pGEX-ZSA68). Applying genomic DNA of *B. burgdorferi* ZS7 under similar conditions, ≥10 copies of *zs7.a68* could be detected with high reproducibility (Table 2), which is in line with previous reports [62, 63,64]. Due to the notion that mutants of *B. burgdorferi* lacking the lp54 plasmid rarely occur, if at all, in nature [30,49] and the assumption, derived from the genomic sequence of *B. burgdorferi* B31 [20], that there is only one copy of *zs7.a68* in the spirochetal genome of strain ZS7 (*B. burgdorferi* s.s.), *zs7.a68* was used in all subsequent experiments in which *B. burgdorferi* was quantified.

Next, the sensitivity of the real-time PCR was assessed in specimens of unprocessed mouse tissue previously spiked with *B. burgdorferi*, to more readily reflect the in vivo situation. When serial numbers of spirochetes ($100–10^5$) were mixed with 30–50 mg of various mouse tissues prior to DNA extraction *zs7.a68* DNA was detectable when PCR samples contained >100 (heart and ear) and >600 (joint) spirochetes, respectively (Table 1). This amounts to a detection limit of approximately 1–7 spirochetes/ng tissue DNA. Notably, no *B. burgdorferi* DNA was detectable in the context of spleen tissue under these conditions. To compare the sensitivity of our assay with a method described in a previous study [62], spiking experiments were also carried out according to the latter protocol (ie: $10–10^5$ spirochetes were spiked into 100 µl mouse heart digestion mixture prior to DNA isolation). With this second spiking protocol, sensitivity

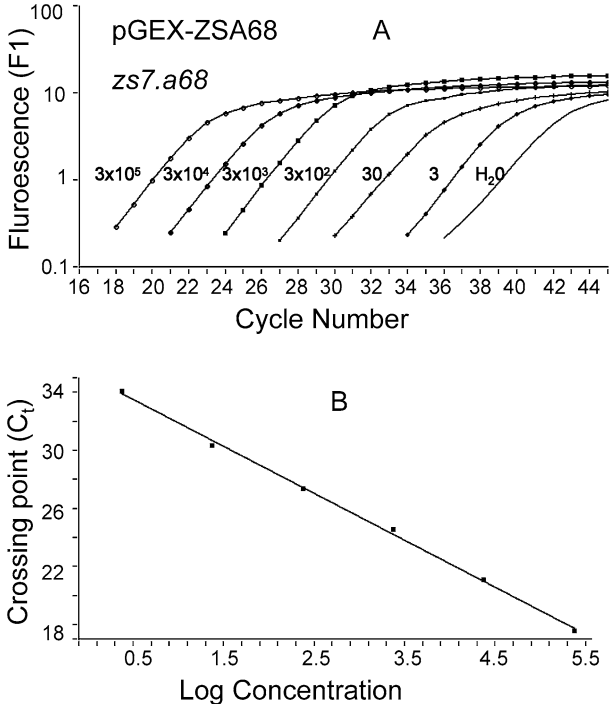

Fig. 1A, B Representative standard curve used in real-time PCR for estimation of *Borrelia burgdorferi*-specific genes in plasmids and mouse specimens. **A** representative titration of plasmid standard pGEX-ZSA68 ($3–3\times10^5$/PCR) using primer pairs for *zs7.a68*. **B** standard curve of C_t versus log concentration of plasmid

was higher than with our first protocol, as we could detect single copies of *B. burgdorferi* using primer pairs for both *zs7.a68* (not shown) and the chromosome-encoded gene *flagellin* (Fig. 2), also with a linear correlation (Fig 2B). However, it is yet to be determined which of the two protocols is more relevant to the in situ conditions (see Discussion).

Quantification of *B. burgdorferi* DNA in tissue specimens of tick-infected mice

Disease-susceptible immunodeficient (SCID) and immunocompetent (AKR/OlaHsd) mice as well as immunocompetent and disease-resistant BALB/c mice [53] were challenged with infected ticks and the number of spirochetes seeding various organs were determined on days 30 and 120 p.i. (two mice/strain/time point). Infection of individual mice was assessed either by the development of clinical arthritis in SCID and AKR mice and/or by seroconversion in AKR and BALB/c mice as described (Table 3 and [53]). Accordingly, infected SCID mice developed arthritis, which was already prominent at day 21 p.i., and further increased in severity with time. As reported before, the development of clinical arthritis was less pronounced in AKR mice, but was clearly visible on day 68 p.i. [53]. AKR and BALB/c, but not SCID mice generated specific antibodies to *B. burgdorferi*, the serum level of which increased with increasing time of infection (Table 3).

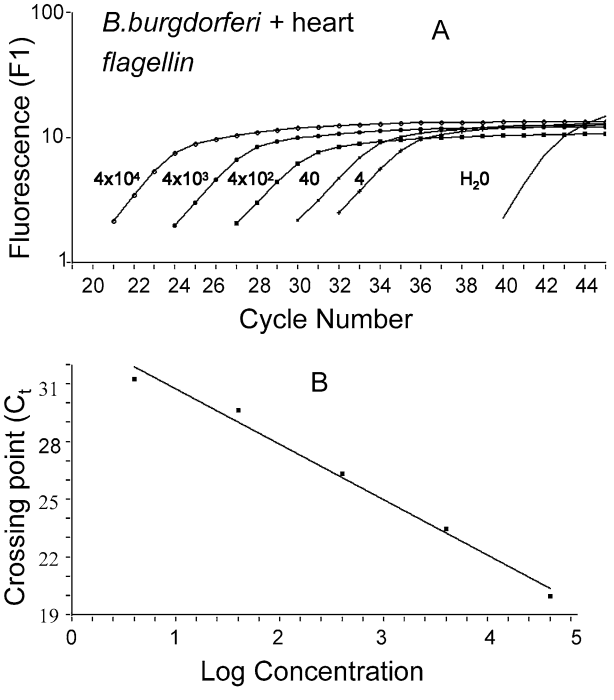

Fig. 2 **A** Representative titration of *B.burgdorferi* (4–4×10^4/PCR) and an aliquot of digested heart tissue (according to Wang (62), 100 ng/PCR) together processed for DNA and analysed employing *flagellin*-specific primers. **B** standard curve of C_t versus log concentration of *B.burgdorferi*

Table 3 Pathology and serology of *B.burgdorferi* (ZS7) infection. The scores for severity of arthritis are given for the right and left tibiotarsal joints according to the following scheme: ++ severe; + moderately severe; ± mild swelling; ± reddening; – no clinical signs. *ND* not detected

Strain	Arthritis (day 21)	*B. burgdorferi* specific Ig (μg/ml), day 30	Arthritis (day 68)	*B. burgdorferi* specific Ig (μg/ml), day 60
C.B-17 SCID	++/+	ND		
C.B-17 SCID	±/±	ND		
C.B-17 SCID			++/++	ND
C.B-17 SCID			++/++	ND
AKR/OlaHsd	–	7		
AKR/OlaHsd	–	8.5		
AKR/OlaHsd			+/±	7.1
AKR/OlaHsd			±/±	11.6
BALB/c	–	7.2		
BALB/c	–	9		
BALB/c			–	20.4
BALB/c			–	27.9

Next, the spirochete burden was tested in heart, ear, joint and spleen tissues of tick-infected SCID, AKR and BALB/c mice at days 30 and 120 p.i.. On day 30 p.i., ~1–7 spirochetes/ng tissue DNA were detected in heart and joint tissue samples of SCID mice (Table 4, Fig. 3). No spirochetes were found in ear or spleen tissues of SCID mice at this time point. By day 120 p.i., spirochete numbers had increased by 3–4 orders of magnitude in heart and joint tissue, and were now also present in similar numbers in ear and spleen tissue of SCID mice (Fig. 3, Table 4). In infected AKR mice, high numbers of spirochetes (300–400 spirochetes/ng tissue DNA) were found on day 30 p.i. in ear and joint tissues, but were undetectable in heart and spleen tissues. On day 120 p.i., spirochetes were undetectable in all tested AKR tissues. Finally, spirochetes were undetectable in the tissues of infected BALB/c mice at both time points. A summary of the kinetics of dissemination of *B. burgdorferi* is given in Fig. 3.

Table 4 Summary of real-time PCR detection of ZS7 *B. burgdorferi* DNA in murine tissues. Copy numbers of *zs7.a68* are given as an average of two mice per group with standard deviations for all samples tested. *ND* Not detected by real-time PCR. Spirochetal DNA was undetectable in BALB/c mice at days 30 and 120 post-infection

Strain	Tissue	Average copies/ng tissue DNA	
		Day 30 p.i.	Day 120 p.i.
C.B-17 SCID	Heart	1	4,821 ± 915
	Spleen	ND	13,890 ± 4667
	Ear	ND	2,154 ± 123
	Joint	6 ± 1	3,412 ± 1788
AKR/OlaHsd	Heart	ND	ND
	Spleen	ND	ND
	Ear	411 ± 128	ND
	Joint	361 ± 57	ND

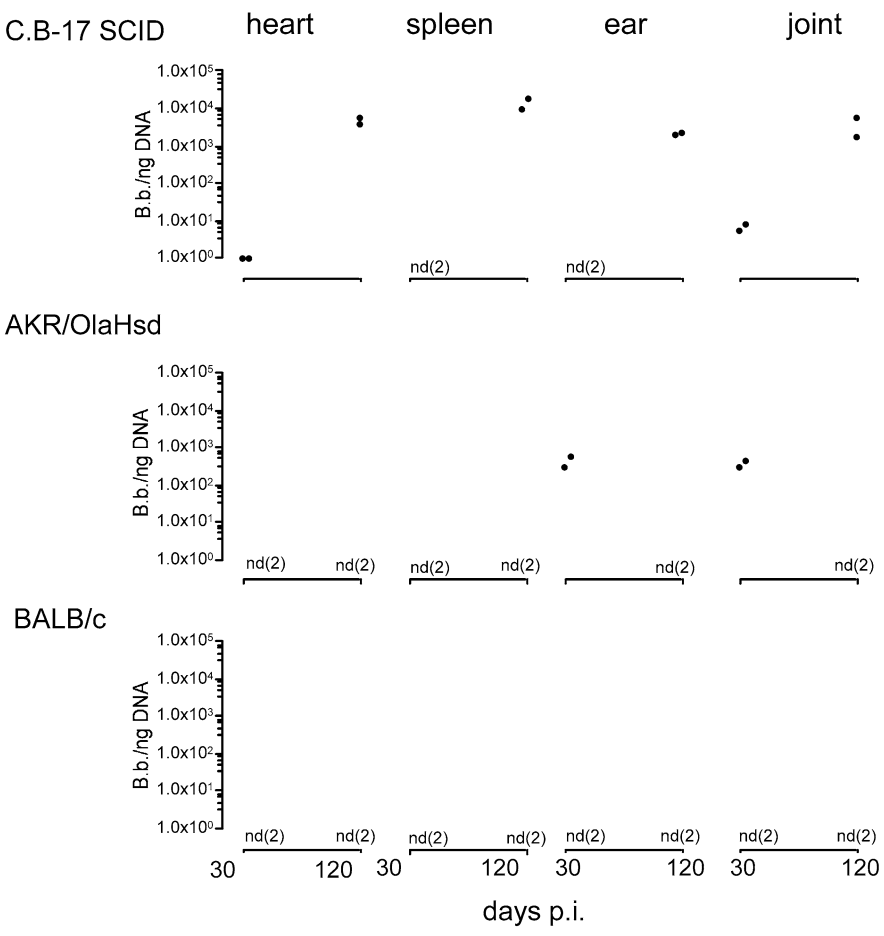

Fig. 3 Number of spirochetes in tissue of mice previously infected with *B. burgdorferi* via ticks. Real-time PCR quantification of *zs7.a68* in naturally infected C.B-17 SCID, AKR/OlaHsd, and BALB/c mice (two mice/group/ time point) were sacrificed on days 30 and 120 p.i. DNA was extracted from the indicated tissues and subjected to real time PCR, using *zs7.a68* as target gene issues. ZS7 *B.b.* were quantified per ng tissue DNA for heart, spleen, ear, and joint in each mouse strain at days 30 and 120 p.i.. *Nd B. burgdorferi* DNA not detectable, mice where *B.burgdorferi* was not detected are indicated in *parentheses*, i.e. (2)

Quantification of *B. burgdorferi* gene transcripts in tissue specimens of tick-infected mice

For quantification of gene transcripts, RNA was isolated from heart, spleen, ear, and joint tissues of two mice from each of the three murine strains at days 30 and 120 p.i.. To control for effectiveness of RNA extraction and cDNA synthesis, HPRT mRNA was used to standardize for transcripts of the five selected *B. burgdorferi* genes, *ospA*, *zs7.a36*, *zs7.a66, zs7.a68* and *ospC*. To account for variation in cell densities of different organs, transcripts were normalized to 100 *hprt* copies.

When SCID mice were analysed on day 30 p.i., *zs7.a36* transcripts were found at comparable levels in heart and joint tissues, and most interestingly, also in ear tissue, in which spirochetal DNA was undetectable (Fig. 4 versus Table 4). Furthermore, *ospC* was expressed, though at lower levels in heart and ear tissue, but was below detection level in joint and spleen tissue. By day 120 p.i., *zs7.a36* and *ospC* transcripts were considerably reduced (up to >50-fold) in heart and ear and were undetectable in joint and spleen tissues of SCID mice (Fig. 4), in spite of the highly increased spirochete burden in heart, joint, ear and spleen ($\approx$1,000 fold compared to day 30 p.i.; Table 4 and Fig. 3). None of the other transcripts, including *ospA*, *zs7.a66* or *zs7.a68*, were detectable in any of the tissues of SCID mice at the two time points.

When testing AKR mice, in which spirochetal DNA was detectable on day 30 p.i. in ear and joint, but not in heart or spleen tissue (Table 4), z *s7.a36* transcripts were found only in ear and heart, but in none of the other tissues, including joint (Fig. 4). Neither *ospA*, *zs7.a66*, *zs7.a68* nor *ospC* transcripts were detectable at this time point in any of the tested organs. On day 120 p.i., when numbers of spirochetes were below the detection limit in all tissues of infected AKR mice, no transcripts of any of the five genes tested were detectable, with the exception of low amounts of *zs7.a36* in the heart tissue of one mouse. Finally, in infected BALB/c mice, where spirochetal DNA could not be detected at any time point, only low numbers of *zs7.a36* transcripts were detectable in the heart at day 30 p.i. and no transcripts were found in any tissue at day 120 p.i..

Discussion

We show here that real-time RT-PCR is useful to quantify *B. burgdorferi* burden and gene expression in naturally infected mice. The main findings are that (1) spirochetes seem to express *ospC* and *zs7.a36* during the early phase of infection, but decrease and/or cease

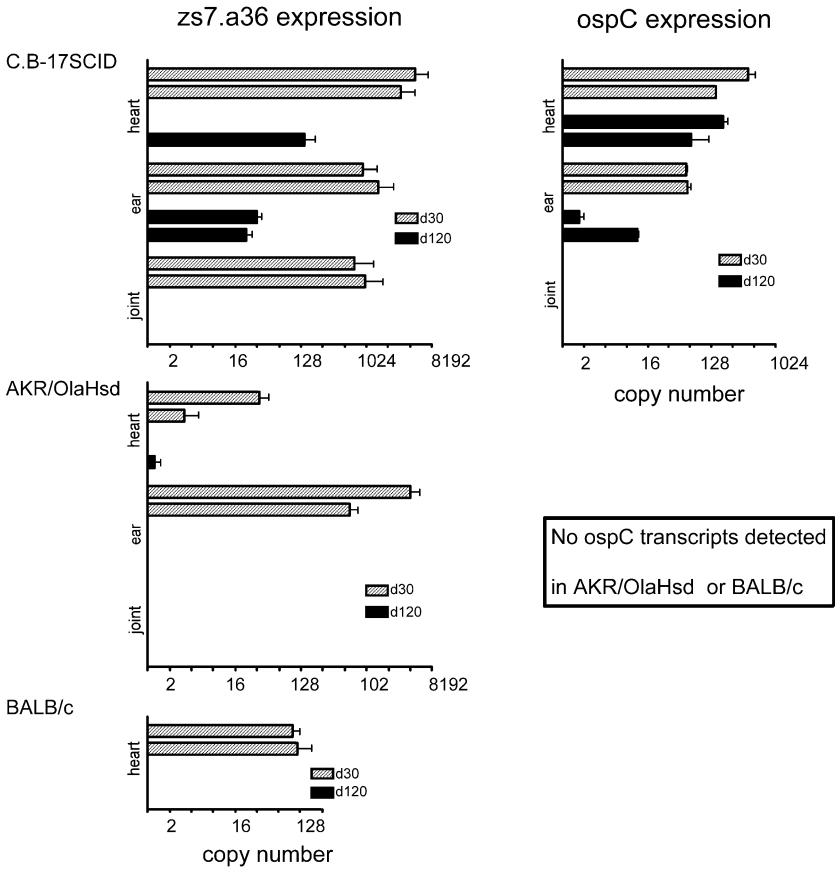

Fig. 4 Quantification of *B. burgdorferi* gene transcripts in tissue specimens of tick infected C.B-17 SCID, AKR/OlaHsd and BALB/c mice. Transcripts specific for *ospA*, *zs7.a36, zs7.a66, zs7.a68,* and *ospC* were quantified by real time RT-PCR in heart, spleen, ear and joint of mice on days 30 and 120 p.i.. Data from two mice/group/time point/tissue are shown with *error bars* from multiple testing of at least two cDNA aliquots. Copy numbers indicated refer to the number of transcripts per 100 *HPRT* copies per PCR reaction. No column <0.1copies or no transcripts detectable

expression by later stages, even when expanding in an immunodeficient environment, (2) individual genes, such as *ospC* and *zs7.a36* may be differentially expressed in distinct organs of the same mouse, (3) spirochetes do not appear to express *ospA*, independent of the immune status of mice and/or the severity of infection including chronic inflammatory processes and (4) specific immunity is a major factor in reducing spirochetal burden.

The present study which quantifies spirochete burden and gene expression in mice infected by the natural route cannot be simply compared with previous reports in which PCR and real time PCR analysis was applied to mice challenged by needle infection for a similar purpose [42, 45,62]. The reason for this is the fact that the two routes of spirochete inoculation, i.e. tick versus needle infection, lead to greatly differing courses of infection, induction of immune responses and onset of disease manifestations [23, 25,55]. However, it is clear that only the natural mode of infection will uncover strategies of either spirochetes or mammals which are of biological significance and thus of relevance for the appropriate treatment of Lyme disease.

Our present experimental protocol, using the most reliable and sensitive primer pairs with specificity for *zs7.a68*, supports the previous findings that as few as 1–10 *B. burgdorferi* organisms can be detected by real-time PCR [27, 47, 48, 63,64], though reproducibility is reduced with low numbers of spirochetes [62]. However, as previously shown, artificial spiking of unprocessed mouse tissue with spirochetes led to a dose-dependent inhibitory effect by tissue material on the amplification of *B. burgdorferi* genes [27, 47, 58,63]. Furthermore, when spirochetes were spiked into spleen tissue, *B. burgdorferi* DNA was undetectable at concentrations used in our protocol for determining real-time PCR sensitivity. This decreased sensitivity of gene amplification in the context of mouse tissue needs some comment. Following a protocol by which spirochetes were spiked into mouse heart tissue aliquots (pre-digested), Wang et al. found a high correlation between the expected numbers of spirochetes and those determined by quantitative PCR in the spiked sample after DNA processing, though with a high degree of variability at low spirochete copy numbers [62]. Although we were able to confirm their results using their protocol with both the plasmid-encoded *zs7.a68* and chromosome-encoded *flagellin* target genes, it is unclear to what extent either of the two methods represent the in vivo situation. This is also true for another report showing that the specificity and sensitivity of detection of spirochetal DNA were not altered when isolated mouse spleen cells rather than tissue specimens were spiked with spirochetes [45]. In short, our results reveal differences in the ease at which spirochetes are detected in distinct tissues and the importance of developing optimal conditions for detection from each particular tissue. Together these studies also emphasize that the number of spiro-

chetes determined in infected tissue by any method is only a minimal estimate.

At day 30 p.i., spirochetes were detected in heart and joint, but not ear and spleen tissues of SCID mice. In disease-susceptible AKR mice, spirochetes were found in high numbers on day 30 p.i. in ear and joint but not in other tissues and were undetectable in all tissues of disease-resistant BALB/c mice. The finding that spirochetes were associated with ear and joint but not heart tissue of AKR mice is consistent with other reports [62,64], and suggests a differential tropism and expansion of *B. burgdorferi* in mice. The observation that *B. burgdorferi* was found in heart and joint, but not ear tissue of SCID mice at day 30 may also support a previous finding on tropism in this particular strain [52]. That no spirochetes were detected in the spleen at this time may be due to low sensitivity for this tissue, though one cannot rule out the possibility of differential colonization. The increase of spirochete numbers in all tissues tested in SCID mice at late stages of infection—a process accompanied by a time-dependent increase in severity of arthritis and tissue destruction ([51] and Table 3)—emphasizes the uncontrolled course of *B. burgdorferi* infection in this immunocompromised mouse strain; it also supports the assumed positive correlation between numbers of tissue-associated spirochetes and severity of pathology [9, 62, 63,64]. The finding that *B. burgdorferi* was undetectable on day 120 p.i. in both AKR and BALB/c mice further supports the notion that the spirochete burden is drastically reduced, but not necessarily eradicated, by specific immunity, in particular antibodies [5, 8,54]. It is presently unclear whether the differential course of early infection in these two immunocompetent mouse strains is due to qualitative differences in their innate and/or adaptive immune responses. However, the selective infection of joint tissue of AKR, but not BALB/c, mice could explain the development of clinical arthritis, including pathological alteration in synovial tissue, in the former but not the latter mouse strain [45,53].

The differential expression of *ospC* and *zs7.36* in SCID versus AKR mice on day 30 p.i. may indicate that factors of the innate and adaptive immune system contribute to this process of phenotypic variation, corroborating related studies, in which *B. burgdorferi* PCR and microarray analyses were employed [27, 36, 37, 38,61]. The involvement of specific antibodies in down-regulation of the Osps in spirochetes is also indicated by the fact that much higher numbers of *ospC* transcripts (between 10–100-fold) were observed in spirochetes seeding most organs, except the ear, of SCID mice as compared to those seeding AKR tissues on day 30 p.i.. That only *zs7.a36* transcripts were detectable in the heart tissue of BALB/C mice on day 30 p.i. argues for a very low spirochetal load in this mouse strain (see Table 4). However, as BALB/c mice produced *B. burgdorferi*-specific antibodies (Table 3), including those to OspC ([66], and data not shown), *ospC* must have been expressed earlier in infection, at concentrations sufficient to be immunogenic.

Spirochetes isolated from heart, joint and/or ear tissues of SCID and AKR mice 30 days p.i. differed markedly in their levels of expression of *ospC* and/or *zs7.a36*, respectively. A similar variation in the numbers of gene transcripts among tissues of individual mice was recently found in C3H mice [28]. These results not only reveal the versatility of *B. burgdorferi* in situ, but also the dilemma of designing efficacious immunotherapeutic regimens. The latter is most convincingly illustrated by the previous report showing that immune serum to the *B. burgdorferi* protein, Arp, is able, upon transfer, to selectively resolve arthritis with no effects on carditis and infection [18].

Transcripts of *zs7.a66*, *ospA* and *zs7.a68* were undetectable in any of the mouse tissues tested, independent of the genetic background and immune status of the recipients during the observation period of 120 days p.i.. The finding that no *ospA* transcripts were produced by spirochetes during the acute phase and even in a highly inflammatory environment of the chronic phase of the infection is at variance with a recent report in which host-adapted spirochetes in mice were found to express *ospA* when challenged with an inflammation-inducing agent, i.e. zymosan in a dialysis chamber [13]. However, in light of the present study it is questionable whether this artificial system represents the genuine in vivo situation. Thus, when considering levels of *B. burgdorferi* gene expression, *zs7.a36* seems to be regulated differently than three other lp54-encoded genes: *zs7.66*, *zs7.68* and *ospA* during infection in mice. This complements the previous notion that from 16 identified lp54-encoded genes all but one, i.e. *bba64*, were down-regulated by mammalian host-specific signals, as revealed by an in vivo study using a membrane cultivation chamber [6].

The novel finding that expression of *zs7.a36* and *ospC* is down-regulated in SCID mice at later stages of infection (day 120 p.i.), in spite of extensive expansion of the spirochete population, indicates that host factors other than those associated with specific immunity interfere with gene expression in spirochetes. Although elusive so far, it is possible that host molecules such as fibronectin [26], proteoglycan [29,35], glycosphingolipids [4], decorin [17], complement regulators [31], plasminogen [12,21] and integrins [11], which serve as receptors for spirochetal outer surface proteins, participate in this process(es).

To our knowledge, this is the first report on quantitative analysis of gene expression in target tissues of tick-infected mice with distinct genotypes and different immune status at early and late stages of infection. It is clear to us that the present study is by no means comprehensive. Further studies using more animals and additional spirochete isolates, are required to verify the present results for *B.burgdorferi*s.s. and other species of the highly heterogeneous [44] *B.burgdorferi*.s.l. complex. However, our data do suggest that besides specific immune processes, factor(s) of the innate immune response force spirochetes to differentially change their phenotype in the mammalian host, even in individual organs of one

recipient. The study also demonstrates that real-time RT-PCR analysis is a powerful tool to elucidate spirochetal traits relevant for persistence and the development of therapeutic regimens against Lyme disease.

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