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Linear and Circular Plasmids in Skin and Cerebrospinal Fluid Isolates of *Borrelia burgdorferi* Agent of Lyme Disease

¹Ali Karami, ²Peter Hindeersson, ³Niels Hoiby, ¹Saeid Morovvati and ¹Akbar Khalilpour

¹Research Center of Molecular Biology, Institute of Military Medicine,
Bagyatallah University of Medical Science, Tehran, Iran

²Department of Clinical Microbiology, National University Hospital (Rigshospitalet)

³Institute of Medical Microbiology and Immunology, Panum Institute, Copenhagen, Denmark

Abstract: Seven strains of the lyme disease agent, *Borrelia burgdorferi* isolated from skin and cerebrospinal fluid of patients with erythema migrans and acrodermatitis chronica atrophicans were examined for presence of linear and circular plasmids. By using two dimensional gel electrophoreses several linear and circular plasmid were identified, each strains contains 5 to 11 plasmid mostly linear, varying in size between 3 to 60 kb pairs, none of the strains has similar plasmid profile. Comparing plasmid profile of Danish isolates with plasmid profile of *B. burgdorferi* from different geographical area revealed similarity to Swedish isolates. After 42 passage of DK1 strain in BSK-II medium it has lost one 5.5 kb linear plasmid. PCR amplification of total plasmid from seven DK strains with primers designed from conserved region of OspA gene produced fragment with 822 bp similar to size of the primers span in published sequence. Cloning and sequencing of OspA gene from DK1 and DK6 strains has shown similarity between 75 to 95% to different *B. burgdorferi*. Electron Microscopy analysis of 25 kb plasmid revealed that this plasmid indeed has linear structure. DNA sequence of some fragments of 25 kb linear plasmid has shown similar sequence to eukaryotic viruses specially Vaccinia and Varriola.

Key words: *Borrelia burgdorferi*, lyme disease, plasmid profile, linear plasmids, OspA, cloning

INTRODUCTION

Borrelia burgdorferi a bacteria from spirochete genus is the agent of tick born Lyme disease with local and systemic manifestation. This bacteria has been isolated from human, rodents other mammals, birds (Barbour and Garona, 1987a, b; Barbour, 1989a, b) and arthropods from different geographical area specially North America, Europe and Asia (Hinnebusch and Barbour, 1991). The clinical course differs from erythema migrans and erythematous rash to arthritis, neurotic and cardiovascular manifestations *Borrelia* may be unique among prokaryote in having a gnome that is mainly linear DNA. Recently physical and genetic map of linear chromosome of *B. burgdorferi* has been published, it consist of 946 to 952 kb Linear DNA (Sherwood and Wai, 1993; Davidson *et al.*, 1992).

This bacteria also contains several circular and specially linear plasmids from 5 to 55 kb. Recently analysis of entire *Agrobacterium tumefaciens* C58 genome revealed presence of one 2.1-Mb linear and one 3-Mb circular plasmid (Servent *et al.*, 1993) and it has

been shown that *rhodococcus fascians* contains 4 Mb linear chromosome (Crespi *et al.*, 1992). Presence of several linear plasmids seems the segmentation of *Borrelia* DNA to several linear pieces has led to the suggestion that the relatively small linear chromosome and the linear plasmids actually are minichromosomes. In *B. hermsii* it has been shown that total cellular DNA organized into several complete gnomes and it suggests that linear plasmids are like small chromosomes (Ferdows and Barbour, 1989). Plasmid profile of *B. burgdorferi* from different geographical area has been revealed significant heterogeneity a feature that can be used for classification of bacteria within given species (Barbour and Garon, 1987a, b; Barbour, 1989a, b). Another related spirochete *B. hermsii* like *B. burgdorferi* has several linear and circular plasmids and the genes responsible for antigenic variation are located in linear plasmids. In *B. burgdorferi* a 49 kb linear plasmid carries the genes for Outer Surface Protein A and B (OspA and OspB) (Barbour and Garon, 1987a, b; Barbour, 1989a, b). It has been shown that passage of *B. burgdorferi* in BSK medium changes the plasmid profile and loss of

plasmids may change the infectivity of organism (Schwan *et al.*, 1988). Structure of linear plasmids of *B. burgdorferi* shows similarity to eukaryotic virus such as vaccinia and African swine fever virus in having covalently closed ends like hairpin loops (Hinnebusch and Barbour, 1991).

MATERIALS AND METHODS

Strains: Seven strains of *Borrelia burgdorferi* DK1 to DK7 isolated from patient obtained from *Borrelia* Laboratories Statens Serum Institute, Copenhagen Denmark. Passage number of each strain are shown in Table 1, strains were cultured in BSKII medium (Barbour, 1989a, b) for 3 weeks at 33°C in microaerophilic condition and after optimal growth that followed by dark field microscopy examination, bacteria were harvested by centrifugation for 30 min at 14,000 g at 4°C. Strains were massaged in BSK-II medium in same condition for studying change in plasmid profile and infectivity by extracting plasmid after each passage.

Plasmid extraction: Plasmid DNA extraction was carried out by a modified alkaline lysis method. The bacterial pellet was suspended in 300 µL of STE buffer (15% sucrose in 50 mM Tris hydrochloride (pH 8.0), 50 mM EDTA). Lysozyme (10 mg mL⁻¹) was added to a final concentration of 100 µg mL⁻¹ and the mixture was incubated for 10 min on ice. Bacteria were lysed with lysis solution (2% SDS, 1% NaOH) for 10 min at room temperature and proteins were precipitated with 300 µL of 3-5M ammonium acetate for 10 min. The supernatant was extracted after centrifugation at 10,000 g for 10 min with phenol/chloroform and DNA was precipitated with 2 volumes of cold 2-isopropanol, incubated for 20 min at -20°C and centrifuged for 25 min at 25,000 g. DNA was dried in a vacuum centrifuge and resuspended in 50 µL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer. DNA concentration were determined spectrophotometrically by measuring the *A*₂₆₀ (Lebbech *et al.*, 1991) (Fig. 1).

Sodium deoxycholate and diethyl piropcarbonate (DEP) plasmid extraction:

For comparing other methods for plasmid extraction we use sodium deoxycholate and DEP (Diethyl piropcarbonate) (Barbour *et al.*, 1989a, b). After suspending bacterial pellet in 240 µL of STE buffer and lysozyme and proteinase K treatment (like alkaline lysis) at this point following reagents were added to the samples, 300 µL of 1% sodium deoxycholate in STE and 7 µL of DEP (Sigma Chemical Co. St Louis, Mo) after shaking for 10 min at room temperature to the lysate was added 7.5 M potassium acetate and tube was centrifuged and precipitated as stated before and dissolved in TE buffer.

Tow dimensional gel electrophoresis: DNA samples were examined by electrophoresis in 0.3% agarose (Sea Kem, FMC Bio Products, Rochland, ME) gel in TAE buffer (0.04 M Tris- acetate, 0.001 M EDTA). Gels were run at 50 V for 5 min and then at 14 V for 18-24 h, followed by staining with 0.5-1 µg mL⁻¹ ethidium bromide. After visualization of plasmid bands with a UV transilluminator

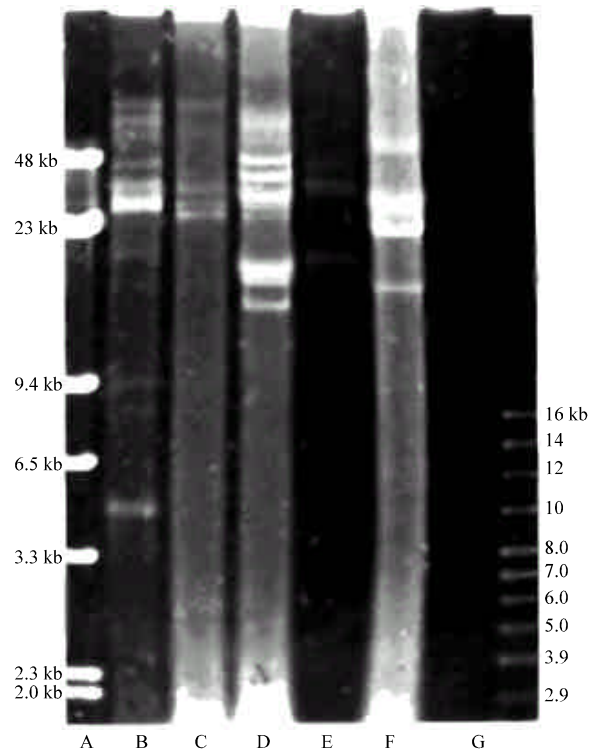


Fig. 1: Plasmids isolated from different strains of *Borrelia burgdorferi*: The DK1 strain (B), Dk5 strain (C), DK7 strain (D), DK2 strain (E), DK6 strain (F) and a super coiled circular molecular weight marker (G). Linear molecular markers (A) (HindIII fragments of Lambda DNA). Samples were separated in 0.3% gel at 14°C for 20 h then stained with ethidium bromide

Table 1: *B. burgdorferi* strains, origin and Passage number

Strain	Passage number	Origin
DK1	32	Skin, Erythema Migrans (EM)
DK2	11	Skin, Acrodermatitis Chronica atrophicans (ACA)
DK3	11	Skin (ACA)
Dk4	10	Skin (EM)
DK5	12	Skin (ACA)
DK6	12	CSF
DK7	30	Skin (ACA)

(TM-20 UV, Inc Sangarbie, CA) the gel was exposed to same UV light for 5 min. The lane of plasmids was cut from the gel and placed in 90 degree position from the first dimension in a new gel holder and the second dimension gel (0.3%) was poured. Ethidium bromide to a final concentration of 0.5-1 $\mu\text{g mL}^{-1}$ was added to the TAE buffer and the gel was run at 14 V for 18 h. As a molecular weight marker, super coiled circular standards containing from 16 to 2 kb (Boehringer Mannheim, Germany) and HindIII digested lambda DNA (Boehringer) as a linear marker were used. (As a characterized control of circular and linear plasmids, super coiled pUC18 and phage lambda DNA digested with HindIII, respectively, were applied) (Fig. 2).

Cloning of linear plasmid fragments: Total plasmid DNA was extracted from DK1 and separated by same method, after visualization of plasmid bands, pAK92 plasmid was extracted from the gel after two time freeze and thaw at -80°C . Plasmid DNA was extracted from supernatant by adding 1/20 volume of 5 M NaCl and 2 volume of cold 2-isopropanol and precipitated at 25,000 g for 30 min, dried and dissolved in 50 μL of TE buffer. After digestion of purified plasmid with EcoRI restriction enzyme resulted fragment were cloned to pUC18 cloning vector (Pharmacia, Sweden) and transformed to competent *E. coli* Jm105 (Pharmacia, Sweden). After confirming of clones by restriction analysis, several clone containing pAK92 fragment were found.

PCR analysis: In order to obtain PCR primers specific for OspA, primers from the conserved region of OspA were designed (GenBank accession number X14407) (Bergstrom *et al.*, 1989).

Pr 5456, 5' GGAGAATATATTATGAAAAAATATTT ATTGGGA 3'.

Pr 5457 5' CGTTTTTAATTCATCAAGT 3'.

PCR was performed in a reaction volume of 100 μL containing 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and 10 ng of the gel purified 25 kb linear plasmid (target DNA), or 10 ng of total plasmid extracted from strains DK1, 2, 3, 4, 5, 6 and DK7, 1 pmol of each of the primers 5456 and 5457 and 10 μL of 10X PCR buffer (10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 0.01% gelatin, 5.5 mM MgCl_2). After incubation for 3 min at 94°C , 2.5 U of Taq DNA polymerase (Boehringer) was added to each tube. Amplification was carried out at 94°C for 1 min, 45°C for 1 min and 72°C for 2.5 min for 40 cycles. Ten microliter of the PCR reactions were analyzed by agarose gel electrophoresis (0.7%) (Sea-Kem FMC) (Fig. 3).

Preparation of DNA probe and hybridization: A 32 mer oligo from the published sequence of the OspA gene (GenBank accession number X14407 (Bergstrom *et al.*, 1989) was designed from base number 139-170 (5' GGAGAATATATTATGAAAAAATATTTATTGGG 3') and synthesized with an Applied Biosystem 381A DNA synthesizer. DNA was labeled with the DIG-labeling system (Boehringer), according to the manufacturers instructions. The labeled probe was stored at -20°C until use. After electrophoresis of the total plasmid preparation

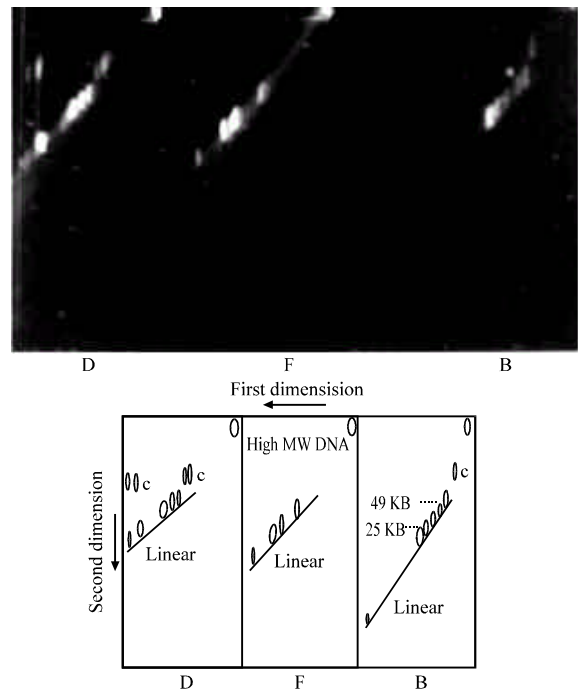


Fig 2: Two dimensional gel electrophoresis of three strain of *B. burgdorferi* Sample (D) the DK7 strain plasmids which can be UV-EtBr nicked is indicated by arrows. These UV-EtBr nicked plasmid forms most likely represents circular plasmid indicated by letter c, where as the plasmid moves on diagonal line are linear as a result in this strain there are 4 plasmids with upper bands revealing the nicked form of circular plasmids and 4 bands without upper bands revealing linearity of the plasmids. Sample (F) The DK6 strain as all the plasmids moving on diagonal line indicating that this strain contains only linear plasmids and there is no upper band in the plasmids and DK1 (sample B) most of the plasmids except one move on the diagonal line indicating low content of circular plasmids, largest plasmid in this strain has circular shape with upper band but linear plasmid including a 49 kb plasmid (encoding the OspA-B genes) have no upper band

and visualization, plasmid bands were transferred from the gel to a nylon membrane (Zeta-probe GT BioRad, Richmond, CA) by alkaline vacuum blotting. Hybridization was carried out at 50°C for 15 h in the same buffer by adding 25 µL of the labeled probe. Post hybridization washes and development of the filter was performed according to the manual of the DIG labeling kit (Fig. 4).

Pathogenicity test: Ten female gerbils of the strain *Meriones unguiculatus* (weight, 50 to 70 g, Shamrock, United kingdom) were infected interaperitoneally with 10⁸ *B. burgdorferi* original low passage infected DK1 (5 gerbil) and high passage (No. 43) that has lost two small linear plasmid (5 gerbil). One gerbil served as control. On day 24 after infection the animal were scarified and kidney, spleen and urinary bladder tissues were removed. Three specimens from each tissue were obtained for culture of *B. burgdorferi*. Cultures were initiated in BSK-II medium by inoculation of a 1 to 2 mm thick tissue slice without further preparation. The remanding tissue was frozen immediately at -80°C in 0.9% NaCl until tissue preparation for PCR. The tissue culture were incubated at 33°C and examined by dark-field microscopy for growth of spirochetes once a week for 5 weeks. Sample preparation for PCR from gerbil tissue was performed as described before (Hansen *et al.*, 1992; Lebbech *et al.*, 1991). PCR of Plasmid extracted from tissue culture and DNA extracted from animal tissues was performed by using OspA specific primers as described above.

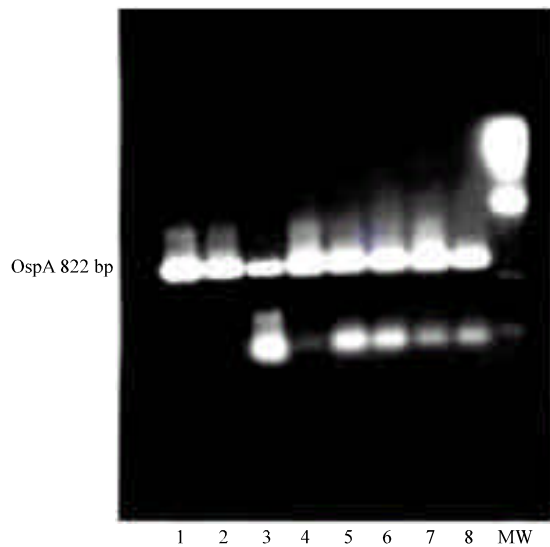


Fig. 3: PCR amplification of plasmid extracted from seven strain of DK with OspA specific primers. All strains give exact fragment size of 822 bp. From left to right DK1, DK2, DK3, DK4, DK5, DK6 and DK 7, Linear MW (Hind III fragments of lambda DNA)

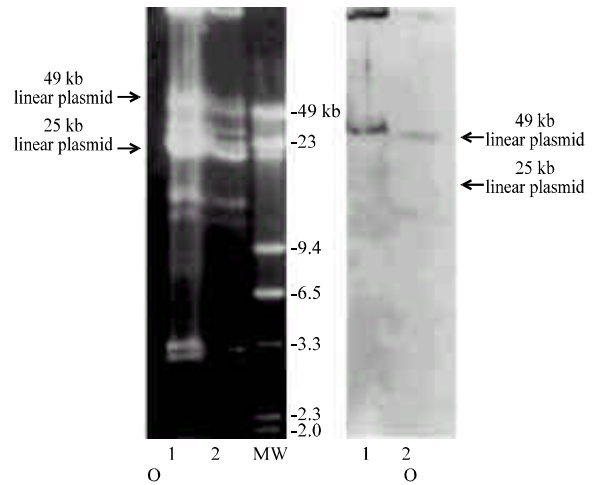


Fig. 4: Hybridization of total plasmid extracted from DK1 strain with OspA specific probe. Left: lane 1 and 2 are total plasmids extracted from DK1 strain, lane 1 contains more DNA material for detection of low copy number plasmids, lane 2 is the same DNA but with less amount for clear separation of plasmid bands. Right: Probe hybridize strongly to the linear plasmid with size of 50 kb, week hybridization is visible in plasmid with size of 25 kb in DK1 strain

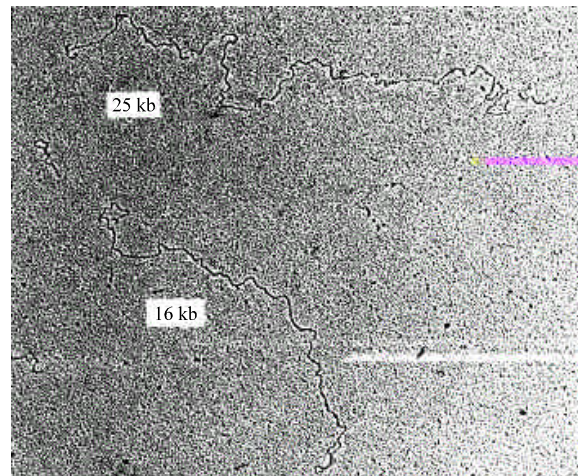


Fig. 5: Electron micrograph of 25 kb plasmid extracted from DK1 strain. One supercoiled plasmid. Magnification 52000x

Electron microscopy of plasmids DNA from DK1 strain: Linear plasmid samples were prepared for electron microscopy as previously described (Barbour *et al.*, 1988) (Fig. 5). Electron microscopic analysis of plasmid samples has been done by professor Cloud. F. Garon at the Rocky Mountain Laboratories, Montana, USA.

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1
B31 MKKYLLGIGLILALIACKQNVSSLDEKNSASVDLPGEMKVLVSKEKDKDG
DK1 -----
DK6 -----
PBi -----
PKO -----

50
B31 KYSCLKLTVDKIELKGTSDKDNNGSGVLEGTKDDKSKAKLTIADDLSKTTFE
DK1 ---K-T---I-----D---V---T-D-----AD-LSK--F-
DK6 ---M-T---L-----S---T---E-S-----SE-LSK--F-
PBi ---M-T---L-----S---T---E-S-----SE-LSK--F-
PKO --D-I-T---L-----N---V---V-A-----SD-LGQ--L-

101
B31 LFKEDGKTLVSRKVSSKDKTSTDEMFNEKVNCLQKPRQEKMEPNLN .IRM
DK1 L-----R--S-----T-TD-M--E-GELSAKMTRENGTKLEYTEM
DK6 I-----K--N---S-IE-K--A-GELSEKILRANGTRLEYTEI
PBi I-----K--N---S-IE-K--A-GELSEKILRANGTRLEYTEI
PKO V-----K--T---S-TE-K--E-GEVSEKIIIRADGTRLEYTGI

151
B31 KSDGTGKAKEVLKNFTLEGKVANDKVTLEVKEGTVTLKSKEIAKSGEVTVA
DK1 -----NFT---KVAND-V--E-K---T---EIAK---VT-A
DK6 -----DFA---TLAAD-T--K-T---V---HIPN---IT-E
PBi -----DFA---TLAAD-T--K-T---V---HIPN---IT-E
PKO -----GYV---TLTAE-T--V-K---T---NISK---VS-E

201
B31 LNDTNTTQATKKTGAWDSKSTLTISVNSKKTQLVFTKQDTITVQKYDS
DK1 ---TNTTQ-----GA-D-K-----S-----TQL---QD-----KYDS
DK6 ---SNSTQ-----GK-D-N-----S-----KNI---ED-----KYDS
PBi ---SNSTQ-----GK-D-N-----S-----KNI---ED-----KYDS
PKO ---TDSSA-----AA-N-G-----T-----KDL---EN-----QYDS

251
B31 AGTNLEGTAVEIKTLDELKNV* * *
DK1 A-TN---T---KT---L--ALK*
DK6 A-TN---N---KT---L--ALK*
PBi A-TN---N---KT---L--ALK*
PKO N-TK---S---TK---I--ALK*

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Fig. 6: Amino acid comparison of OspA protein from DK1 and DK6 strains with OspA protein from other *Borrelia burgdorferi* strains

RESULTS

Analysis of plasmid profile from different Danish isolate of *Borrelia burgdorferi* isolated from skin and cerebra spinal fluid of lyme disease patients reveals several linear and circular plasmids with strong heterogeneity. For distinguishing between linear and circular plasmids we used two dimensional gel electrophoresis, by this simple technique we could see that circular plasmids moves slower than linear plasmids in second dimension after exposing to ethidium bromide and UV irradiation. DK1 strain contains 1 large circular plasmid with 60 kb and 10 linear plasmids with size of 50, 45, 28, 25, 18, 9.5, 8.5, 5, 4.5 and 4 kb. Twenty five kilo base linear plasmid in this strain always appears as a sharp and strong band when comparing to other plasmids.

Because linear plasmids in other organisms always have DNA binding proteins in ends of the plasmids we compared effect of proteinase K and some sample without proteinase K, results shows that this linear plasmids dose not have proteins binded to ends of the plasmids. Comparing plasmid profile of strain DK1 with passage number of 31 an passage number 46 in BSK-II medium reveals that this bacteria has lost two linear plasmid with size of 4.5 and 5.5 kb and in passage no 42 two new 3 and 2 kb linear plasmids could bee seen. DK2 contains linear and circular plasmids with the size of 50, 38, 30, 27 and 23 kb. DK3 have plasmids with size of 48, 40, 35, 28, 25, 14, 16 and 18 kb. DK4 strains have plasmids with size of 30, 28 and 19 kb. DK5 contains linear and circular plasmid with size of 31, 26 and 18 kb. DK6 that is isolated from CSF of patient have only linear plasmid with size of 18, 25, 29 and

38 kb. DK7 have plasmids with size of 17, 19, 21, 29, 33, 35 and 41 kb. In second dimension, this strain shows 2 large circular plasmids of 41 and 35 and two smaller circular plasmids with 17 and 19 kb. Plasmid profile of different passage number of bacteria in BSK II medium reveals loss of some plasmids and in some case new plasmids appears (data not shown).

Comparing plasmid profile from different Danish isolate with published plasmid profile of *Borrelia burgdorferi* from different geographical area revealed close similarity Swedish and German isolates. After several passages in BSK medium Plasmid profile of with DK1 strain reveals that smaller plasmids are unstable. After purification of 25 kb linear plasmids from DK1 strain and completely digested by restriction enzymes the total size of fragments were 50 kb, twice of size of the original linear plasmid. One explanation for this result is that two different linear plasmid with the same size of 25 kb is present in this strain. Electron microscopies of 25 kb linear plasmid reveal that this plasmid has linear structure and two dimensional gel electrophoresis is able to distinguish between linear and circular plasmids. Digested fragment were cloned in pUC18 vector and one of the clones containing 1.5 of 25 kb linear plasmid from strain DK1 were sequenced. Sequence of this clone shows very AT rich DNA and comparing with other DNA sequence in database showed high degree of similarity to certain eukaryotic virus like vaccinia and other pox viruses. These viruses have telomeric ends like linear plasmids of *Borrelia*.

PCR amplification of all DK1 to 7 with OspA specific primers has produced the fragment of expected size. Digestion of these fragments by different restriction enzyme showed that there is difference between OspA restriction map of skin and CSF isolates. Hybridization of plasmid extracted from DK1 strain to OspA specific probe shows strong hybridization to 49 kb linear plasmid and weak hybridization to 25 kb linear plasmid (Fig. 4). For comparing sequence variation between skin and CSF isolate, we sequenced OspA gene of DK1 (skin Isolate) and DK6 (CSF isolate) this sequence shows variation in the middle of the gene. OspA sequence of Dk6 strain is 100% similar to PBI strain previously isolated from CSF of patient in Germany. OspA from DK1 (skin isolates) is 86% similar to PBI strain and only 83% similar to B31 strain (Fig. 5a and 6). It has been shown that low passage DK1 strain was infectious for gerbils of the strain *Meriones unguiculatus* (Davidson *et al.*, 1992; Jonsson *et al.*, 1992). We compared infectivity of low passage containing all linear and circular plasmids with high passage that has lost to small linear plasmids, in animal model. Tissue culture and PCR of samples from infected has shown that

low passage *B. burgdorferi* is infective and culture and PCR is positive but high passage *Borrelia* that has lost plasmid has shown no sign of infectivity and both tissue culture and PCR are negative. It has been shown before that several passage of *B. burgdorferi* in BSK-II medium can cause loss of plasmid and infectivity (Hinnebusch and Barbour, 1991).

DISCUSSION

The number of circular and linear plasmids in different geographical isolates of *B. burgdorferi* are very different and after even long invitro passage of bacteria in BSK medium profile of bacteria in same strain is very similar to original isolates even with lose of some plasmids mainly small size plasmids. In seven isolates of *B. burgdorferi* from lyme disease patient in Denmark none of two strain has similar plasmid profile. Majority of plasmids in these strains are linear. Comparing results from two dimensional system with electron microscopy of plasmids shows that all of the linear and circular plasmids recognized by two dimensional gel electrophoresis has the same structures in electron microscopy analysis and we could distinguish between linear and circular plasmids in *Borrelia* with this very simple technique.

Hybridization of total plasmids extracted from DK1 strain with OspA specific probe shows very strong hybridization to linear plasmid corresponding to 50 kb in size revealing that in DK1 strain OspA and OspB genes are located in 50 kb linear plasmid. Weak hybridization also has been detected to 25 kb linear plasmid.

Inactivity test in Gerbils with low and high passage of DK1 strain that has lost linear plasmids reveals that small linear plasmid that has been lost from this strain may have role in infectivity. Other experiments has shown the role of plasmids in infectivity of *B. burgdorferi* but lost plasmid in were circular not linear (Schwan *et al.*, 1988).

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