BACTERIAL CHARACTERISATION AND PATHOGENICITY

Expression in *Escherichia coli* of *flaB*, the gene coding for a periplasmic flagellin of *Leptospira interrogans* serovar pomona

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A periplasmic flagellin gene, *flaB*, of *Leptospira interrogans* serovar pomona (strain Pomona) was expressed in *Escherichia coli* for the production and antigenic characterisation of the protein. The *flaB* structural gene, which was previously cloned into pUC118, was derived by PCR from the recombinant plasmid and used to generate an expression construct with the *trc* promoter-driven pProEx HT system. Under the conditions employed, the *flaB* was expressed as inclusion bodies formed within *E. coli*, yielding c. 120 mg of the recombinant protein/L of culture. A polyhistidine tag introduced at the amino-acid terminus of the FlaB protein allowed for the purification of the protein by nickel-chelate affinity chromatography. The expressed protein reacted with both mouse and bovine antisera to *L. interrogans* on Western blots, indicating that it could be of use in the diagnosis of leptospirosis. The recombinant leptospiral flagellin may also be of value in studying its role in the pathogenesis of leptospirosis.

Introduction

Leptospirosis is a zoonosis caused by pathogenic serovars within the genus *Leptospira*. It is a spirochaetal disease of both animals and man, causing abortion, stillbirth, infertility, decreased milk production, and sometimes death in domestic livestock [1]. Infection in man is not common except in tropical regions and occurs mostly through occupational or accidental contact with infected animals or through the ingestion of water contaminated with their urine [2, 3]. The control of leptospirosis relies on serological identification of infected animals with crude or purified antigens, or both, exhibiting a varying degree of diagnostic sensitivity. Leptospiral antigens (proteins, carbohydrates, lipids) are isolated from whole organisms or fractions thereof [4–10]. Such procedures present a potential health hazard in handling infectious live organisms, the difficulty of producing a large quantity of leptospires and a complication in purifying the antigens to homogeneity. Recombinant DNA technology offers an alternative approach to overcome such problems encountered during the preparation of leptospiral antigens for diagnostic purposes.

Pathogenic leptospires, previously classified as one

species (*Leptospira interrogans*), are now subdivided into seven species: *L. borgpetersenii*, *L. inadai*, *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. santarosai* and *L. weilii*, on the basis of DNA relatedness [11, 12] and DNA polymorphic patterns [13]. These species comprise >200 serovars that are grouped into 23 serogroups [14]. Leptospire motility is mediated by two periplasmic flagella (PF) located within the periplasmic space, between the outer membrane sheath and the helically shaped cytoplasmic cylinder [15]. The PF is composed of a basal body, hook and filament [16]. The filaments from spirochaetes [17–24] comprise two distinct classes of proteins, FlaA and FlaB, that are present in the outer sheath and core layers of the PF filament, respectively. Monoclonal and polyclonal antibodies have been used recently to identify a strong immunoreactive protein of 35 kDa in *L. interrogans* serovar pomona (strain Pomona), which was shown by N-terminal sequencing and gene cloning to be the FlaB protein [25].

A structural role has been proposed for the N-terminal and C-terminal regions of a flagellin, i.e., they point into the interior of the filament to form a hairpin loop structure with exposure of the central part on the outer surface of the filament [26, 27]. The terminal regions are also believed to be involved in filament assembly [28–30]. Comparison of the serovar pomona FlaB with flagellins from various bacterial species showed that the central region is the most variable in amino-acid

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sequence [25]. In contrast, the FlaB proteins are highly conserved among the pathogenic *Leptospira* serovars and do not appear to contain serovar-specific determinants in the central regions of the proteins [31]. According to the structural model proposed by Homma *et al.* [26] and Namba *et al.* [27], it has been suggested that the variable central region exposed on the outer surface of the filament is involved in the induction of a species-specific immune response in an infected host [25, 32]. Thus, expression of a *flaB* gene in a heterologous host will provide a valuable protein material in large amounts for evaluation of its diagnostic potential, study of its roles in pathogenesis and immunity, and elucidation of its structure and function. An earlier study cloned and sequenced the *flaB* gene of strain Pomona [25]. In the present, a gene construct was generated with a pProEx HT vector to express the pomona *flaB* in *Escherichia coli*. Purification and antigenic characterisation of the recombinant FlaB protein are reported as a part of the initial investigation aimed at the application of this protein as an immunological reagent in the detection of leptospiral infection.

Materials and methods

Chemicals, vectors and bacterial strains

Isopropyl- β -D-thiogalactoside (IPTG), phenylmethylsulphonyl fluoride (PMSF) and ampicillin were purchased from Sigma. *Taq* DNA polymerase, protein expression vectors (pProEx HT) and *E. coli* DH5 α competent cells were obtained from Life Technologies (Burlington, Ontario, Canada). Deep Vent DNA polymerase, restriction endonucleases, the M13/pUC reverse primer (5'AGCGGATAACAATTTTCACACAGG3') and other molecular biology reagents were purchased from New England Biolabs (Mississauga, Ontario, Canada). Nickel-nitrilotriacetic acid (Ni-NTA) agarose and monoclonal anti-histidine tag antibody (anti-His MAb) were supplied by Qiagen (Santa Clarita, CA, USA). All other chemicals were of commercially available analytical grade.

Construction of expression vector

All DNA manipulations were performed according to established procedures [33]. The *flaB* gene was derived by PCR from pUC118pomFla-1 [25] with a thermostable DNA polymerase mixture of *Taq* and Deep Vent as described previously [31] with the primers (5'ACAGGATCCATGATTATCAATCACAACCT3' and 5'GTGGAGCTCTCAGATGTGCTGCAGAAGCT-T3', which have the *Bam*HI or *Sac*I restriction sites incorporated at their 5' ends (underlined). The PCR-amplified *flaB* gene was inserted as a sticky-ended fragment into the *Bam*HI and *Sac*I cloning sites of pProEx HT. The recombinant plasmid thus constructed, designated pHTFlaB, was used to transform the expression host *E. coli* strain DH5 α to ampicillin

resistance. The presence of an insert in recombinant plasmids was confirmed by double digests with *Bam*HI/*Sac*I. The pHTFlaB was sequenced as previously described [25] with the M13/pUC reverse primer (5'AGCGGATAACAATTTTCACACAGG3') to ensure that the *flaB* gene was in-frame at the fusion point.

SDS-PAGE and Western blotting

SDS-PAGE was performed by the method of Laemmli [34] with stacking 4% gels and resolving 12% gels in a mini-gel apparatus (BioRad, Mississauga, Ontario, Canada) [35]. The separated proteins were either stained with Coomassie Blue or analysed by Western blots [35] probed with mouse or bovine polyclonal antisera to *L. interrogans*. Immunostaining was done with an alkaline phosphatase or horseradish peroxidase substrate kit (BioRad).

Expression of the flaB gene in E. coli

The cells harbouring pHTFlaB were cultured at 37°C in 10 ml of Luria-Bertani broth supplemented with ampicillin 100 μ g/ml. After growth to an A₅₉₅ of between 0.6 and 1.0, the culture was induced to express the recombinant protein by addition of 0.6 mM IPTG. The level of protein expression was monitored over a time course of 3 h by SDS-PAGE. For routine purification of the recombinant FlaB protein, a 500-ml culture was grown. After induction with IPTG for 2 h, cells were harvested by centrifugation at 10 000 *g* for 20 min. The cell pellets were stored frozen (–80°C) until used.

Analysis of solubility of the expressed protein

The IPTG-induced cell pellets (*c.* 2 g fresh weight) were resuspended in 10 ml of phosphate-buffered saline (PBS) containing 1 mM PMSF and passed through a French press cell at 14 000 psi. The homogenate was collected and centrifuged at 27 000 *g* for 30 min. The whole cell extract, supernate and insoluble fractions (pellets) were analysed by SDS-PAGE for the presence of the expressed protein.

Purification of the recombinant FlaB protein

The buffers used in protein purification were buffer A (8 M urea, 0.1 M Na₂HPO₄, 1 mM PMSF and 0.01 M Tris-HCl, pH 8.0), buffer B (8 M urea, 0.1 M Na₂HPO₄, 1 M NaCl and 0.01 M Tris-HCl, pH 6.3) and buffer C (8 M urea, 0.1 M Na₂HPO₄, 1 M NaCl and 0.01 M Tris-HCl, pH 5.9).

Frozen cells (*c.* 2 g fresh weight) were resuspended in 10 ml of buffer A with stirring at room temperature for 1 h and then at 4°C overnight. The cell extract was centrifuged at 30 900 *g* for 40 min. The supernate was collected and loaded, at 0.5 ml/min, on to a Ni-NTA

agarose column (1 × 7.5 cm) that had been pre-equilibrated with buffer A. The column was serially washed with 40 ml of buffer A, 40 ml of buffer B and 40 ml of buffer C. Proteins were eluted from the column with 80 mM imidazole in buffer C. Fractions (2 ml) were collected and the absorbance was monitored at 280 nm. Peak fractions were pooled and stored at -20°C. The protein concentration was determined by the method of Bradford [36], with bovine serum albumin as the standard reference protein.

Protein N-terminal amino-acid sequencing

The affinity-purified recombinant protein preparation was separated by SDS-PAGE and electroblotted on to a polyvinylidene difluoride membrane. Protein N-terminal amino-acid sequence was determined by Edman degradation with a Model 470A gas-phase sequencer equipped with an on-line model 120A PTH analyser (Applied Biosystems, Foster City, CA, USA).

Results and discussion

Expression of *L. interrogans* *flaB* gene

PCR amplification of pUC118pomFla-1 [25] gave a product of 870 bp, comprising the *flaB* structural gene and a restriction endonuclease recognition sequence at each end to allow cloning into the vector, pProEx HT; this plasmid contains the *lacI*^q gene, thereby enabling IPTG-induced expression of the inserted gene from the *trc* promoter. By inserting the *flaB* structural gene downstream in frame with the initiation codon of a coding sequence for MSYYHHHHHDYDIPTTENLYFOGAMGS — underlined are a six-histidine tag and a Tobacco Etch Virus (TEV) protease cleavage site, respectively — an expression vector (designated pHTFlaB) producing a FlaB fusion protein with a polyhistidine tag at the N-terminus was constructed. The histidine tag facilitates affinity purification of the desired protein and may be removed by proteolysis with TEV protease.

Expression of the *L. interrogans* *flaB* gene in *E. coli* was followed for 3 h by SDS-PAGE after the addition of IPTG (Fig. 1). Upon induction, a strong protein band of *c.* 31 kDa was seen; this was slightly smaller than the predicted size of the FlaB fusion protein (34.686 kDa). This protein was detected on Western blots probed with anti-His MAb (data not shown). The non-induced cells containing pHTFlaB (Fig. 1, lane 2) and the non-induced or induced cells harbouring pProEx HT (not shown in Fig. 1) showed no protein band in this region. Therefore, these results indicated expression of the recombinant protein. Differences between the apparent and deduced molecular masses of proteins have been generally recognised; possibly molecular mass estimation with SDS-PAGE is not precise. Maximal expression of the recombinant protein was seen after induction for 2 h (Fig. 1, lane 4). To

Fig. 1. Expression of the *L. interrogans* *flaB* gene in *E. coli*. The cells harbouring pHTFlaB, following induction with 0.6 mM IPTG, were analysed by SDS-PAGE (12%) for presence of the recombinant FlaB in a 3-h experiment. The cells were lysed with the SDS/ β -mercaptoethanol sample buffer and heated at 100°C for 5 min before loading on to the gel. Lane 1, molecular mass standards; 2, proteins from non-induced cells; 3–5, proteins from induced cells (1, 2 and 3 h, respectively). Each lane contains total cell proteins from 1 ml of culture with an A_{590} of 0.2. The induced recombinant protein is indicated (←).

determine whether FlaB was expressed as a soluble or insoluble protein, cells were disrupted with a French press and both soluble fraction and pellets were analysed by SDS-PAGE. The expressed protein was not seen in the culture supernate (Fig. 2, lane 1) or in the soluble cellular fraction (lane 4), but was present in the insoluble fraction (lane 3). This suggested that the recombinant protein had formed inclusion bodies within the the cytoplasm of *E. coli*.

Purification and antigenic characterisation of the recombinant FlaB protein

Because it was expressed in an insoluble form within *E. coli*, the recombinant FlaB was purified under denaturing conditions. The recombinant protein, extracted and solubilised from the cells with urea, was purified by affinity chromatography on a Ni-NTA agarose column. SDS-PAGE analysis of the final preparation of the recombinant protein showed a purity of >95% (Fig. 3). The purified 31-kDa protein was analysed by N-terminal amino-acid sequencing, which confirmed it as the FlaB fusion protein. An additional minor protein band of 35-kDa was present in the FlaB fusion protein preparation (Fig. 3, lane 5). This co-purifying 35-kDa protein is believed to be a partially denatured form of the FlaB fusion protein because it reacted with a mouse polyclonal antiserum to *L. interrogans* (Fig. 4a, lane 2). Assuming 100% recovery of the FlaB protein from affinity chromatography and

Fig. 2. Analysis of solubility of the recombinant *L. interrogans* FlaB protein expressed in *E. coli*. The sample was prepared as described in *Materials and methods*. Cells containing pHTFlaB were induced with IPTG for 2 h, disrupted with a French press, and analysed by SDS-PAGE (12%) for soluble and insoluble proteins. Lane 1, culture supernate; 2, proteins from non-induced cells; 3, proteins from the insoluble fraction; 4, proteins from the soluble fraction; 5, molecular mass standards.

95% purity in the final FlaB preparation, it was estimated that expression of the *flaB* gene in *E. coli* yielded *c.* 120 mg of the recombinant protein/L of cell culture. Thus, the expression construct pHTFlaB, in combination with one-step affinity chromatography, resulted in a large quantity of purified recombinant FlaB protein. The recombinant leptospiral flagellin may

be of value in developing a recombinant protein-based serodiagnostic test and in studying its roles in pathogenesis and immunity.

The serovar pomona FlaB was previously shown to react with a specific mouse MAb and antisera from leptospire-infected cattle [25]. To evaluate the antigenicity of the expressed FlaB protein, whole cell extract prepared from the IPTG-induced cells harbouring pHTFlaB was analysed by Western blots which were probed with mouse polyclonal antiserum directed against strain Pomona. As shown in Fig. 4a (lane 2), mouse antiserum detected a protein band of 31 kDa, which was previously confirmed to be the recombinant FlaB fusion protein by Western blots with anti-His MAb and by N-terminal amino-acid sequencing. The affinity-purified recombinant protein was further analysed by Western blotting with field sera from cattle infected (13 animals) or not infected with (12 animals) with *L. interrogans*. The antisera from all infected animals used were capable of recognising the recombinant FlaB, whereas the sera from non-infected animals did not show specific immunological reaction with the protein. Fig. 4b illustrates representative results for four infected (lanes 1–4) and four non-infected (lanes 5–8) animals. It may also be noted that an ELISA with the purified protein immobilised on the plate gave results consistent with the Western blotting data. All these results indicate that the recombinant protein is immunologically active and is similar in antigenicity to the native protein. Thus, the recombinant FlaB may be a useful reagent for the serological detection of infection with pathogenic leptospires, particularly in an ELISA format.

Fig. 3. SDS-PAGE analysis of the recombinant *L. interrogans* FlaB fusion protein purified from *E. coli* by affinity chromatography on a Ni-NTA agarose column. Lane 1, molecular mass standards; 2, whole cell proteins from non-induced cells; 3, whole cell proteins from IPTG-induced cells; 4, urea-extracted proteins from IPTG-induced cells; 5, affinity-purified FlaB preparation. The recombinant protein is indicated (←).

Fig. 4. (a) Detection of the recombinant *L. interrogans* FlaB protein expressed in *E. coli* by Western blots probed with mouse polyclonal antiserum (1 in 1000 dilution) to *L. interrogans* serovar pomona (strain Pomona). Lane 1, molecular mass standards; 2, whole cell proteins from IPTG-induced cells containing pHTFlaB. (b) Western blot analysis of the recombinant FlaB of *L. interrogans* serovar pomona with field sera from *L. interrogans*-infected or non-infected cattle. The affinity-purified FlaB was immobilised on nitrocellulose membranes and probed with sera from four infected (lanes 1–4) and four non-infected (5–8) cattle at a dilution of 1 in 1000. The recombinant protein is indicated (←).

Spirochaetal gene expression studies have mostly centred on *Borrelia burgdorferi* [32, 37–42] and *Treponema pallidum* [21, 43–49], and there have been limited studies on leptospires. For example, Yamaguchi *et al.* [50] and Doherty *et al.* [51] demonstrated the expression in *E. coli* of leptospiral antigen genes by using specific antisera on colony immunoblots, although the sequence identity of these genes was not determined. More recent studies on the expression of genes of pathogenic *Leptospira* spp., including those coding for surface proteins OmpL1 and LipL41 [52, 53] and a periplasmic flagellin of serovar grippityphosa [24], have been reported. The present study has extended the previous work by showing another example of expression in *E. coli* of a leptospiral antigen gene, i.e., the serovar pomona *flaB*, and by reporting purification and antigenic characterisation of the recombinant product.

In summary, an expression construct, pHTFlaB, has been generated for effective production of a leptospiral flagellin protein (FlaB), in *E. coli*. Antigenic characterisation of the recombinant protein indicates that it may have value in the serodiagnosis of leptospirosis.

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References

1. Thiermann AB. Leptospirosis: current developments and trends. *J Am Vet Med Assoc* 1984; **184**: 722–725.
2. Heath SE, Johnson R. Leptospirosis. *J Am Vet Med Assoc* 1994; **205**: 1518–1523.
3. Farr RW. Leptospirosis. *Clin Infect Dis* 1995; **21**: 1–8.
4. Chang A, Faine S. Electron-microscopic evidence for reactions of axial filaments of *Leptospira* with IgM and IgG antibodies. *Bull World Health Organ* 1970; **43**: 571–577.
5. Palit A, Gulasekharan J. Genus-specific leptospiral antigen and its possible use in laboratory diagnosis. *J Clin Pathol* 1973; **26**: 7–16.
6. Myers DM, Coltorti EA. Broadly reacting precipitating and agglutinating antigen of Leptospirae. *J Clin Microbiol* 1978; **8**: 580–590.
7. Chapman AJ, Adler B, Faine S. Genus-specific antigens in leptospira revealed by immunoblotting. *Zentralbl Bakteriol Mikrobiol Hyg A* 1987; **264**: 279–293.
8. Srivastava SK, Verma R, Gupta BR. Characterization of protein antigens of *Leptospira interrogans* serovar pomona. *Indian J Animal Sci* 1989; **59**: 328–334.
9. Ballard SA, Faine S, Adler B. Purification and characterization of a protein antigen from *Leptospira interrogans* serovar hardjo, common to a wide range of bacteria. *J Gen Microbiol* 1990; **136**: 1849–1857.
10. Brown JA, LeFebvre RB, Pan MJ. Protein and antigen profiles of prevalent serovars of *Leptospira interrogans*. *Infect Immun* 1991; **59**: 1772–1777.
11. Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers F, Brenner DJ. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. *Int J Syst Bacteriol* 1987; **37**: 407–415.
12. Ramadass P, Jarvis BDW, Corner RJ, Penny D, Marshall RB. Genetic characterization of pathogenic *Leptospira* species by

- DNA hybridization. *Int J Syst Bacteriol* 1992; **42**: 215–219.
13. Ralph D, McClelland M, Welsh J, Baranton G, Perolat P. *Leptospira* species categorized by arbitrarily primed polymerase chain reaction and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *J Bacteriol* 1993; **175**: 973–981.
14. Kmety E, Dikken H. Revised list of *Leptospira* serovars. Groningen, The Netherlands, University Press. 1988.
15. Goldstein SF, Charon NW. Motility of the spirochete *Leptospira*. *Cell Motil Cytoskeleton* 1988; **9**: 101–110.
16. Smibert RM. Spirochaetales, a review. *Crit Rev Microbiol* 1973; **2**: 491–552.
17. Norris SJ, Charon NW, Cook RG, Fuentes MD, Limberger RJ. Antigenic relatedness and N-terminal sequence homology define two classes of periplasmic flagellar proteins of *Treponema pallidum* subsp. *pallidum* and *Treponema phagedenis*. *J Bacteriol* 1988; **170**: 4072–4082.
18. Brahamsha B, Greenberg EP. Cloning and sequence analysis of *flaA*, a gene encoding a *Spirochaeta aurantia* flagellar filament surface antigen. *J Bacteriol* 1989; **171**: 1692–1697.
19. Isaacs RD, Hanke JH, Guzman-Verduzco L-M *et al.* Molecular cloning and DNA sequence analysis of the 37-kilodalton endoflagellar sheath protein gene of *Treponema pallidum*. *Infect Immun* 1989; **57**: 3403–3411.
20. Pallesen L, Hindersson P. Cloning and sequencing of a *Treponema pallidum* gene encoding a 31.3-kilodalton endoflagellar subunit (FlaB2). *Infect Immun* 1989; **57**: 2166–2172.
21. Champion CI, Miller JN, Lovett MA, Blanco DR. Cloning, sequencing and expression of two class B endoflagellar genes of *Treponema pallidum* subsp. *pallidum* encoding the 34.5- and 31.0-kilodalton proteins. *Infect Immun* 1990; **58**: 1697–1704.
22. Mitchison M, Rood JI, Faine S, Adler B. Molecular analysis of a *Leptospira borgpetersenii* gene encoding an endoflagellar subunit protein. *J Gen Microbiol* 1991; **137**: 1529–1536.
23. Parales J, Greenberg EP. N-terminal amino acid sequences and amino acid compositions of the *Spirochaeta aurantia* flagellar filament polypeptides. *J Bacteriol* 1991; **173**: 1357–1359.
24. Woodward MJ, Redstone JS. Deoxynucleotide sequence conservation of the endoflagellin subunit protein gene, *flaB*, within the genus *Leptospira*. *Vet Microbiol* 1994; **40**: 239–251.
25. Lin M, Surujballi O, Nielsen K, Nadin-Davis S, Randall G. Identification of a 35-kilodalton serovar-cross-reactive flagellar protein, FlaB, from *Leptospira interrogans* by N-terminal sequencing, gene cloning, and sequence analysis. *Infect Immun* 1997; **65**: 4355–4359.
26. Homma M, Fujita H, Yamaguchi S, Iino T. Regions of *Salmonella typhimurium* flagellin essential for its polymerisation and excretion. *J Bacteriol* 1987; **169**: 291–296.
27. Namba K, Yamashita I, Vonderviszt F. Structure of the core and central channel of bacterial flagella. *Nature* 1989; **342**: 648–654.
28. Joys TM. The covalent structure of the phase-1 flagellar filament protein of *Salmonella typhimurium* and its comparison with other flagellins. *J Biol Chem* 1985; **260**: 15758–15761.
29. Wei L-N, Joys TM. Covalent structure of three phase-1 flagellar filament proteins of *Salmonella*. *J Mol Biol* 1985; **186**: 791–803.
30. Trachtenberg S, DeRosier DJ. Three-dimensional reconstruction of the flagellar filament of *Caulobacter crescentus*. A flagellin lacking the outer domain and its amino acid sequence lacking an internal segment. *J Mol Biol* 1988; **202**: 787–808.
31. Lin M. Molecular analysis of *flaB*, a periplasmic flagellar core protein gene in pathogenic leptospires. *J Biochem Mol Biol Biophys* 1999; **2**: 181–187.
32. Wallich R, Moter SE, Simon MM, Ebnet K, Heiberger A, Kramer MD. The *Borrelia burgdorferi* flagellum-associated 41-kilodalton antigen (flagellin): molecular cloning, expression, and amplification of the gene. *Infect Immun* 1990; **58**: 1711–1719.
33. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual; 2nd edn. Cold Spring Harbor, Cold Spring Harbor Laboratory Press. 1989.
34. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
35. Lin M, Sugden EA, Jolley ME, Stilwell K. Modification of the *Mycobacterium bovis* extracellular protein MPB70 with fluorescein for rapid detection of specific serum antibodies by fluorescence polarization. *Clin Diagn Lab Immunol* 1996; **3**: 438–443.
36. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
37. Fikrig E, Barthold SW, Kantor FS, Flavell RA. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. *Science* 1990; **250**: 553–556.
38. Gassmann GS, Jacobs E, Deutzmann R, Göbel UB. Analysis of the *Borrelia burgdorferi* GeHo *fla* gene and antigenic characterization of its gene product. *J Bacteriol* 1991; **173**: 1452–1459.
39. Fikrig E, Barthold SW, Marcantonio N, Deponte K, Kantor FS, Flavell RA. Roles of OspA, OspB, and flagellin in protective immunity to lyme borreliosis in laboratory mice. *Infect Immun* 1992; **60**: 657–661.
40. Luft BJ, Pawagi S, Jiang W, Fiseene S, Gorevic PD, Dunn J. Analysis and expression of the *Borrelia burgdorferi* P/Gau *fla* gene: identification of heterogeneity with the B31 strain. *FEMS Microbiol Lett* 1992; **93**: 63–68.
41. Jauris-Heipke S, Fuchs R, Motz M *et al.* Genetic heterogeneity of the genes coding for the outer surface protein C (OspC) and the flagellin of *Borrelia burgdorferi*. *Med Microbiol Immunol* 1993; **182**: 37–50.
42. Ge Y, Charon NW. FlaA, a putative flagellar outer sheath protein, is not an immunodominant antigen associated with Lyme disease. *Infect Immun* 1997; **65**: 2992–2995.
43. Stamm LV, Folds JD, Bassford PJ. Expression of *Treponema pallidum* antigens in *Escherichia coli* K-12. *Infect Immun* 1982; **36**: 1238–1241.
44. Walfield AM, Hanff PA, Lovett MA. Expression of *Treponema pallidum* antigens in *Escherichia coli*. *Science* 1982; **216**: 522–523.
45. Norgard MV, Miller JN. Cloning and expression of *Treponema pallidum* (Nichols) antigen genes in *Escherichia coli*. *Infect Immun* 1983; **42**: 435–445.
46. van Embden JD, van der Donk HJ, van Eijk RV *et al.* Molecular cloning and expression of *Treponema pallidum* DNA in *Escherichia coli* K-12. *Infect Immun* 1983; **42**: 187–196.
47. Norgard MV, Chamberlain NR, Swancutt MA, Goldberg MS. Cloning and expression of the major 47-kilodalton surface immunogen of *Treponema pallidum* in *Escherichia coli*. *Infect Immun* 1986; **54**: 500–506.
48. Isaacs RD, Radolf JD. Expression in *Escherichia coli* of the 37-kilodalton endoflagellar sheath protein of *Treponema pallidum* by use of the polymerase chain reaction and a T7 expression system. *Infect Immun* 1990; **58**: 2025–2034.
49. Blanco DR, Champion CI, Exner MM *et al.* Recombinant *Treponema pallidum* rare outer membrane protein 1 (Tromp 1) expressed in *Escherichia coli* has porin activity and surface antigenic exposure. *J Bacteriol* 1996; **178**: 6685–6692.
50. Yamaguchi T, Ono E, Yanagawa R. Expression of antigen genes of *Leptospira interrogans* serovar canicola in *Escherichia coli*. *Microbiol Immunol* 1988; **32**: 1179–1187.
51. Doherty JP, Adler B, Rood JI, Billington SJ, Faine S. Expression of two conserved leptospiral antigens in *Escherichia coli*. *J Med Microbiol* 1989; **28**: 143–149.
52. Shang ES, Exner MM, Summers TA *et al.* The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. *Infect Immun* 1995; **63**: 3174–3181.
53. Shang ES, Summers TA, Haake DA. Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. *Infect Immun* 1996; **64**: 2322–2330.