Expression of two conserved leptospiral antigens in *Escherichia coli*

J. P. DOHERTY, B. ADLER, J. I. ROOD, S. J. BILLINGTON and S. FAINE

Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia

Summary. The genes encoding two protein antigens of *Leptospira interrogans* serovar *pomona* were cloned and expressed in *Escherichia coli*. Rabbit antisera raised against the cloned proteins, designated p12 and p20, were used to identify the antigens in Western blots of disrupted leptospiral cells. The proteins p12 and p20 were conserved within the genus *Leptospira* and were not detected in *Leptonema illini*. Although both proteins were present in leptospiral outer envelope preparations they did not elicit the production of agglutinating or opsonising antibodies.

Introduction

Leptospirosis is an acute, febrile, bacteraemic disease caused by serovars of *Leptospira interrogans*. Leptospiral antigens are clearly important in the classification of the organism (Johnson and Faine, 1984), the diagnosis of the disease (Faine, 1982), and immunity to the disease (Adler and Faine, 1978, 1983). Many different antigens have been extracted from leptospires (Faine, 1974) but these have mostly been in crude preparations containing complex mixtures of antigens. With the exceptions of lipopolysaccharide (LPS) (Ono *et al.*, 1982, 1984; Jost *et al.*, 1986; Farrelly *et al.*, 1987), the individual antigens have not been identified or characterised. As with many other gram-negative bacteria (Gregory, 1986), it has proved extremely difficult to obtain preparations of individual antigens which are not contaminated with LPS (Kelson *et al.*, 1988).

Molecular cloning and expression of leptospiral antigens in *Escherichia coli* offers the potential of a reproducible source of individual leptospiral antigens. Therefore, we chose this approach to obtain antigens for subsequent study of their roles in classification, diagnosis and immunity.

Materials and methods

Bacterial strains and plasmids

Serovars of *L. interrogans*, *L. biflexa* and *Leptonema illini* (see table) described previously (Adler and Faine, 1983) were cultured in Tween 80-bovine albumin (EMJH)

medium with added pyruvate (Johnson *et al.*, 1973). All *E. coli* recombinants were derivatives of strain JFM65 (Rood *et al.*, 1980) or strain DH5 α (BRL, USA) and were cultured in 2 $\times$ YT medium (Vieira and Messing, 1982). All recombinant plasmids were derivatives of the cloning vector pUC18 (Norrand *et al.*, 1983) and were designated with a pLBA prefix. The plasmids pLBA1 and pLBA4 were selected by their ability to code for the production of leptospiral antigens. These plasmids contain 8.2-kb and 5.6-kb *Pst*I fragments of *L. interrogans* serovar *pomona* DNA, respectively. Plasmid pLBA7 is a deletion derivative of pLBA1. Plasmids pLBA8 and pLBA9 are pUC18-derived recombinants which contain a fragment of pLBA4.

Antigen preparation

Preparations of *E. coli* were made in a French press as follows. Cultures (800 ml) of the recombinant strains were grown overnight at 37°C. After centrifugation at 3000 *g* for 10 min at 4°C, the cells were resuspended in 16 ml of 0.01 M sodium phosphate buffer, pH 7.2, containing NaCl 0.85% w/v (PBS) and then disrupted by protrusion through a pre-cooled (4°C) French Pressure Cell (Aminco, Silver Spring, MD, USA) at about 138 MPa. Cell debris was removed by centrifugation at 500 *g* for 15 min at 4°C and the supernatant fluid stored at –20°C in 2-ml volumes.

Ammonium sulphate fractionation of French-pressed *E. coli* preparations was done at 4°C. A saturated solution of (NH₄)₂SO₄ was added drop by drop to a 2-ml volume of extract, until the required concentration was obtained. The solution was stirred for 30–60 min and then centrifuged at 7700 *g* for 15 min. The supernate was decanted and subjected to further addition of (NH₄)₂SO₄ as required. The precipitates obtained from each sequential fractionation step were resuspended in a minimal volume of PBS, dialysed extensively against PBS diluted 1 in 10, adjusted to a final volume of 2 ml, and stored at –20°C.

Table. Detection of p12- and p20-related antigens in serovars of *L. interrogans*, *L. biflexa* and *L. illini* by immunoblotting with rabbit antisera against cloned antigens

Serogroup of <i>L. interrogans</i>	Serovar	Strain no.	Immunospecific staining with antiserum to	
			p12	p20
Pomona	<i>pomona</i>	L10	+	+
Pomona	<i>kennewicki</i>	L245	+	+
Pomona	<i>mozdok</i>	L306	+	+
Pomona	<i>proechimys</i>	L304	+	+
Australis	<i>australis</i>	L168	+	+
Autumnalis	<i>bim</i>	L267	+	+
Ballum	<i>arborea</i>	L264	+	+
Bataviae	<i>bataviae</i>	L263	+	+
Canicola	<i>canicola</i>	L128	+	+
Cynopteri	<i>cynopteri</i>	L243	+	+
Celledoni	<i>celledoni</i>	L242	+	+
Djasiman	<i>djasiman</i>	L181	+	+
Grippotyphosa	<i>grippotyphosa</i>	L174	+	+
Hebdomadis	<i>hebdomadis</i>	L182	+	+
Icterohaemorrhagiae	<i>copenhageni</i>	L136	+	+
Javanica	<i>javanica</i>	L183	+	+
Mini	<i>szwajizak</i>	L198	+	+
Panama	<i>panama</i>	L184	+	+
Pyrogenes	<i>zanoni</i>	L178	—	+
Sarmin	<i>sarmin</i>	L224	+	+
Sejroe	<i>hardjo</i>	L13	+	+
Shermani	<i>shermani</i>	L244	+	+
Tarassovi	<i>tarassovi</i>	L169	+	+
Serovars of <i>L. biflexa</i>				
	<i>andamana</i>	L286	+	+
	<i>bebrich</i>	L284	—	+
	<i>maritza</i>	L283	—	—
	<i>patoc</i>	L41	—	—
	<i>sobradinho</i>	L287	+	+
<i>Leptonema illini</i>		L89	—	—

Leptospiral sonicates were prepared as before (Adler *et al.*, 1980b). Leptospiral outer envelope was isolated from *L. interrogans* serovar *copenhageni* strain L136 by the method of Auran *et al.* (1972).

Serological methods

Microscopic agglutination tests (MAT) were performed as described by Adler and Faine (1976). The methods for generation and measurement of the chemiluminescence response by phagocytic cells ingesting opsonised leptospire were as described by McGrath *et al.* (1984) with mouse peritoneal macrophages. Macrophages were harvested and prepared as described by Jost *et al.* (1986). Antiserum against whole leptospire of serovar *pomona* served as a positive control in the agglutination and opsonisation tests.

Preparation and analysis of DNA

Whole-cell DNA was prepared from *L. interrogans* serovar *pomona* strain L10 as described by Marshall *et al.* (1981). After extraction with phenol and chloroform, the DNA was precipitated with ethanol (2 volumes, -20°C) and purified by ultra-centrifugation in a caesium chloride-ethidium bromide density gradient. The ethidium bromide was removed by extraction with isopropanol saturated with NaCl and the DNA was dialysed against tris (hydroxymethyl) aminomethane-ethylene diamine tetra-acetic acid (tris-EDTA) buffer, pH 8.0 (Maniatis *et al.*, 1982). *E. coli* plasmid DNA was purified as described by Young *et al.* (1978). Small scale plasmid preparations were made by the rapid boil procedure (Holmes and Quigley, 1981).

Ligation and transformation methods were as described by Burns *et al.* (1983) and Dagert and Ehrlich (1979). Electrophoresis of DNA samples was performed in 0.8–1.2% agarose in a horizontal gel apparatus with a tris acetate (pH 7.8) electrode buffer system (Young *et al.*, 1978). Restriction endonucleases and calf intestinal alkaline phosphatase (molecular biology grade) from Boehringer-Mannheim (Penzberg, FRG) were used throughout. T4 DNA ligase was obtained from New England Biolabs (Beverly, MA, USA).

Colony immunoassay

Recombinant clones were grown on 2×YT agar containing ampicillin 100 µg/ml, replicated on to nitrocellulose filters (BA85, Schleicher and Schuell) and lysed by exposure to sodium dodecyl-sulphate (SDS) 1% w/v and chloroform vapour (Kemp *et al.*, 1983). After vigorous washing in 50 mM tris-saline buffer, pH 7.4 (TBS), the filters were treated with rabbit antiserum as described for the immunoblotting procedure. The antiserum was prepared against whole, live *L. interrogans* serovar *pomona* strain L10 as described previously (Chapman *et al.*, 1987), absorbed twice with whole *E. coli* strain JFM65 for 2 h on ice and used at a dilution of 1 in 400 in Tween 20 0.05% v/v in TBS.

Electrophoresis and immunoblotting

Leptospiral and *E. coli* components were separated by electrophoresis on discontinuous 15% w/v SDS-polyacrylamide gels (Laemmli, 1970). French-pressed *E. coli* preparations and leptospiral sonicates were prepared in 0.04 M tris-HCl buffer, pH 6.8, containing SDS 1.3% w/v, bromophenol blue 0.002% w/v and glycerol 4% w/v and immersed in boiling water for 2 min. Samples of protein, 25 µg/lane, were electrophoresed at 30 mA/gel until the dye was near the bottom of the gel. The resolved material was transferred to nitrocellulose (0.45-µm pore size; Schleicher and Schuell) in a Biorad Transblot cell by the method of Towbin *et al.* (1979) with a 1 in 2 dilution of transfer buffer.

Antigenic components were detected by immunostaining as described by Chapman *et al.* (1987), except

that the incubation with primary antibody was performed overnight at room temperature. This treatment was followed by incubation at 37°C for 4 h with the appropriate horseradish peroxidase-conjugated secondary antibody—either goat anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) or rabbit anti-human IgG (Silenus, Melbourne, Australia). 4-Chloro-1-naphthol (Merck, Darmstadt, FRG) was used as the chromogen (Hawks *et al.*, 1982).

To determine whether the antigens were sensitive to proteinase, nitrocellulose filters containing SDS-PAGE-separated leptospiral sonicates were incubated in PBS containing Proteinase K (Sigma, St Louis, MO, USA; 100 mg/ml) for 24 h at 37°C. After thorough washing in PBS they were immunostained as described previously. To determine if the antigens were sensitive to reduction, dithiothreitol 10 mg/ml was added to the electrophoresis samples just before boiling.

Preparation of rabbit antisera against cloned antigens

French-pressed preparations of *E. coli* strains JFM65 (pLBA1) and DH5 α (pLBA9) were fractionated by sequential ammonium sulphate precipitation and fractions were tested by Western blot analysis to determine if they were enriched for the cloned antigens. The 0–20% and 0–30% ammonium sulphate fractions, respectively, were emulsified with Freund's incomplete adjuvant (1:1.2, v:v) and used to immunise New Zealand White rabbits. The corresponding fractions from *E. coli* strains JFM65 or DH5 α , containing only the vector pUC18, were used to immunise control rabbits. The rabbits were given intradermal injections of 0.1 ml of emulsified antigen at six sites and were bled 4 weeks later.

Antiserum was also prepared against the cloned antigen of 20 kDa (p20) by the method of Boulard and Lecroisey (1982). The equivalent Coomassie blue-stained regions were excised from gels of SDS-PAGE-separated preparations of the *E. coli* strain DH5 α containing pLBA9 or pUC18. The excised bands were macerated, emulsified with adjuvant and injected into rabbits as described previously. Rabbits were given a booster injection without adjuvant 4 weeks after the first injection and were bled after a further 3 weeks.

Cloning of leptospiral antigens

DNA purified from *L. interrogans* serovar *pomona* strain L10 was digested with *Bam*HI, ligated to *Bam*HI-digested, alkaline phosphatase-treated vector plasmid pUC18, and used to transform *E. coli* JFM65 to ampicillin resistance. The resultant gene bank of 1000 recombinant clones was then screened by colony immunoassay. Two recombinant plasmids, pLBA1 and pLBA4, which coded for the production of leptospiral antigens were obtained. Plasmids pLBA7, a deletion derivative of pLBA1, and pLBA8 and pLBA9, subclones of pLBA4, were constructed as described in fig. 2.

Fig. 1. Immunoblot of *E. coli* clones stained with rabbit antiserum against *L. interrogans* serovar *pomona* strain L10. Cell extracts were prepared from *E. coli* JFM65 cells which contained the following plasmids: lanes A and D, pUC18; lanes B and E, pLBA1; lanes C and F, pLBA4. Samples in lanes D, E and F were pretreated with dithiothreitol. The positions of molecular weight markers (Kda) are indicated.

Results

Cloning of leptospiral antigens

Screening of the *E. coli* gene bank revealed the presence of four clones which expressed antigens that reacted with rabbit antiserum to *L. interrogans* serovar *pomona*. Two of these clones, containing plasmids pLBA1 and pLBA4, were studied further. Cell extracts of the two recombinants were prepared in a French press and were examined by immunoblotting (fig. 1). The results showed that plasmid pLBA1 coded for a dithiothreitol-sensitive antigen which yielded a multiple banded profile and that plasmid pLBA4 coded for an antigen of 20 Kda, designated p20. Restriction maps of pLBA1 and pLBA4 were prepared by digestion with endonucleases and are shown in fig. 2. Subcloning of pLBA1 localised the gene encoding the expressed

Fig. 2. Derivation and restriction maps of recombinant plasmids. The blocked area represents the pUC18 portion of the plasmids. Sizes are in kilobase pairs (kb). The deletion plasmid pLBA7 was isolated (A) by digestion of pLBA1 with *Pst*I, religation, and transformation of strain DH5 α to ampicillin resistance. The subclones pLBA8 and pLBA9 each contain the same 2.2-kb *Pst*I fragment from pLBA4 but in opposite orientations (B). These plasmids were isolated after digestion of pLBA4 with *Pst*I and ligation of the resultant DNA fragments into *Pst*I-digested pUC18 DNA. Ampicillin resistant derivatives of strain DH5 α were selected.

leptospiral antigen to a 3.2-kb *Pst*I fragment contained on pLBA7. pLBA8 and pLBA9, two subclones of pLBA4, contained a 2.2-kb *Pst*I fragment in opposite orientations (fig. 2). The p20 antigen was detected in both subclones by immunoblotting. However, plasmid pLBA9, which contained the fragment in the opposite orientation to pLBA4, showed a marked increase in the level of expression of the antigen, to the extent that p20 was clearly visible on Coomassie blue-stained polyacrylamide gels (fig. 3).

Antisera against cloned antigens

Rabbit antisera were prepared against p20, the pLBA1-encoded antigens, and the appropriate controls, as described in methods. These antisera were used to immunostain transblots of *L. interrogans* serovar *pomona* sonicate (fig. 4). Antisera raised against the cloned antigen encoded by pLBA1 recognised a single band of 12 Kda in the leptospiral sonicate. This antigen was designated

p12. Each of the antisera raised against p20 recognised a band of 20 Kda in leptospiral extracts. Prior treatment of the leptospiral sonicate with proteinase K abolished the reactivity of both p22 and p20 (fig. 4) indicating that they were proteins. The p12 antigen was also sensitive to treatment with dithiothreitol.

Immunoblot analysis demonstrated that both antigens were present in an outer-envelope extract of *L. interrogans* serovar *copenhageni* strain L136. As potential surface antigens, p12 and p20 could be involved in immunity to infection by eliciting agglutinating or opsonising antibodies. However, the antisera raised against these antigens did not agglutinate whole leptospires in microscopic agglutination tests, nor did they opsonise leptospires for phagocytosis by mouse macrophages.

The antisera were used to immunostain transblots of sonicates of representative serovars of *L. interrogans*, *L. biflexa* and *L. illini* (table); 23 serovars from 20 serogroups of *L. interrogans* and five serovars from five serogroups of *L. biflexa* were

Fig. 3. SDS-PAGE of cell extracts from *E. coli* clones: lane A, JFM65 (pUC18); lane B, JFM65 (pLBA4); lane C, DH5 α (pLBA8); lane D, DH5 α (pLBA9); lane E, DH5 α (pUC18). The positions of molecular weight markers (Kda) are indicated.

Fig. 4. Immunoblot of *L. interrogans* serovar *pomona* strain L10 sonicate stained with rabbit antisera raised against the antigens, p12 and p20: lane A, 0–20% ammonium sulphate fraction of JFM65 (pUC18); lanes B and C, 0–20% ammonium sulphate fraction of JFM65 (pLBA1); lane D, 20-kda region from SDS-PAGE gel of DH5 α (pUC18); lanes E and F, 20-kda region from SDS-PAGE gel of DH5 α (pLBA9); lane G, 0–30% ammonium sulphate fraction of DH5 α (pUC18); lane H, 0–30% ammonium sulphate fraction of DH5 α (pLBA9). Lanes C and F were treated with proteinase K before immunostaining. Molecular weight markers (Kda) are indicated.

examined. The antigen p20 was detected in all 23 *L. interrogans* serovars and in three of the five *L. biflexa* serovars. Similarly, antigen p12 was detected in all but one, serovar *zanoni* strain L178, of the 23 *L. interrogans* serovars, and in two of the five *L. biflexa* serovars. The apparent molecular weight of the antigen designated p12 varied from 11 Kda to 14 Kda in different serovars. Neither of the antigens p12 or p20 was detected in *L. illini*.

Discussion

Others have cloned and obtained expression of leptospiral genes in *E. coli*, but they did not investigate the antigenic properties of the cloned products. For example, Yelton and Cohen (1986) cloned a tryptophan synthesis gene from the saprophyte *L. biflexa*, and Dain *et al.* (1985) cloned the haemolysin gene of *L. interrogans* serovar *pomona*. These authors did not investigate the antigenic properties of their cloned products but

examined their role in metabolism or pathogenesis, respectively.

In this study two leptospiral protein antigens were cloned and expressed in *E. coli*. Rabbit antisera raised against the cloned antigens were used to identify the native antigens in Western blots of leptospiral sonicate. Both antigens were proteins as demonstrated by their inactivation by proteinase K. The antigen encoded by the recombinant plasmid pLBA4 had a molecular weight of 20 000 in both its cloned and native forms. Subcloning of pLBA4 localised the p20 structural gene to a 2.2-kb *Pst*I fragment contained in opposite orientations in plasmids pLBA8 and pLBA9. Both subclones expressed the p20 antigen; however, strains with pLBA9, which contained the fragment in the opposite orientation to pLBA4, showed a marked increase in the level of expression of the antigen. These results suggest that in pLBA4 and pLBA8 the gene encoding the p20 leptospiral antigen is expressed from its own promoter, whereas

in pLBA9 the gene is orientated such that its expression is controlled by a promoter carried on the pUC18 portion of the plasmid. The second antigen (p12) had a molecular weight of 12 000 in its native form, but appeared to either adhere non-specifically to *E. coli* components, or to be produced in aggregated or fragmented forms in *E. coli*. In both cloned and native forms this antigen was sensitive to treatment with dithiothreitol, indicating that disulphide bonding was necessary to maintain its antigenicity.

Both p12 and p20 were shown to be present in the outer envelope of *L. interrogans* and could, therefore, play potential roles in inducing immunity to infection. Immunity to leptospirosis is humorally mediated and appears to depend on the production of agglutinating and opsonic antibodies (Adler and Faine, 1977; Adler *et al.*, 1980a, b; McGrath *et al.*, 1984; Jost *et al.*, 1986). However, rabbit antisera raised against the antigens p12 and p20 did not have agglutinating and opsonising properties. Although both antigens were present in the outer envelope preparation they may not be exposed on the surface of the organism but embedded at the base of the LPS, as has been postulated for some outer membrane proteins in other gram-negative bacteria (Sawada *et al.*, 1984). Indeed, an outer membrane protein not exposed on the bacterial surface has been identified by monoclonal antibodies in leptospires of serovar *copenhageni* (Jost *et al.*, 1988). We do not yet have sufficient information to assign definite structural locations or roles to p12 or p20.

The rabbit antisera were also used to determine the distribution of the two antigens within the genus *Leptospira* and in the closely related *Leptonema* species, *L. illini*, formerly classified as *Leptospira*. Antigen p20 was detected in all serovars and p12 was detected in all but one of the serovars tested of the pathogenic species *L. interrogans*. Both antigens were detected in some but not all serovars of the non-pathogenic species *L. biflexa*; neither was detected in *L. illini*. Thus, both p12 and p20 appear to be conserved antigens within the genus *Leptospira* and, as such, may have structural or metabolic roles. The absence of p12 in *L. interrogans* serovar *zanoni* and of both antigens in some serovars of *L. biflexa* may be due to the functions of these

components being performed by antigenically different proteins in these serovars. As the current method of classification of leptospires relies solely on the agglutinating antigens, little is known about the relationship and specificity of non-agglutinating antigens within this classification.

The broad distribution of the antigens within the genus would seem to preclude a role in immunity following natural infections, as immunity to leptospirosis is at best serogroup specific. The lack of agglutinating or opsonic activity in antisera against either p12 or p20 is consistent with this hypothesis. However, results with rabbit antisera may not necessarily give an accurate indication of the specificity or significance of the antigens in naturally acquired infections of man and other species. Therefore, we are examining sera from human leptospirosis patients for antibodies against the two cloned antigens. Preliminary results indicate that some naturally-infected patients produce antibodies against p12 and p20.

Serodiagnosis of leptospirosis usually depends upon the MAT, which requires the use of whole, viable leptospires. Genus-specific antigen preparations have been used for diagnosis in various procedures such as passive haemolysis (Cox *et al.*, 1957), passive haemagglutination (Palit and Gula-sekharam, 1973) and enzyme immunoassay (Terpstra *et al.*, 1980). A reported advantage of these tests was their tendency to become positive earlier than the MAT; however others have reported high false-positive rates due to cross-reactions with saprophytic leptospires (Bragger and Adler, 1976). Furthermore, the prerequisite of growing large quantities of leptospires for antigen has limited the use of some of the tests. It is possible that both these problems may be overcome by the use of cloned antigens which are found either exclusively or predominantly in the pathogenic species *L. interrogans*. Further studies are aimed at determining whether the cloned antigens p12 and p20 could be used for this purpose.

This work was supported by a research grant from the National Health and Medical Research Council, Canberra, Australia. We thank B. H. Jost for preparation of leptospiral outer envelope.

REFERENCES

- Adler B, Faine S 1976 Susceptibility of mice treated with cyclophosphamide to lethal infection with *Leptospira interrogans* serovar *pomona*. *Infection and Immunity* **14**: 703-708.
- Adler B, Faine S 1977 Host immunological mechanisms in the resistance of mice to leptospiral infections. *Infection and Immunity* **17**: 67-72.
- Adler B, Faine S 1978 The antibodies involved in the human immune response to leptospiral infection. *Journal of Medical Microbiology* **11**: 387-400.

- Adler B, Faine S 1983 A *pomona* serogroup-specific, agglutinating antigen in *Leptospira*, identified by monoclonal antibodies. *Pathology* **15**: 247–250.
- Adler B, Faine S, Muller H K, Green D E 1980a Maturation of humoral immune response determines the susceptibility of guinea-pigs to leptospirosis. *Pathology* **12**: 529–538.
- Adler B, Murphy A M, Locarnini S A, Faine S 1980b Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **11**: 452–457.
- Auran N E, Johnson R C, Ritz D M 1972 Isolation of the outer sheath of *Leptospira* and its immunogenic properties in hamsters. *Infection and Immunity* **5**: 968–975.
- Boulard C, Lecroisey A 1982 Specific antisera produced by direct immunization with slices of polyacrylamide gel containing small amounts of protein. *Journal of Immunological Methods* **50**: 221–226.
- Bragger J M, Adler B 1976 A card test for the serodiagnosis of human leptospirosis. *Journal of Clinical Pathology* **29**: 198–202.
- Burns D M, Abraham L J, Beacham I R 1983 Characterization of the *ush* gene of *Escherichia coli* and its protein products. *Gene* **25**: 343–353.
- Chapman A J, Adler B, Faine S 1987 Genus-specific antigens in *Leptospira* revealed by immunoblotting. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene A* **264**: 279–293.
- Cox C D, Alexander A D, Murphy L C 1957 Evaluation of the hemolytic test in the serodiagnosis of human leptospirosis. *Journal of Infectious Diseases* **101**: 210–218.
- Dagert M, Ehrlich S D 1979 Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**: 23–28.
- Dain A A, Rozinov M N, Golltsmaier T A, Gershanovich V N, Chemukha I u G 1985 Cloning and the expression of the hemolysin gene of *Leptospira pomona* in *Escherichia coli*. *Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii* (7), 7–10.
- Faine S 1974 Leptospiral antigens. *Folia Facultatis Medicae, Universitatis Comenianae Bratislaviensis* **12**: Suppl 57–74.
- Faine S (ed) 1982 Guidelines for the control of leptospirosis. WHO Offset Publication No. 67, World Health Organization, Geneva.
- Farrelly H E, Adler B, Faine S 1987 Opsonic monoclonal antibodies against lipopolysaccharide antigens of *Leptospira interrogans* serovar *hardjo*. *Journal of Medical Microbiology* **23**: 1–7.
- Gregory R L 1986 Microbial ribosomal vaccines. *Reviews of Infectious Diseases* **8**: 208–217.
- Hawks R, Niday E, Gordon J 1982 A dot immunobinding assay for monoclonal and other antibodies. *Analytical Biochemistry* **119**: 142–147.
- Holmes D S, Quigley M 1981 A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**: 193–197.
- Johnson R C, Walby J, Henry R A, Auran N E 1973 Cultivation of parasitic leptospires: effect of pyruvate. *Applied Microbiology* **26**: 118–119.
- Johnson R C, Faine S 1984 *Leptospira* Noguchi, 1917. In: Krieg N R, Holt J G (eds) *Bergey's Manual of systematic bacteriology*, 9th edn, Vol. 1. Williams and Wilkins, Baltimore, pp 62–67.
- Jost B H, Adler B, Vinh T, Faine S 1986 A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. *Journal of Medical Microbiology* **22**: 269–275.
- Jost B H, Adler B, Faine S 1988 Reaction of monoclonal antibodies with species specific determinants in *Leptospira interrogans* outer envelope. *Journal of Medical Microbiology* **27**: 51–57.
- Kelson J S, Adler B, Chapman A J, Faine S 1988 Identification of leptospiral flagellar antigens by gel electrophoresis and immunoblotting. *Journal of Medical Microbiology* **26**: 47–53.
- Kemp D J, Coppel R L, Cowman A F, Saint R B, Brown G V, Anders R F 1983 Expression of *Plasmodium falciparum* blood-stage antigens in *Escherichia coli*: detection with antibodies from immune humans. *Proceedings of the National Academy of Science of the USA* **80**: 3787–3791.
- Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Maniatis T, Fritsch E F, Sambrook J 1982 Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, New York, pp. 93–94.
- Marshall R B, Wilton B E, Robinson A J 1981 Identification of *Leptospira* serovars by restriction-endonuclease analysis. *Journal of Medical Microbiology* **14**: 163–166.
- McGrath H, Adler B, Vinh T, Faine S 1984 Phagocytosis of virulent and avirulent leptospires by guinea-pig and human polymorphonuclear leukocytes *in vitro*. *Pathology* **16**: 243–249.
- Norrander J, Kempe T, Messing J 1983 Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**: 101–106.
- Ono E, Naiki M, Yanagawa R 1982 Production and characterization of monoclonal antibodies to lipopolysaccharide antigen of *Leptospira interrogans* serovar *kremastos* and *canicola*. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene A* **252**: 414–424.
- Ono E, Naiki M, Yanagawa R 1984 Isolation of antigenic oligosaccharide fraction from *Leptospira interrogans* serovar *canicola* with a monoclonal antibody. *Journal of General Microbiology* **130**: 1429–1435.
- Palit A, Gulasekharan J 1973 Genus-specific leptospiral antigen and its possible use in laboratory diagnosis. *Journal of Clinical Pathology* **26**: 7–16.
- Rood J I, Laird A J, Williams J W 1980 Cloning of the *Escherichia coli* K-12 dihydrofolate reductase gene following MU-mediated transposition. *Gene* **8**: 255–265.
- Sawada S, Suzuki M, Kawamura T, Fujinaga S, Masuho Y, Tomibe K 1984 Protection against infection with *Pseudomonas aeruginosa* by passive transfer of monoclonal antibodies to lipopolysaccharides and outer membrane proteins. *Journal of Infectious Diseases* **150**: 570–576.
- Terpstra W J, Lighthart G S, Schoone G J 1980 Serodiagnosis of human leptospirosis by enzyme-linked-immunosorbent-assay (ELISA). *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene A* **247**: 400–405.
- Towbin H, Staehelin T, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Science of the USA* **76**: 4350–4354.
- Vieira J, Messing J 1982 The pUC plasmids an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259–268.
- Yelton D B, Cohen R A 1986 Analysis of cloned DNA from *Leptospira biflexa* serovar *patoc* which complements a deletion of the *Escherichia coli* *trpE* gene. *Journal of Bacteriology* **165**: 41–46.
- Young I G, Jaworowski A, Poulis M I 1978 Amplification of the respiratory NADH dehydrogenase of *Escherichia coli* by gene cloning. *Gene* **4**: 25–36.