

Reaction of monoclonal antibodies with species specific determinants in *Leptospira interrogans* outer envelope

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Summary. A set of 24 monoclonal antibodies (MABs) was produced against an outer envelope preparation from *Leptospira interrogans* serovar *copenhageni*. The MABs reacted in enzyme immunoassay with species-specific determinants of an antigen in the leptospiral outer envelope (OE) of pathogenic but not of saprophytic species of *Leptospira*. The MABs did not agglutinate whole leptospires, nor could they opsonise homologous leptospires for phagocytosis by mouse macrophages or protect new-born guinea-pigs against lethal infection. The MABs reacted by Western blotting with a 35×10^3 -mol-wt band in OE separated on SDS-polyacrylamide gels, and also reacted with other bands to a lesser extent. The determinants to which the MABs were directed were localised in the leptospiral OE by immunogold labelling techniques.

Introduction

Leptospires are slender, helically coiled organisms surrounded by an outer envelope (OE) or sheath that encloses the protoplasmic cylinder. The protoplasmic cylinder contains the nuclear and cytoplasmic contents of the leptospire (Johnson and Faine, 1984). Thus an intact OE is essential for survival of the organism. The OE is approximately 11 nm in width and is composed of three to five electron-dense layers (Johnson, 1977). Although it is a structurally important component of the leptospire, a definite role for the OE in immunity to leptospirosis has not been established. OE contains lipid 35.7%, protein 45.8% and carbohydrate 6.0% (Auran *et al.*, 1975).

As agglutinating antibodies are important in immunity to leptospirosis (Adler and Faine, 1978a, b; Jost *et al.*, 1986; Farrelly *et al.*, 1987), it is probable that surface components of the leptospiral cells elicit the production of a protective humoral immune response. Auran *et al.* (1972) and Bey *et al.* (1974) described the isolation of leptospiral OE and demonstrated that this preparation was both immunogenic and able to confer protection against experimental infection. The OE preparation is very heterogeneous (Auran *et al.*, 1975) and nothing is known about the nature, specificity or contribution of individual antigens to its protective capacity.

Anti-OE antibodies may be protective because the antibody-complement reaction has been shown to cause damage to the OE that leads to death of the organism (Anderson and Johnson, 1968), but the identity of these target antigens has not been elucidated.

Leptospires are classified into serovars on the basis of cross-absorption agglutination reactions with rabbit antisera. Two serovars are considered antigenically different if 10% or more of antibody titre remains after absorption with homologous serum. Clearly, agglutinating antigens are involved in these reactions, but nothing is known about the nature of the antigens.

This paper reports the production and characterisation of monoclonal antibodies (MABs) directed against components in the leptospiral OE.

Materials and methods

Leptospires

Leptospira interrogans serovar *copenhageni* strain H45 was isolated from a rat (Faine and van der Hoeden, 1964). *Leptonema illini* strain 3055 was obtained from A. D. Alexander, Walter Reed Army Medical Center, Washington DC, USA. The other leptospiral serovars used in this study were provided by N. Stallman, WHO Leptospira Reference Laboratory, Brisbane, Australia. Cultivation and enumeration of leptospires were as described by Adler and Faine (1976).

Antigens, antisera and serological methods

Leptospiral OE was prepared by the method of Auran *et al.* (1972) and sonicated leptospires were prepared as described by Adler *et al.* (1980). The preparation of mouse antisera was as previously described (Tu *et al.*, 1982), as were the methods for performing the microscopic agglutination test (MAT), gel immunodiffusion and enzyme immunoassay (EIA) (Adler and Faine, 1983a). EIA with serovar *copenhageni* OE as the antigen was performed by coating the wells of the plates with OE 10 µg/ml in phosphate-buffered saline, pH 7.2 (PBS), instead of sonicated leptospires. Subclasses of mouse immunoglobulin were determined by reaction in gel immunodiffusion with specific anti-mouse immunoglobulins (Nordic Immunologicals, Tilburg, The Netherlands).

Production of monoclonal antibodies

Adult female BALB/c mice were immunised intraperitoneally (i.p.) with 200 µg (0.25 ml) of serovar *copenhageni* OE emulsified with an equal volume of Freund's incomplete adjuvant. After 28 days, the mice received an i.p. booster injection of 50 µg of serovar *copenhageni* OE in 0.5 ml of water. Spleen cells were harvested 4 days later and fusion and subsequent culture and cloning of the hybrid cells were performed as described by Jost *et al.* (1986). After large-scale culture, cells were removed by centrifugation and culture supernate was concentrated in an Amicon CH2 concentrator fitted with a HIP30-43 cartridge. IgG immunoglobulins were purified by affinity chromatography with Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Protein concentration was determined by absorption at 280 nm.

Opsonisation assays and passive protection experiments

Opsonisation and passive protection experiments were performed as previously described (Jost *et al.*, 1986).

Polyacrylamide gel electrophoresis and Western blotting

Leptospiral antigens were electrophoresed on discontinuous sodium dodecyl sulphate (SDS)-polyacrylamide 15% w/v gels for 4 h in a Protean II gel box (Bio-Rad, USA). Electrophoresed gels were stained for protein by the silver staining method of Morrissey (1981) and for carbohydrate by the method of Hitchcock and Brown (1983). Resolved antigens were electrophoretically transferred on to nitrocellulose membranes (Bio-Rad, USA) by the method of Towbin *et al.* (1979) in a Bio-Rad Transblot Cell for 3 h at 60V, a 1 in 2 dilution of their described transfer buffer being used. Reactions of MABs with blotted antigens were detected by means of a peroxidase-conjugated second antibody with chloro-1-naphthol (Merck, West Germany) as the chromogen (Hawkes *et*

al., 1982). Blotted antigens were digested with proteinase K 100 µg/ml (Sigma) in PBS at 37°C for 24 h. Control blots were incubated in PBS. Periodate oxidation of blotted antigens was as described by Woodward *et al.* (1985). The production of an anti-LPS MAB designated F1-1 was reported previously (Jost *et al.*, 1986).

Affinity purification of outer envelope protein

Twenty mg of purified (F9-10) IgG_{2a} was coupled to 1.0 g of Tresyl-activated Sepharose CL-4B (Pharmacia) according to the supplied directions. The coupled gel matrix was packed into a Pharmacia PD-10 column with PBS; 25 mg of serovar *copenhageni* OE in PBS was loaded on to the column and the unbound antigen washed through with PBS. Elution of bound OE protein was achieved with 0.1M glycine-HCl buffer, pH 2.7, and the eluted fraction was measured by absorption at 280 nm. The fraction containing purified OE protein was concentrated with an Amicon Ultrafiltration Stirred Cell fitted with a PM-10 membrane.

Immune electronmicroscopy

Leptospires were centrifuged at 3000 g for 30 min and resuspended in PBS. Immunogold labelling was performed as described by Barbour *et al.* (1984), with the modifications that grids were incubated for 20 min at room temperature in bovine serum albumin (Sigma) 1% w/v in PBS before incubation with antibody at 37°C for 1 h. PBS containing Tween 20 (Sigma) 0.1% v/v was used for all washes. A 1 in 10 dilution of gold-labelled goat anti-mouse immunoglobulins (Auroprobe EM GAM G5, Janssen Pharmaceutica, Beerse, Belgium) was used for the labelling.

Results

Characterisation of OE reactive MABs

MABs were of the IgG₁ or IgG_{2a} subclass and reacted in EIA with sonicated leptospires or OE preparation, but did not agglutinate live leptospires. As the serological reactions were identical for all 24 MABs produced, the results presented will be those derived from the use of one MAB, designated F9-10. The MABs reacted with all serovars of the Icterohaemorrhagiae serogroup and with representative serovars of all other *L. interrogans* serogroups, but did not react with several serovars of *L. biflexa* tested, nor with *Leptonema illini* (table).

The MABs did not precipitate sonicated leptospiral antigen or OE preparation in gel immunodiffusion experiments, nor did they opsonise homologous leptospires for phagocytosis by murine macrophages, as determined by chemiluminescence and indirect immunofluorescence assays (re-

Table. Reaction of MAB F9-10 with leptospiral antigens of different serovars as measured by enzyme immunoassay (EIA)

Species	Serogroup	Serovar	EIA* result
<i>L. interrogans</i>	Icterohaemorrhagiae	<i>copenhageni</i>	100.0
	Australis	<i>australis</i>	60.7
	Autumnalis	<i>autumnalis</i>	83.2
	Ballum	<i>ballum</i>	72.6
	Bataviae	<i>bataviae</i>	59.2
	Butembo	<i>butembo</i>	52.6
	Canicola	<i>canicola</i>	72.3
	Celledoni	<i>celledoni</i>	78.8
	Cynopteri	<i>cynopteri</i>	98.5
	Djasiman	<i>djasiman</i>	84.4
	Grippotyphosa	<i>grippotyphosa</i>	72.3
	Hebdomadis	<i>hebdomadis</i>	63.7
	Javanica	<i>javanica</i>	77.8
	Panama	<i>panama</i>	74.1
	Pomona	<i>pomona</i>	77.0
	Pyrogenes	<i>zanoni</i>	65.2
	Sejroe	<i>hardjo</i>	101.5
	Shermani	<i>shermani</i>	118.2
	Tarassovi	<i>tarassovi</i>	88.9
<i>L. biflexa</i>	Andamana	<i>andamana</i>	—
	Semarang	<i>patoc</i>	—
		<i>semarang</i>	—
		<i>sao paulo</i>	—
<i>Leptonema illini</i>			—

* Expressed as percentage of OD at 488nm obtained with heterologous EIA compared with homologous *copenhageni* EIA (OD = 1.35); — = EIA OD < background.

sults not shown). The MABs could not passively protect newborn guinea-pigs from lethal challenge, even when amounts of 1 mg of MAB/day were administered for periods of up to 5 days after challenge.

Nature of the antigen

OE prepared from *L. interrogans* serovars *hardjo*, *pomona* and *copenhageni* was electrophoretically resolved on SDS-polyacrylamide 15% w/v gels and stained with silver, or Western blotted and stained with dilutions of MAB. Silver staining of resolved OE revealed the presence of numerous bands in the antigen preparations (fig. 1a). Some were common to all 3 serovars whereas others were unique. There was also a diffuse band in the (20–30) × 10³-mol. wt region of the gel which resembled the profile obtained when purified serovar *copenhageni* LPS was resolved and stained with a carbohydrate-specific silver stain (fig. 1b). This diffuse band in the OE preparation was shown to be LPS by

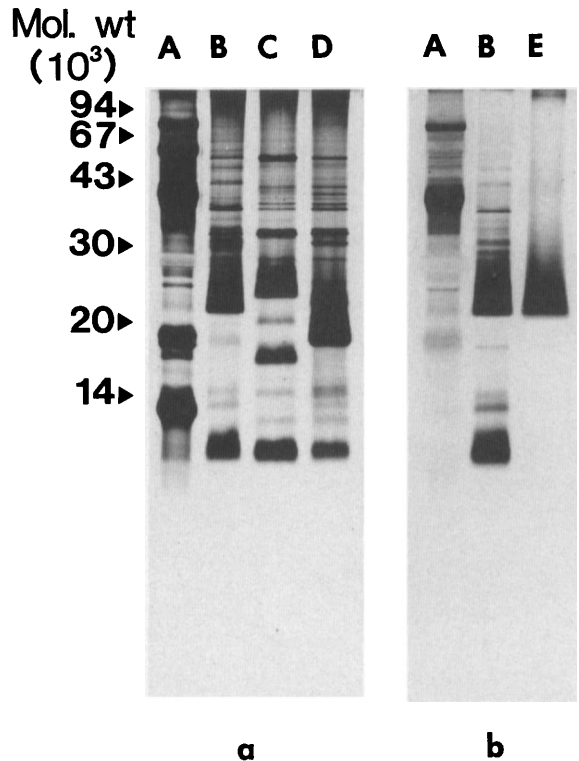


Fig. 1. SDS-PAGE of leptospiral antigens: (a) profile obtained by protein silver-staining; (b) profile obtained by carbohydrate silver-staining. Lanes: (A) Mol. wt marker proteins; (B) 10µg serovar *copenhageni* OE; (C) 10µg serovar *pomona* OE; (D) 10µg serovar *hardjo* OE; (E) 5µg serovar *copenhageni* LPS.

immunostaining with the anti-LPS MAB (Jost *et al.*, 1986; fig. 2b). Purified F9-10 IgG 50 µg/ml was incubated with blotted OE. The MAB reacted strongly with a 35 × 10³-mol. wt band in the OE preparation and also to a lesser extent with bands at mol. wts (10³) 51 and 62 (fig. 2a). Reaction of F9-10 with the 35 × 10³-mol. wt band was also seen in leptospiral sonicate antigen (fig. 2a). The other 23 MABs reacted in identical manner to F9-10 with the OE in Western blotting experiments. The profiles obtained with these MABs resembled that of a previously reported anti-leptospiral MAB (Adler and Faine, 1983b). To determine the chemical nature of the epitopes to which the MABs were directed, blotted OE was treated with proteinase K or periodate and stained with F9-10 as previously described (fig. 3). Treatment with proteinase K completely eliminated reaction of F9-10 with the major 35 × 10³-mol. wt band whereas the control blot was unaffected. Treatment of the blotted OE with periodate did not alter the binding of antibody indicating that protein and not terminal reducing sugars are important in the determinants.

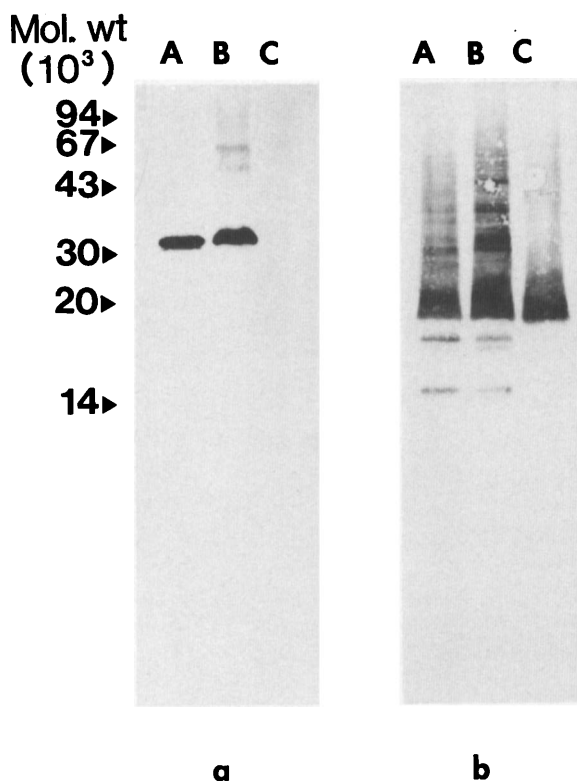


Fig. 2. Western blot analysis of resolved serovar *copenhageni* antigens: (a) profile obtained by staining with F9-10 IgG (anti-OE) 50 µg/ml; (b) profile obtained by staining with F1-1 IgA (anti-LPS) 50 µg/ml. Lanes: (A) 20 µg sonicate antigen; (B) 20 µg OE; (C) 5 µg LPS.

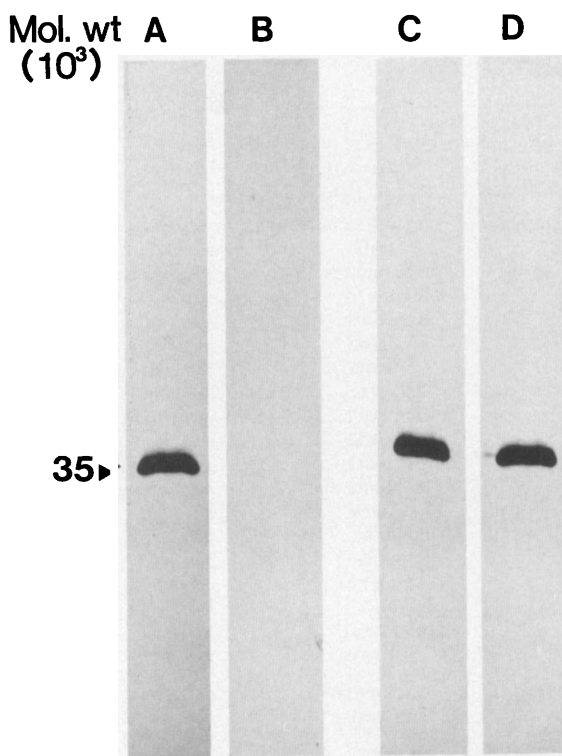


Fig. 3. Western blot analysis of 20 µg of serovar *copenhageni* OE resolved on a 10% w/v SDS-polyacrylamide gel and stained with F9-10 IgG 50 µg/ml following treatment with: Lanes (A) buffer (control); (B) proteinase K; (C) buffer (control); (D) periodate.

Reaction of F9-10 with blotted leptospiral sonicate antigens from other *L. interrogans* serovars produced a similar pattern of staining (not illustrated), regardless of the identity of the *L. interrogans* serovar used, demonstrating the species-specificity of the epitope with which F9-10 reacts. F9-10 did not react in Western blotting experiments with either *L. biflexa* serovar *patoc* or *Leptonema illini* sonicate antigens.

Affinity purification of the 35 × 10³-mol. wt OE protein

By means of affinity chromatography, a small amount of purified OE protein (designated p35) was obtained. This antigen was electrophoresed, blotted and immunostained with F9-10 MAB as previously described. Staining with the MAB revealed a single band at 35 × 10³ mol. wt which corresponded to the 35 × 10³-mol. wt band in the untreated OE (fig. 4). There was insufficient

material available to determine whether the bands at mol. wts (10³) 51 and 62 were co-purified.

Location of the 35 × 10³-mol. wt antigen on the leptospire

Immunogold labelling of leptospires and ultra-thin leptospiral sections were used to define the structural location of the epitopes with which the MABs reacted. During centrifugation of the leptospires before the immunogold labelling experiments, much of the outer layer of the leptospires was removed. However, in several areas the outer layer remained intact and F9-10 bound specifically to this structure but not to the underlying protoplasmic cylinder (fig. 5). No reaction of the MAB was noted with the flagellum (fig. 5) or interior components of the cell (not illustrated).

Discussion

Although there have been extensive studies on leptospiral LPS antigens (Shinagawa and Yana-

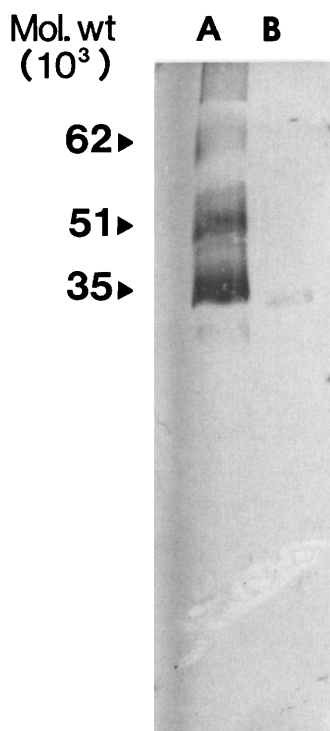


Fig. 4. Western blot analysis of serovar *copenhageni* OE proteins stained with F9-10 50 µg/ml. Lanes (A) 20 µg OE; (B) 1 µg affinity-purified OE protein.

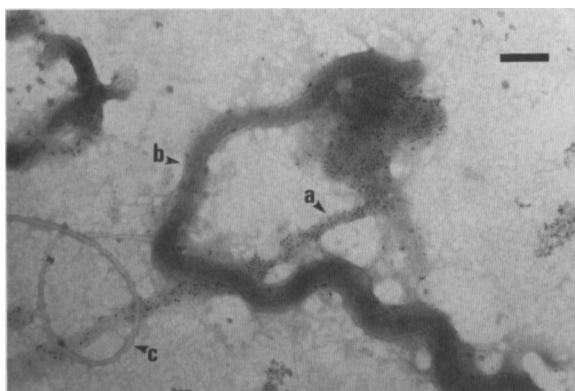


Fig. 5. Immunogold labelling of serovar *copenhageni* with F9-10 200 µg/ml showing reaction with detached outer envelope (a) but not with protoplasmic cylinder (b) or flagellum (c); bar = 200 nm.

gawa, 1972; Adler *et al.*, 1980; Ono *et al.*, 1984; Vinh *et al.*, 1986) there is little information available about the protein antigens of *Leptospira* and their role in immunity. Previous work has shown that antigens in the leptospiral OE are immunogenic and confer immunity to leptospirosis (Auran *et al.*, 1972; Bey *et al.*, 1974; Glosser *et al.*, 1974; Bey and

Johnson, 1978*a, b*, 1982; Broughton and Scarnell, 1985). The antibodies produced in response to this immunogen were agglutinating and produced only serovar-specific (Bey and Johnson, 1982) or limited intra-serogroup immunity (Auran *et al.*, 1975). The antigens responsible for the protective capacity were not characterised and we have herein attempted to define some of the OE antigens by use of MABs.

The 24 MABs selected for study were species-specific, did not agglutinate leptospires nor passively protect animals from infection with the homologous serovar. The MABs all reacted identically with a 35×10^3 -mol. wt protein found in all serovars of pathogenic, but not saprophytic leptospires tested. These MABs reacted identically with a previously reported anti-leptospiral MAB, D5 (Adler and Faine, 1983*b*) which was shown to be species-specific but the reacting antigen was not identified. The determinant with which the MABs reacted was also found to lesser extents in protein species of mol. wts (10^3) 51 and 62 which correspond approximately to some of the putative surface-exposed proteins of Nunes-Edwards *et al.* (1985). It is not possible to conclude whether these proteins share a common epitope or whether the higher-mol. wt proteins were complexed or contaminated with trace amounts of the 35×10^3 -mol. wt protein.

From the PAGE profile of leptospiral OE, it is apparent that the preparation was not pure. As well as containing many different protein species, it contained a significant amount of contaminating LPS. It has already been shown that LPS and outer membrane proteins (OMPs) from other bacteria can form close, possibly covalent, associations that make the antigens difficult to separate for analysis (Poxton *et al.*, 1985).

It is also well documented that agglutinating antibodies directed against the leptospiral LPS are opsonic and protective (Adler and Faine, 1978*a, b*; Jost *et al.*, 1986; Farrelly *et al.*, 1987), unlike the MABs reacting with the leptospiral OE. A similar situation was observed with other organisms, such as *Pseudomonas aeruginosa* (Hancock *et al.*, 1982; Sawada *et al.*, 1984), in which the MABs directed against the OMPs were also non-agglutinating and were species specific or, at least, more widely cross-reactive than the anti-LPS MABs. In passive protection experiments, the anti-LPS MABs were more protective than those MABs directed against the OMPs (Sawada *et al.*, 1984), even though both LPS and OMPs are the major exposed components in gram-negative cells. Although the OMPs of *P. aeruginosa* and the OE proteins of *L. interrogans* appear to be located in the outer cell walls of these

organisms, there is no evident explanation as to why they do not elicit agglutinating antibodies. Possibly the OMPs are embedded deeper in the cell wall than the LPS or, as suggested by Sawada *et al.* (1984), the OMPs may be situated at the base of the protruding LPS.

OE preparations have been described and used for immunisation of various animal species in bacterins currently used as veterinary vaccines (Glosser *et al.*, 1974; Bey and Johnson, 1978*a, b*, 1982; Broughton and Scarnell, 1985). Takashima and Yanagawa (1975) attempted to define the protective fractions in leptospiral cell walls. They found that the protein fraction of OE was relatively ineffective as a protective antigen. From our study of these protein antigens with MABs, it appears that the proteins might not be important in protection. It seems likely that the LPS contaminating the OE preparations elicits the agglutinating, protective, serovar-specific antibodies found in animals immunised with these bacterins. Although there may be other protective antigens in the leptospiral OE, previous work by Nunes-Edwards *et al.* (1985) suggests that OE proteins detected by the MABs may be the major surface-exposed OMPs. It is not known whether the other putative protective antigens would elicit significant protective immune responses. Production of MABs against these other antigens would be necessary for investigation of this possibility.

It is possible that the most immunodominant antigen in the leptospiral OE is LPS, because the

antibodies produced in animals immunised with OE bacterins are agglutinating and predominantly serovar-specific, although in these studies, as the antibody response is measured only by MAT, antibodies against other non-agglutinating antigens would not be detected. This corresponds to the type of antibody response seen in LPS-immunised rabbits (Adler and Faine, 1978*b*) and hamsters (B. H. Jost, unpublished observations). The lack of species immunity in naturally acquired leptospirosis may be due to lack of major species-specific antigens that elicit protective antibodies. There are no data available on the human response to OE bacterins, although sera from infected human patients do react with the major OE proteins by Western blotting (B. H. Jost, unpublished observation).

Although our results do not show a role for the 35×10^3 -mol. wt protein species antigen in immunity, the antibodies directed against it may be useful in providing a simple, rapid method for the identification of clinical isolates as pathogenic *L. interrogans* without the need for time-consuming biochemical or conventional serological methods. Indeed such an application has already been reported (Jost *et al.*, 1987).

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