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EXPRESSION OF LEPTOSPIRA BIFLEXA SEROVAR PATOC
ANTIGENS IN ESCHERICHIA COLI JA221

BY

JOANN WILLGOHS MOGARD

A thesis submitted in partial fulfillment
of the requirements for the degree
Master of Science
Major in Microbiology
South Dakota State University
1988

EXPRESSION OF LEPTOSPIRA BIFLEXA SEROVAR PATOC
ANTIGENS IN ESCHERICHIA COLI JA221

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Helen N. Westfall
Major Advisor

Date

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Head, Microbiology Dept.

Date

ACKNOWLEDGEMENTS

I would like to thank Dr. Robert Todd and the Microbiology Department for supporting my two years at SDSU with a graduate research assistantship.

I would like to thank the students, faculty, and staff of the Microbiology Department for sharing their expertise, supplies and equipment with me.

To Dr. David Benfield: "Thank you for allowing me to use your research facilities and supplies at the Veterinary Science Department throughout this past year." Also, thank you for your time and expertise in discussions of my research. Special thanks to Eric Nelson for his technical assistance and theoretical discussions while I learned the techniques of polyacrylamide gel electrophoresis and western blotting.

To the members of my committee I am indebted for their analysis, discussions and contributions throughout the execution of this research. Thank you for your time and efforts.

I am deeply indebted to Dr. Helen Westfall, my major advisor, for her personal kindness, support, and understanding. She has given generously of her time, knowledge and encouragement throughout my research. Her advice, patience, and comments have been invaluable during the writing of this thesis. Thank you!

Finally, thank you to Vickie Molengraaf for her valuable assistance in printing this thesis.

DEDICATION

To the memory of my parents, Hugo and Marie Willgohs, for instilling in me the value of an education, as well as the work ethic and determination to reach my educational goals.

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INTRODUCTION

In 1886, Weil described an infectious disease characterized by sudden onset of malaise, muscular pain, fever, jaundice, albuminuria, and in severe cases epistaxis, subcutaneous hemorrhages and lymphadenitis (113). The disease is still often named after him, although most correctly it refers to the often fatal form of human leptospirosis caused by Leptospira interrogans serovar icterohaemorrhagiae. By 1914, Inada and his colleagues (59) had successfully transmitted the disease to guinea pigs by inoculating them with the blood of patients suffering from the Japanese form of Weil's disease. These investigators named the organism Spirochaeta icterohaemorrhagiae (59). Concomitantly, according to Noguchi (84), Hubener and Reiter identified the spirochete in the tissues of experimental animals, thus confirming the causative agent of Weil's disease, and named it Spirochaeta nodosa. Studies published in 1917 by Noguchi (84) demonstrated (i) the relatedness of these two organisms as well as several other strains, (ii) the presence of a morphologically and pathologically similar spirochete in the kidneys of wild rats, and (iii) morphologic differences suggesting the need for a new genus. Leptospira was the name given to these morphologically distinct organisms (84).

The genus Leptospira belongs to the order Spirochaetales and the family Leptospiraceae (63). Currently, there are two Leptospira species: L. interrogans, which includes the pathogenic or parasitic organisms; and L. biflexa, all of which are saprophytic, free-living

leptospire (63). Approximately 240 different serovars, the final taxonomic subdivision of these organisms, have been reported based on antigenic analysis via microscopic agglutination and cross-agglutination absorption tests (63). These serovars are categorized into serogroups (approximately 60) due to antigenic cross-reactivity (63). In addition to antigenic analysis, a number of biological and biochemical tests are used to distinguish the two species including: growth at low temperatures (65), sensitivity to 8-azaguanine (66) and/or the purine derivative 2,6-diaminopurine (64), the presence or absence of lipase activity (64), the ability to grow in trypticase soy broth (63), and the requirement of 1-2% NaCl for growth (63). These biological and biochemical tests unfortunately do not distinguish between serovars, therefore serology remains the primary means of identification of the specific serovar (6).

The organisms are flexible, right-handed (23) helical rods that are 0.1 μm in diameter and 6-12 μm in length. The hooks seen at both ends of this negatively stained Leptospira (Figure 1) are typical of the organism. Leptospire is entirely enclosed in the outer cell envelope or sheath (OS). Inside this OS is the protoplasmic cylinder which contains both cytoplasmic and nuclear regions enclosed by the cytoplasmic membrane-cell wall complex (21,22). Around the helical protoplasmic cylinder are wound 2 periplasmic flagella (PF), also commonly called axial filaments or endoflagella (23). Each PF is subterminally attached to the protoplasmic cylinder (25). The flagella do not overlap; they extend



Figure 1. Electronmicrograph of Leptospira biflexa serovar patoc negatively stained with phosphotungstic acid (PTA). 20,000X. Line scale equals 1 um.

Stuart (102) developed an early culture medium in which the nutritional requirements of leptospires were provided in the form of a rabbit serum supplement. One major drawback of this medium is that variability in rabbit serum leads to inconsistency in maximal growth of the leptospires (10). In 1965, Ellinghausen and McCullough (36) developed an albumin medium for leptospires, which Johnson and Harris improved upon in 1967 (65). Modified Ellinghausen, McCullough, Johnson and Harris (EMJH) medium or the modified albumin medium of Bey and Johnson (11) are the most commonly used media in the United States (10). The above described albumin media require preparation of separate albumin supplements and chemically defined basal media which makes media preparation both difficult and time consuming. Medium that supports the growth of leptospires is now commercially available, but its expense often precludes its routine use.

Members of the species Leptospira interrogans are the causative agents of leptospirosis in man and animals, a zoonoses of worldwide proportion. Originally named Weil's disease, it has also been known as seven-day fever, autumn fever, swamp fever, canicola fever, pea-picker's fever and many others (34). These alternative names have caused problems in diagnosis of leptospirosis, because they all represent the same disease, with variations in the infecting serovar only. Clinical manifestations can range from mild influenza-like illness to aseptic meningitis as well as severe kidney and liver involvement (28,33,47,109,116). A Brazilian study (31) recently demonstrated evidence of cardiovascular involvement

esophagus, and the conjunctiva of the eye being the most common portals of entry of the organism (6,29). These organisms can also be sexually transmitted in pigs, wild animals, and possibly man (37,39,47,55,109).

Regardless of the infecting strain or serovar, the disease is biphasic. The first phase is characterized by leptospiremia; the second by leptospiruria and the presence of detectable serum antibodies (6,28,108,109). The presence of septicemia during the acute, first phase can lead to involvement of any tissue or organ (109). In most tissue, the phagocytes effectively remove the organisms. They do, however, tend to colonize the proximal convoluted tubules of the kidneys (6), possibly due to the absence of phagocytic cells, and are shed in the urine producing leptospiruria (109). This carrier or shedder state may persist from several days to months or years depending on the host (6,109), and is the most important factor in spreading the infection (109).

Until 1960, human leptospirosis was primarily an occupation-associated disease found in veterinarians, dairymen, swineherdsman, slaughterhouse workers, coal miners, and fish or poultry processors (6,47). Since that time many outbreaks of leptospirosis have resulted from recreational exposure to contaminated water (20,32,37,47,75). Recreational exposure is especially common when livestock have access to the water supply, or the leptospire are washed into the water after accumulating in the soil of livestock breeding areas (92).

Since 1970, epidemics of human leptospirosis have been virtually absent, and the number of cases has been reduced substantially in all developed countries (52). At the present time the number of leptospirosis cases reported annually is 50-150 in the United States, 80-180 in Great Britain, 100-180 in France, 25-80 in West Germany and 20-70 in Switzerland (52). The incidence of human leptospirosis caused by serovars icterohaemorrhagiae and canicola has been reduced due to improved hygiene and working conditions, rat control, and effective vaccination of dogs (37). However, serovar hardjo has emerged in recent years as a human pathogen (37). Many of these cases are not reported, because the disease lacks pathognomic features of leptospirosis and is not clinically as severe as that caused by serovar icterohaemorrhagiae. It can, however, result in lymphocytic meningitis, hepato-renal failure and death in 38, 9, and 2% of the cases, respectively (37). Thus, the reported annual cases of leptospirosis is most likely significantly lower than the actual incidence of the disease.

Livestock and dogs are important reservoirs of pathogenic leptospires and can be severely affected by certain serovars. The incidence of affected cattle and swine in the United States has been conservatively estimated at 18 million and 5 million cases, respectively (54). In Central and South America, where the disease is more prevalent, an estimated 62 million cattle and 7.5 million swine are affected by leptospirosis (54). These figures, indicating

nearly 100 million affected livestock in the Western hemisphere alone, illustrate the widespread nature of this disease.

Serovar hardjo causes severe economic loss, especially in the dairy industry. Two clinical conditions are recognized: (i) agalactia, the acute form of leptospirosis resulting in a sudden drop in milk yield that may persist for prolonged periods of time (37), and (ii) reproductive wastage resulting in late term abortions, stillbirth, and premature or term live, weak calves (37). Serovar hardjo has been implicated in 20-69% of aborted bovine fetuses and represents the most severe financial loss in the cattle industry due to leptospirosis (37, 41-43,71). While cattle are the natural host of serovar hardjo, other serovars responsible for bovine leptospirosis include serovars icterohaemorrhagiae, canicola, pomona, grippotyphosa, szwajizak, and balonica (54).

Leptospirosis is not a commonly recognized clinical disease in sheep (54), although severe to fatal disease caused by serovars pomona and hardjo have been reported in New Zealand, Italy, Australia, and the United States (72). Hardjo is the major infecting serovar in sheep (37,72). They appear to be less susceptible than other livestock except during the immunocompromised periods: i.e. 2 weeks pre-lambing through 1 week post-lambing for ewes and the first week of life for neonatal lambs (36,37). Agalactia and reproductive decline are once again the primary problems (37).

Pigs are the newly discovered host of serovars muenchen, and bratislava (37). They also remain the primary natural carrier of

serovar pomona (54). Reproductive losses and infertility are the primary manifestations of the disease, causing severe problems in the swine industry (37). Serovars tarassovi, australis, grippotyphosa, canicola, icterohaemorrhagiae, hardjo, and autumnalis have also either been isolated from or serologically detected in the swine population (37,54,68).

In horses, bratislava is the most common infecting serovar, although there is some geographical variation in the predominant serovar (35). While the majority of leptospiral infections are asymptomatic (35), abortion and fatal jaundice in foals are prominent features of the disease (37). Periodic ophthalmia (moon-blindness) has been reported as a common sequela in equine leptospirosis (109). A significant association between serologic evidence of leptospiral antibodies and periodic ophthalmia has been found (35).

Acute and chronic nephritis due to serovar canicola is now rare in the canine population due to effective vaccination of dogs (8,37,78). Infertility and abortions caused by bratislava, however, can be a severe problem in breeding colonies (37). Other serovars that cause canine leptospirosis include australis, autumnalis, ballum, bataviae, grippotyphosa, and pomona (8).

The prevalence of leptospire in livestock, dogs, humans, and wildlife presents problems in control of the disease and makes eradication virtually impossible. No human vaccine currently exists (5), thus prevention of human leptospirosis relies on public awareness; improvement of hygienic conditions in farmyards,

slaughterhouses, and laboratories; and avoidance of recreational exposure in livestock-contaminated water (47). The most common method of control in livestock and dogs is vaccination (10,105). Improved control can be achieved when vaccination is used in combination with streptomycin for prophylactic chemotherapy (10,71,100,105). Since currently available vaccines are serovar-specific (10,91,105), the best protection is afforded with multivalent vaccines containing the serovars that are endemic in a given geographic area (10,91,105). Additional control can be achieved by isolation of infected animals, rodent control, and adequate fencing, especially around watering areas (10,91).

Presumptive laboratory diagnosis of leptospirosis is based on dark-field microscopic demonstration of Leptospira in blood, urine, or tissue samples (6,107,109). They appear as very thin, rather long, finely coiled helices. Since the concentration of leptospire in the sample is usually low, the organisms are often difficult to detect (6). Alternatively, concentration of the organisms via centrifugation can result in false positives due to misidentification of fibrils or other cellular extrusions as leptospire (6). Special staining techniques also are of limited use. Fluorescent antibody techniques, enzyme-linked immunosorbent assay (ELISA) (4), chemiluminescent immunoassay (111), and the radiometric method (76) for detection of leptospire while successfully used by several researchers, are not routinely used in diagnosis of leptospirosis (6).

Definitive diagnosis requires either culture of the organism or serologic identification (6,107,108). Since leptospires have a relatively long generation time (6-16 hours) (63), cultures are often not positive for 5-7 days and are not considered negative until after 6 weeks of incubation (6). The microscopic agglutination test (MAT) uses live, pathogenic leptospires, is relatively serovar-specific, and remains the standard method of serologic identification (6,26). The MAT detects IgM as well as IgG, making it useful in diagnosis of both acute and chronic leptospiral infections (27). Performance of this test requires maintenance of at least 5 serovars, all of which are pathogenic, and its use is limited primarily to reference laboratories (6,26).

In fetal infections caused by serovar hardjo, serologic diagnosis is often misleading or inaccurate, making culture of the organism critical for definitive diagnosis (3). A major problem in culturing leptospires is contamination with other, faster growing organisms, especially from nonsterile samples such as aborted fetal tissues (3). Recently, Adler and colleagues (3) developed an antibiotic-containing leptospire medium that circumvents this problem. This semi-solid antibiotic medium contains actidione, bacitracin, 5-fluoro-uracil, neomycin sulphate, polymixin B and rifampicin, and is superior to EMJH medium in reducing contamination problems (3). Currently, neither serologic tests nor isolation procedures provide definitive diagnosis prior to the end of the first week of the disease (6). Diagnosis of leptospirosis is, therefore,

rarely of clinical value; since doxycycline, penicillin, erythromycin, or tetracycline therapy should begin within 10 days of symptom onset to effectively reduce the duration of fever, the extent of hospitalization, and the likelihood of relapse (18,28,47).

Management and treatment of leptospirosis is independent of the infecting serovar (6). Therefore, a genus-specific test for leptospirosis would be sufficient for the clinician, and the serovar-specific nature of the microscopic agglutination test is necessary only from an epidemiological standpoint. The microscopic agglutination test presents a safety hazard for laboratory personnel, because maintenance of live, pathogenic leptospires is required (6,45,109,114). About 70 cases of leptospirosis among laboratory personnel have been reported, making it the sixth most common laboratory-acquired bacterial infection (16). The primary source of these infections is cultures of leptospires, while a few cases are due to bites of infected laboratory animals (16). Thus, a genus-specific serologic test, using nonpathogenic leptospiral antigens, would avoid the safety hazard.

Two genus-specific antigens that have been successfully used for serologic testing include the 50% ethanol soluble-90% ethanol insoluble serovar patoc antigen (Pat E) of Baker and Cox (7,45,85,104) and the formalin-treated, sonicated serovar patoc antigen (Pat F) of Myers & Coltorti (45,80). The Pat E antigen has been used in a hemagglutination test using sheep erythrocytes (7,85), and in the ELISA (45). Pat F is a precipitating protein antigen (80)

that has been detected via immunodiffusion and immunoelectrophoresis, as well as the ELISA (45,80). Fairbrother (46) has shown Pat E and Pat F to be closely related but not serologically identical. In addition, a boiled serovar patoc antigen of Nicolescu and Andreescu (82) has been used for the serologic diagnosis of human leptospirosis by the complement fixation test. Preparation of these antigens involves some type of extraction or other preparatory procedure.

The presence of at least one antigen common to all leptospires has been conclusively shown by Adler and Faine (1) using monoclonal antibodies (Mab) and enzyme immunoassay (EIA). Sakamoto and his colleagues (96) have characterized a partially purified leptospiral genus-specific protein antigen. This antigen was obtained by Triton X-100 treatment of serovars kremastos and canicola followed by fractionation with DEAE-cellulose column chromatography and ethanol precipitation (96). Chemical analysis revealed that the antigen is comprised of protein, neutral and amino sugars, but no detectable lipids (96). The genus-specificity of this antigen may reside in the protein since heating to 100° C or treatment with proteases destroyed the genus-specific activity (96). This antigen has been used for detection of antibodies in human and animal sera by an indirect hemagglutination test (97).

Since ample evidence suggests the presence of genus-specific leptospiral protein antigens, diagnosis of leptospirosis could be simplified if the genes coding for leptospiral antigens were expressed in a rapidly growing, easily cultivated bacterium. This

would eliminate:

- 1) the need for preparation of the relatively complex medium for cultivation of these organisms;
- 2) the time lag due to the long generation time of leptospire, and;
- 3) the safety hazard to laboratory personnel due to the need to maintain live, pathogenic organisms for the microscopic agglutination test.

The presence of leptospiral antigens might be detected by the filter-binding EIA procedure that was successfully used by Engleberg et al (44) for Legionella pneumophila antigens expressed in Escherichia coli. Stamm and associates (101) used a similar binding procedure to detect Treponema pallidum antigens in Escherichia coli K-12. These investigators (44,101) used modifications of the procedures of Meyers (79) and Henning (58) involving in situ cell lysis described by Raetz (94). The procedure involves transfer of transformant colonies (containing donor DNA) to nitrocellulose filters followed by chemical cell lysis and the detection of antigens via an enzyme immunoassay. The treated filter is incubated with serum containing antibodies that are specific for donor antigens, followed by a second incubation with an enzyme-labeled antibody directed against the first antibody (57). A substrate is added next which will detect the presence of bound enzyme (and, therefore, leptospiral antigens) by the formation of an insoluble colored product on the nitrocellulose filter. Lysed colonies that are positive can then be rescreened without chemical lysis to detect expression of donor antigens on the surface of E. coli cells. An important advantage of using

nitrocellulose in this procedure is its excellent protein-binding properties; this allows detection of 1 ng of purified immunoglobulin using a direct method, or 10 ng using an indirect method (14).

A similar procedure that is commonly called nitrocellulose-ELISA (NC-ELISA), dot immunoblotting, or dot enzyme-linked immunosorbent assay (Dot-ELISA) has been used by numerous investigators in diagnosis of leishmaniasis (87-89), leptospirosis (86,112), toxoplasmosis (90), detection of adenovirus and rotavirus (51), and enzyme assays (99). Additional advantages of using nitrocellulose filters are (i) the simplification of the washing steps, and (ii) its specificity which is comparable to the conventional ELISA using polystyrene as the attachment material (14,51).

The powerful protein binding capacity of nitrocellulose is also exploited in Western blotting, a procedure developed by Towbin et al (106). Western blotting involves the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper, allowing radiographic (19,106) or immunologic detection (30) and characterization of discrete protein bands. Molecular weight determinations of these bands can be calculated using the procedure of Neville (81). Additional information about the presence of carbohydrates attached to the proteins can be obtained by Schiff staining of polyacrylamide gels (49).

The availability of two gene libraries (courtesy of Nyles Charon, David Yelton, and Richard Zuener, West Virginia University,

Morgantown, WV) containing Leptospira biflexa serovar patoc DNA allowed investigation of the expression of leptospiral antigens by Escherichia coli JA 221. The filter binding-EIA can be utilized to screen these gene libraries for leptospiral antigens expressed by Escherichia coli. Western blotting can then be employed to characterize these antigens.

Three questions were posed:

- (i) would antigens of Leptospira biflexa serovar patoc be expressed in Escherichia coli JA221,
- (ii) could genus-, species-, and/or serovar-specific antigens expressed in E. coli be used in the serologic diagnosis of leptospirosis, and
- (iii) what is the nature of the leptospiral antigens expressed by E. coli?

The questions above formed the foundation for the research reported in this thesis.

MATERIALS AND METHODS

Organisms

Leptospira biflexa serovar patoc and Escherichia coli JA221 were obtained from Nyles Charon (West Virginia University) for use as positive and negative controls, respectively. Two recombinant genomic libraries of Leptospira biflexa serovar patoc were obtained from David Yelton, Richard Zuerner, and Nyles Charon, West Virginia University. One library was produced using the restriction endonuclease Bam HI (118), while the other was a Hind III library (Nyles Charon, personal communication). The donor DNA was inserted into the tetracycline gene of the plasmid pBR322 (where single restriction endonuclease sites for Bam HI and Hind III are located) (77) and Escherichia coli JA221 was transformed (118). All cells containing recombinant DNA should be ampicillin-resistant (the ampicillin gene of pBR322 remains intact) and tetracycline-sensitive due to insertional inactivation of the tetracycline gene.

Growth Medium, Conditions, and Preservation

Leptospira biflexa serovar patoc was maintained on semi-solid (0.35% agar, w/v) PLM5 medium (Armour Biochemical, Terrytown, NY). Serovar patoc was maintained by transferring 1 ml of inoculum to 10 ml sterile semi-solid medium every 2-4 months. As necessary, a 1 ml inoculum from the semi-solid medium was added to 1000 ml liquid PLM5 in a Corning 490 cm² tissue culture roller flask (Corning Glass

Works, Corning, NY) and incubated at 30° C for 5-7 days with rotation (c.a. 100 RPM) for use as a positive control. The parent cells plus the Bam HI and the Hind III gene libraries were preserved in the following manner: E. coli JA221 and the two gene libraries were cultivated overnight in 20 ml Luria-Burtani (LB) and LB plus 35-50 ug ampicillin/ml (LB-amp) broth, respectively, as described by Mantiatis et al (77). A stock solution of the sodium salt of ampicillin (25 mg/ml) (Sigma Chemical Co. St. Louis, MO) was prepared in water (77), filter sterilized, aliquoted, and frozen at -20°C for future use. The cells were harvested by centrifugation at 16,000 X g for 10 min using a Sorvall RC-5B Refrigerated Superspeed centrifuge. The pellet was resuspended in 5 ml LB or LB-amp containing 15% glycerol, as appropriate. Aliquots (0.5 ml) were placed in sterile glass ampules, sealed and frozen at -70° C. All positive clones (colonies from the gene libraries expressing leptospiral antigens as detected by FB-EIA) were preserved in a similar manner using LB-amp culture medium and sterile microcentrifuge tubes as containers. As necessary, E. coli JA221 or the positive clones were removed from the freezer, and cultivated on the appropriate broth or agar medium for experimental use.

Antigen Preparation

Whole cell antigens were prepared by cultivating L. biflexa serovar patoc in 500 ml PLM5 medium, and E. coli JA221 or the positive clones in 200 ml of the appropriate medium. Cells were

harvested by centrifugation at 13,000 X g for 15 min, and washed in phosphate-buffered saline (PBS) (0.15 M KH_2PO_4 , 0.9% NaCl, pH 7.2). The pellets were resuspended in PBS (1/50th of the original volume for serovar patoc, and 1/20th of the original volume for E. coli JA221 and the positive clones), and frozen at -70°C .

Cells for sonication were cultivated in 500 ml of the appropriate medium, harvested, washed, and resuspended in 20 ml PBS. Samples were then sonicated in an ice-water bath using the large probe of a Branson Sonic Power Sonifier (Branson Instruments, Inc., Danbury, CT). Two 20-30 sec bursts of high frequency sound waves (4.5 mA) with 1 min cooling in between were used to rupture the cells. Unbroken cells were removed by centrifugation at 10,000 X g for 10 min at room temperature. The supernatant (sonicate) was stored either at 4°C or -70°C . Alternatively, the membrane fraction of the sonicate was obtained by centrifugation at 30,000 X g for 20 min, resuspending the pellet in 0.15 M NaCl, and storing at 4°C for use in western blotting.

Protein Determination

Protein concentrations were determined by the Bradford method (17), as marketed by Bio-Rad, Richmond CA, and outlined in the Bio-Rad Protein Assay Instruction Manual (12). Either lyophilized bovine plasma gammaglobulin (Bio-Rad Laboratories, Richmond, CA), or albumin-globulin protein (Sigma) was used as the standard.

Preparation of Antiserum

Four New Zealand White rabbits were injected with whole Leptospira biflexa serovar patoc cells that had been frozen at -70°C in phosphate-buffered saline (PBS) (0.15 M KH_2PO_4 , 0.9% NaCl, pH 7.2). The immunogen (2 mg protein/ml) was mixed with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI) until an emulsion was formed. On Day 0 five sites per rabbit were injected (0.2 ml per site): intramuscularly in the nape of the neck and both hips, and subcutaneously in the axillary regions. On Days 13, 27, 41, and 54, each rabbit was injected in the ear vein with 1 mg protein. Rabbits were terminally bled by cardiac puncture on Day 60. The blood was allowed to clot for several hours and then centrifuged at $500 \times g$ in a Sorvall RT 6000 Refrigerated centrifuge to separate cells from the serum. Five ml aliquots of the serum were frozen and stored at -20°C .

Absorption of *E. coli* JA221 Antibodies

The immune serum was extensively absorbed to remove anti-E. coli JA221 antibodies. For this purpose, E. coli JA221 was grown to stationary phase, as determined by preliminary growth curves, in 250 ml flasks containing 175 ml LB broth with rotation (c.a. 200 RPM) at 35°C . The cells were harvested and washed twice in 50-75 ml PBS. Cell pellets were placed in sterile microcentrifuge tubes (1.5 ml), mixed with 0.5-0.7 ml serum, rotated gently at 4°C for 1 hr, centrifuged at $17,600 \times g$ for 5 min in a Sorvall RC-5B Refrigerated

Superspeed centrifuge using the HSMT rotor to recover the serum, and the serum was transferred to a fresh cell pellet. These steps were repeated 3 times. The absorbed serum was pooled, heat inactivated at 56° C for 30 min, and stored at -20° C in 1 ml aliquots.

ELISA

The titer of the rabbit antiserum was determined using a whole cell antigen of Leptospira biflexa serovar patoc. The concentrated antigen was diluted to yield 20 ug protein/ml in a 15 mM carbonate-35 mM bicarbonate buffer (pH 9.6) without sodium azide (coating buffer) (110). Ninety-six well microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 0.1 ml of the diluted antigen, and dried overnight at 42° C. Control wells were coated with coating buffer only. Coated plates were washed three times with gentle agitation using PBS containing 0.5% Tween 20 (working buffer). Two-fold serial dilutions of a 1:200 dilution of the antiserum were prepared in working buffer; 0.1 ml was applied to the appropriate wells and the plate was incubated at room temperature for 2 hr. Plates were again washed as above, followed by addition of 0.1 ml of a 1:1500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG, IgM, and IgA heavy and light chains) (CooperBiomedical, Malvern, PA). After another 2 hr incubation at room temperature, the wash step was repeated, with a final wash in 0.1 M citrate-phosphate substrate buffer, pH 5.0 (50). One tenth of a ml of ortho-phenylenediamine (OPD) (34 mg OPD/100 ml citrate-

phosphate buffer plus 40 ul 30% H₂O₂) (110) was added to all wells and the plate was incubated in the dark for 30 min at room temperature. The substrate reagent was stored at 4° C in a dark bottle and discarded when a color change was observed. The optical density at 490 nm (OD₄₉₀) was read on a Microelisa Minireader MR590 (Dynatech Laboratories, Alexandria, VA) ELISA reader. The instrument was blanked on citrate-phosphate buffer and the average OD₄₉₀ value of the control wells was subtracted from all results. Wells with a corrected absorbance greater than 0.5 OD₄₉₀ were considered positive. After determining the approximate titer of the antiserum, the actual titer range was established using 1:3000, 1:4000, 1:5000, 1:6000, 1:7000, and 1:8000 dilutions of antiserum.

Evaluation of absorption effectiveness was accomplished by coating an ELISA plate with E. coli JA221 whole cells (20 ug protein/ml). The ELISA was performed as above using 1:10, 1:100, 1:500, 1:1000, 1:2000, and 1:4000 antiserum dilutions.

Positive clones were evaluated by coating ELISA plates with whole or sonicated cells (20 ug protein/ml) and using 1:100, 1:500, and 1:1000 antiserum dilutions.

FB-EIA

Colonies from the gene libraries that express leptospiral antigens were detected via a filter-binding enzyme immunoassay (FB-EIA) in which the filter blots (FB) were produced by preparing spread plates of ampicillin-resistant transformants on LB-amp agar

and incubating them overnight at 35° C. Isolated colonies were then spotted in duplicate onto LB-amp agar plates either with sterile toothpicks or with disposable colony transfer pads (FMC Corp., Rockland, ME), and incubated overnight at 35° C. Colonies were then blotted onto round (85 mm diameter), dry nitrocellulose (NC) filters (0.45 µm) (Millipore Corp., Bedford, MA). On two separate NC filters, 1-2 µl of serovar patoc or E. coli JA221 whole cell suspensions were spotted. Colonies were lysed in situ by the method of Meyers et al (79). Briefly, NC filters were sequentially placed, colony side up for one min each, in four large petri dishes containing 3MM Whatman chromatography paper (Whatman Ltd., Maidstone, England) that was saturated with (i) 0.1 N NaOH, (ii) 1.5 M Tris-hydrochloride (pH 7.4), (iii) 300 mM NaCl-30 mM sodium citrate, and (iv) 70% ethanol. The filters were then vacuum dried at 56° C for 2 hr.

E. coli colonies expressing leptospiral antigens were detected by an enzyme immunoassay (EIA). To prevent nonspecific binding of proteins to the nitrocellulose, dried filters were placed in tris-buffered saline (TBS) (50 mM Tris-hydrochloride, 150 mM NaCl, pH 7.4) containing 3% gelatin (44) to block extraneous binding sites. After rotation at room temperature for 2 hr, the NC filters were transferred to 16 ml of a 1:800 dilution of rabbit anti-patoc serum in TBS plus 1.5% gelatin and incubated overnight at room temperature. The NC filters were then rinsed with distilled water, followed by 4 washes in 20 ml TBS with gentle rotation for 15 min at

room temperature. Washed NC filters were incubated for 2 hr at 30° C in a 1:3000 dilution of the same peroxidase-labeled goat anti-rabbit conjugate used in the ELISA. The washing step, described above, was repeated, and the NC filters were immersed in 0.05% 4-chloro-1-naphthol with 0.015% H₂O₂ for 10-15 min at room temperature. The observation of an insoluble purple product around a colony indicated the presence of leptospiral antigens. Any positive colonies were retrieved from the duplicate LB-amp plate and rescreened without chemical lysis. Positive clones were cultured on tetracycline-containing LB agar (12.5-15.0 ug/ml) (77) to test for tetracycline sensitivity. A stock tetracycline solution (12.5 mg/ml) was prepared using tetracycline hydrochloride in ethanol/water (50% v/v) (77), filter sterilized, aliquoted, and frozen at -70° C.

Plasmid Isolation

Colonies that were positive both in lysed and unlysed FB-EIA tests were selected for plasmid isolation using the protocol of D. Ish-Horowicz (77) for the alkaline lysis method of Birnboim and Doly (13). In this laboratory, the protocol was modified as follows: (i) 20 mg lysozyme was added to a 2 ml solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-chloride (pH 8.0) to facilitate digestion of the cell wall; (ii) the plasmid DNA was precipitated using 95% ethanol at -20° C for 2 hr; and (iii) RNase was not used in the 10 mM Tris-chloride, 1 mM EDTA (pH 8.0) buffer (TE) (77) to digest the RNA. Plasmid DNA in TE buffer was stored at 4° C for future use.

Agarose Gel Electrophoresis

Plasmids isolated from the positive clones were analyzed by agarose gel electrophoresis to determine their relative size in comparison to pBR322. Electrophoresis equipment consisted of a horizontal bed by Aquebogue Machine Shop, Aquebogue, NY, and a Buchler Instruments Voltage and Current Regulated D.C. Power Supply. A 1% agarose gel was prepared using 10X TPE (0.08 M Tris-phosphate, 0.002 M EDTA; pH 8.0) (77). The extracted plasmid DNA samples (30 ul) were added to 3 ul of 10X sample buffer [0.02% (w/v) Orange G (Fluka AG, Switzerland), 0.02% (w/v) xylene cyanol (Sigma), 0.02% (w/v) bromophenol blue, and 0.1mM EDTA in a 50% sucrose solution], and 20 ul of each sample preparation was added to the appropriate lane on the gel. Uncut pBR322 (Sigma) and untransformed *E. coli* JA221 were run as controls. Electrophoresis was carried out at room temperature for 13 hr using 70 volts of current. Ethidium bromide (1 ug/ml) was added to the electrophoresis buffer (TPE) approximately 5 min after electrophoresis was begun. The completed gel was washed in distilled water to remove any unbound ethidium bromide, examined under UV light (366 nm), and photographed with a Polaroid camera.

Polyacrylamide Gel Electrophoresis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli (73) on a 160 X 140 X 0.75 mm vertical slab gel. The composition of the running gel was 7.5% (w/v) acrylamide

(Bio-Rad), 0.19% (w/v) N, N, methylenebisacrylamide (bis) (Bio-Rad), 372 mM Tris (pH 8.7), 0.1% SDS, 0.03% ammonium persulfate (APS) (Sigma), and 0.00083% N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad). The stacking gel was 5% (w/v) acrylamide, 0.14% bis, 124 mM Tris (pH 6.8), 0.1% SDS, 0.05% APS, and 0.0025% TEMED. Twenty-five μ l of a 1:2 dilution of the sample in 2X sample buffer consisting of 8% SDS (w/v), 40% glycerol (v/v), 20% 2-mercaptoethanol (Bio-Rad), plus approximately 2 mg bromophenol blue as the tracking dye; this was heated to 80°C for 3 minutes. These sample preparations were frozen at -20°C and reused 3-4 times with repetition of the heating step prior to each use. Four SDS molecular weight markers (Sigma) [beta galactosidase (116K); bovine albumin (66K); egg albumin (45K); and, carbonic anhydrase (29K)] were reconstituted according the product insert and included in each gel. The running buffer consisted of 0.25 mM Tris-base, 192 mM glycine, and 1% SDS (pH 8.3). An SE600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA) with a PS1200 DC power supply providing a constant current of 35 mA/gel allowed separation of proteins into discrete bands. Following electrophoresis, the gel was stained for 4-12 hr at room temperature with rotation (c.a. 40 RPM). The staining solution was 0.11% Coumassie Brilliant Blue G (Sigma) in a mixture of 450 ml water:450 ml methanol:100 ml acetic acid. Gels were destained for 3-4 hr with a solution of 5% methanol and 10% acetic acid. A permanent record of the gel was obtained by drying the gel onto 3 MM Whatman chromatography paper using a Model SE540 gel dryer (Hoeffer).

Nondenatured polyacrylamide gels were run as above, with the elimination of SDS and beta-mercaptoethanol from the sample buffer, running buffer, and gels. Sample preparations were heated for 1 min at 60° C, then stored and reused as above. Five nondenatured molecular weight markers (Sigma) [bovine albumin (dimer-132K, monomer-66K); egg albumin (45K); carbonic anhydrase (29K); and, alpha-lactalbumin (14.2K)] were reconstituted according the product gel and included in each gel.

Western or Immuno-Blotting

Unstained gels prepared as above, with samples added in duplicate to produce 2 identical "minigels", were blotted onto nitrocellulose (NC) paper (.45 um Schleicher & Schuell Inc, Keene, NH) using the procedure of Towbin et al (106) as modified by Burnette (19). Blotting was carried out in 0.20 mM Tris, 0.15 M glycine buffer in 20% methanol for 3-4 hr at 125 mA using a Heath Regulated HV power supply. Subsequently, half of the NC paper was stained with 0.1% aniline blue (Allied Chemical Corp.) in a solution of 45% methanol and 10% acetic acid followed by destaining with a 2% acetic acid solution in 90% methanol, the other half was immunoblotted using rabbit polyclonal antiserum. NC paper for immunoblotting was incubated at room temperature for 1-2 hrs in PBS containing 0.5% Tween 20 to prevent nonspecific binding of proteins. The NC paper was then incubated overnight at 4° C in anti-leptospiral antiserum diluted 1/50 in PBS with 0.5% Tween 20. The blots were washed four

times for 5 min each in PBS, followed by a 1-2 hr incubation in a 1:400 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin. The wash step was repeated as above, and 4-chloro-1-naphthol (as in FB-EIA) was the substrate used to detect leptospiral antigens.

SDS molecular weight markers on the NC paper stained with aniline blue were used to construct a calibration curve. On semi-log paper, molecular weight was plotted on the y-axis and relative mobility (distance of protein migration/distance of tracking dye migration) (98) was plotted on the x-axis. Molecular weights of proteins on the immunoblots were determined by measuring the distance between the bovine albumin molecular weight marker and the unknown, and referring to the calibration curve.

Genus-, Species-, and Serovar-Specificity Testing

Rabbit antiserum, specific for several serovars of both L. interrogans and L. biflexa, was obtained from David Miller, National Veterinary Services Laboratories, Ames, IA. L. interrogans serovars pomona, bratislava, autumnalis, copenhageni, australis, grippotyphosa, pyrogenes, canicola, ballum, tarassovi, hardjo, and bataviae were used for genus-specific testing of the positive clones. L. biflexa serovars codice, LT430, gent, wa reiden, and waz P438 were used for species-specific analysis of the clones. FB-EIA, ELISA, and western blotting were used, as described above, in this analysis.

RESULTS

Isolated colonies from the Bam HI and Hind III gene libraries were screened for evidence of leptospiral antigens expressed by E. coli JA221 that had been transformed with the plasmid pBR322 containing leptospiral DNA. This analysis was possible only after anti-E. coli JA221 antibodies had been exhaustively absorbed from rabbit anti-patoc serum.

Preliminary growth curves of E. coli JA 221 were necessary so that cells could be harvested in stationary phase for absorption of E. coli antibodies from the anti-patoc serum. Figure 2 indicates that E. coli JA221 was in the stationary phase of growth after 22.5 hrs of incubation at 35° C with rotation (c.a. 200 RPM). E. coli cells harvested after 24 hrs incubation under these conditions were used for absorption of E. coli antibodies from the patoc antiserum. Four absorbed, pooled antisera samples (1:6000 titer by ELISA) were used throughout the experiment. ELISA results indicated evidence of effective removal of E. coli antibodies at dilutions greater than or equal to 1:500 ($OD_{490} < 0.1$).

Approximately 9,600 and 400 isolated colonies from the Bam HI and Hind III gene libraries, respectively, were screened by FB-EIA. All positive colonies (clones) were rescreened without chemical lysis. Initially five positive clones were found, 1 from the Hind III library and 4 from the Bam HI library. H-1 and H-2 are isolated colonies from the single positive colony that Kirsten Hall, SDSU

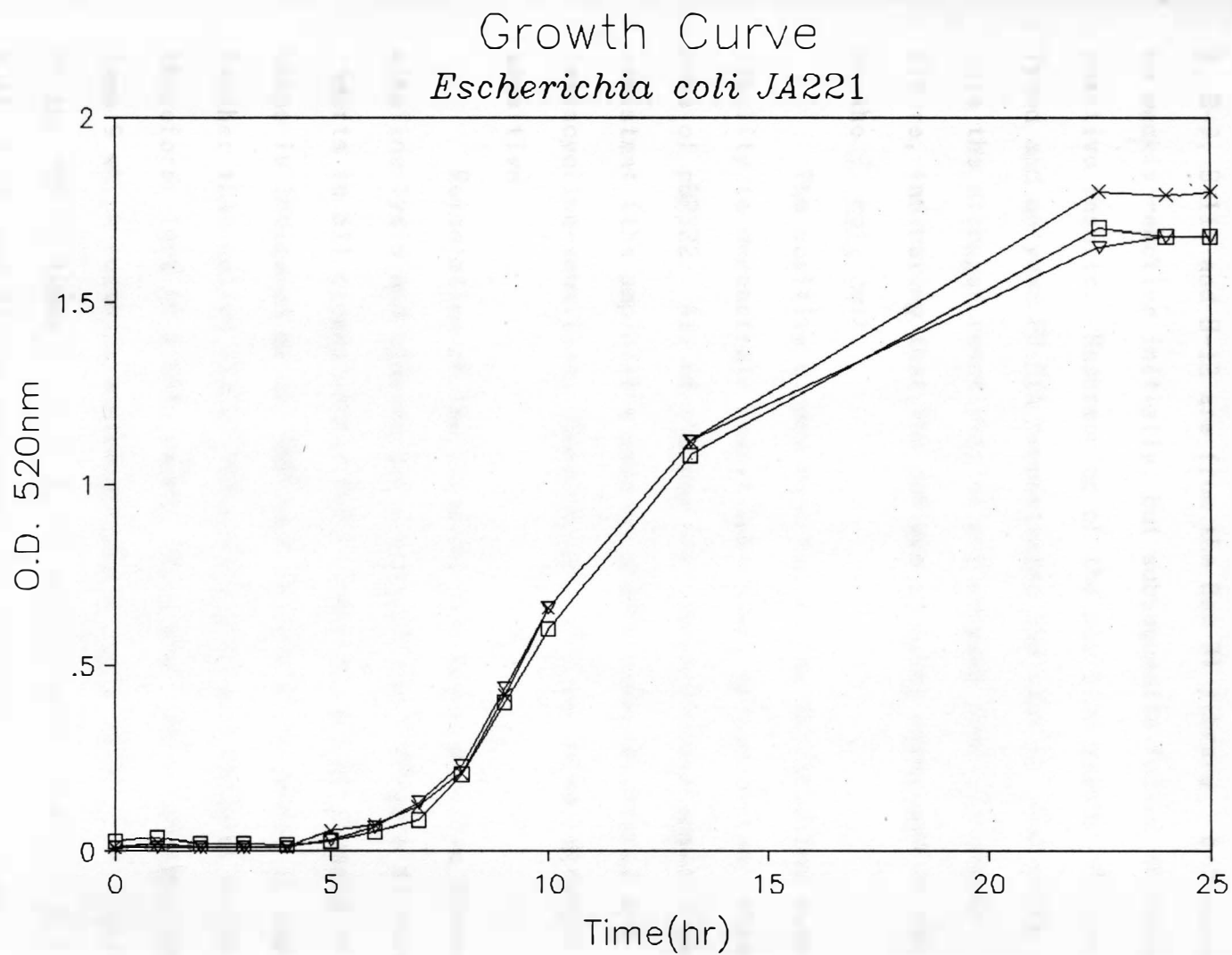


Figure 2. Twenty-five hr growth curves of Escherichia coli JA221 at 35° C with rotation (c.a. 200 RPM).

undergraduate, identified during a Microbiology Problems course. B-7, B-9, B-11, and B-12 are from the Bam HI library. B-9 appeared to be weakly reactive initially, but subsequently failed to demonstrate positive results. Rescreening of the positive clones (Figure 3) by lysed and unlysed FB-EIA demonstrated the lack of reactivity of B-9. Note the stronger reactivity of all unlysed positive clones in this figure, indicating that the antigen is being expressed on the surface of the E. coli cell.

The positive clones were tested for tetracycline susceptibility to demonstrate insertional inactivation of the tetracycline gene of pBR322. All of the positive colonies were ampicillin-resistant (the ampicillin gene of pBR322 remains intact) and tetracycline-sensitive. Surprisingly, B-9 was also tetracycline sensitive.

Reisolation of the plasmids from these positive clones via alkaline lysis and agarose gel electrophoresis (Figure 4) revealed inserts in all clones except B-9. The presence of plasmid in the B-9 clone is indicated by the DNA band in lane 5. However, it migrates farther than native pBR322 indicating a lower molecular weight, and therefore, lack of a DNA insert. Note the lack of plasmid DNA in lane 9 which contains nontransformed E. coli JA221. The larger size of the DNA in lanes 1, 2, 4, 6, 7, and 10 containing H-1, H-2, B-7, B-11, B-12, and H1, respectively, is indicated by the slower migration of the DNA when compared to native pBR322 (lane 8). The

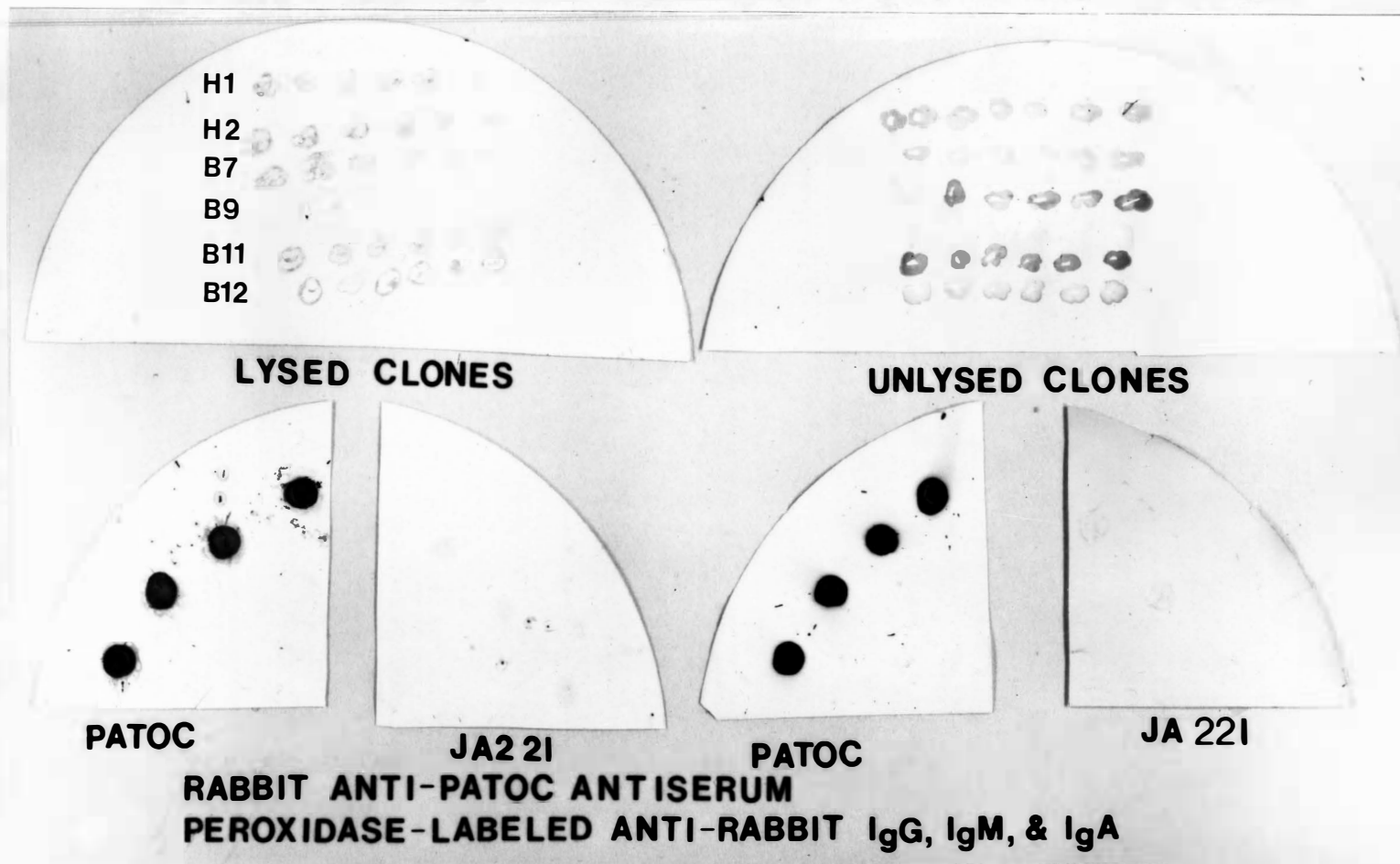


Figure 3. FB-EIA rescreening of positive clones with and without chemical lysis. Rows of unlysed clones correspond to labeling on lysed clones. Positive control, PATOC; negative control, JA 221.

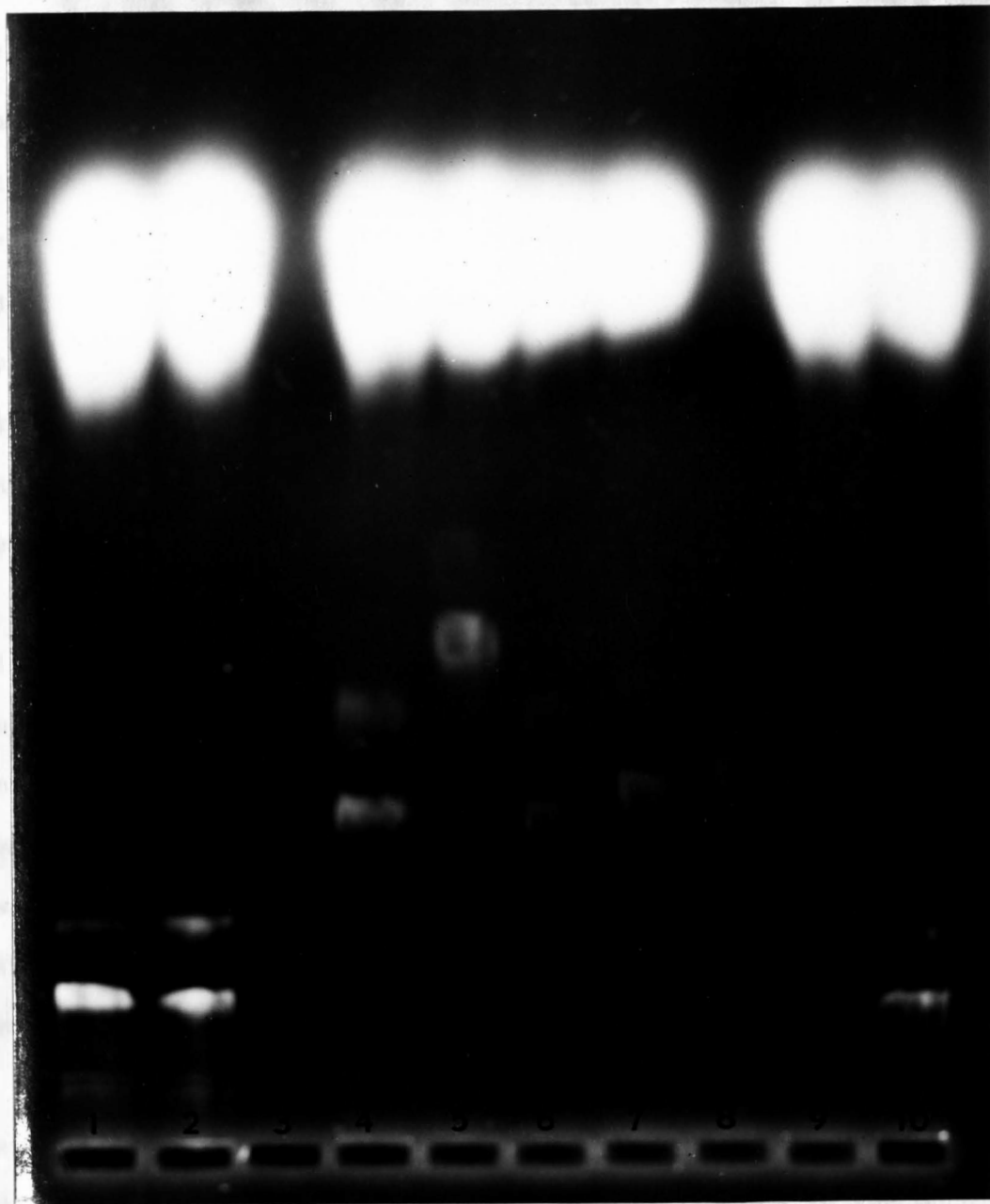


Figure 4. Agarose gel (1%) of plasmids reisolated from the positive clones. Lanes 1 through 10 (left to right) contain H-1, H-2, no DNA, B-7, B-9, B-11, B-12, purified pBR322, *E. coli* JA221, and H-1.

large, bright band near the top of Figure 4 is RNA, which deliberately was not removed in the extraction process.

To test the usefulness of the positive clones in the diagnosis of bovine leptospirosis, serum from a field case of leptospirosis caused by serovar pomona (courtesy of Clyde Kirkbride, South Dakota State University, Brookings, SD) was absorbed as described above and analyzed by both FB-EIA and ELISA (data not shown). Both tests failed to yield satisfactory results, due primarily to the inability to remove the anti-E. coli antibodies present in the bovine serum. Increased nonspecific binding was also a problem, a common occurrence in working with bovine systems (Clyde Kirkbride, personal communication).

Characterization of sonicated whole cells of the positive clones via SDS-PAGE followed by western blotting revealed two antigenic protein bands in clones B-7 and B-11 that had identical molecular weights of 40K and 30K (Figure 5). Western blotting of membrane fractions derived from these clones (Figure 6) also revealed two proteins bands in the same clones, but the molecular weights were 44K and 31K. Antigenic protein bands were not detected in clones H-1 and B-12 (Figure 6).

Testing the positive clones by FB-EIA using L. interrogans serovar pomona and autumnalis antisera (Figure 7) suggested that B-7 and B-11 were expressing genus-specific antigens due to the much stronger reaction of these unlysed clones as compared to the other

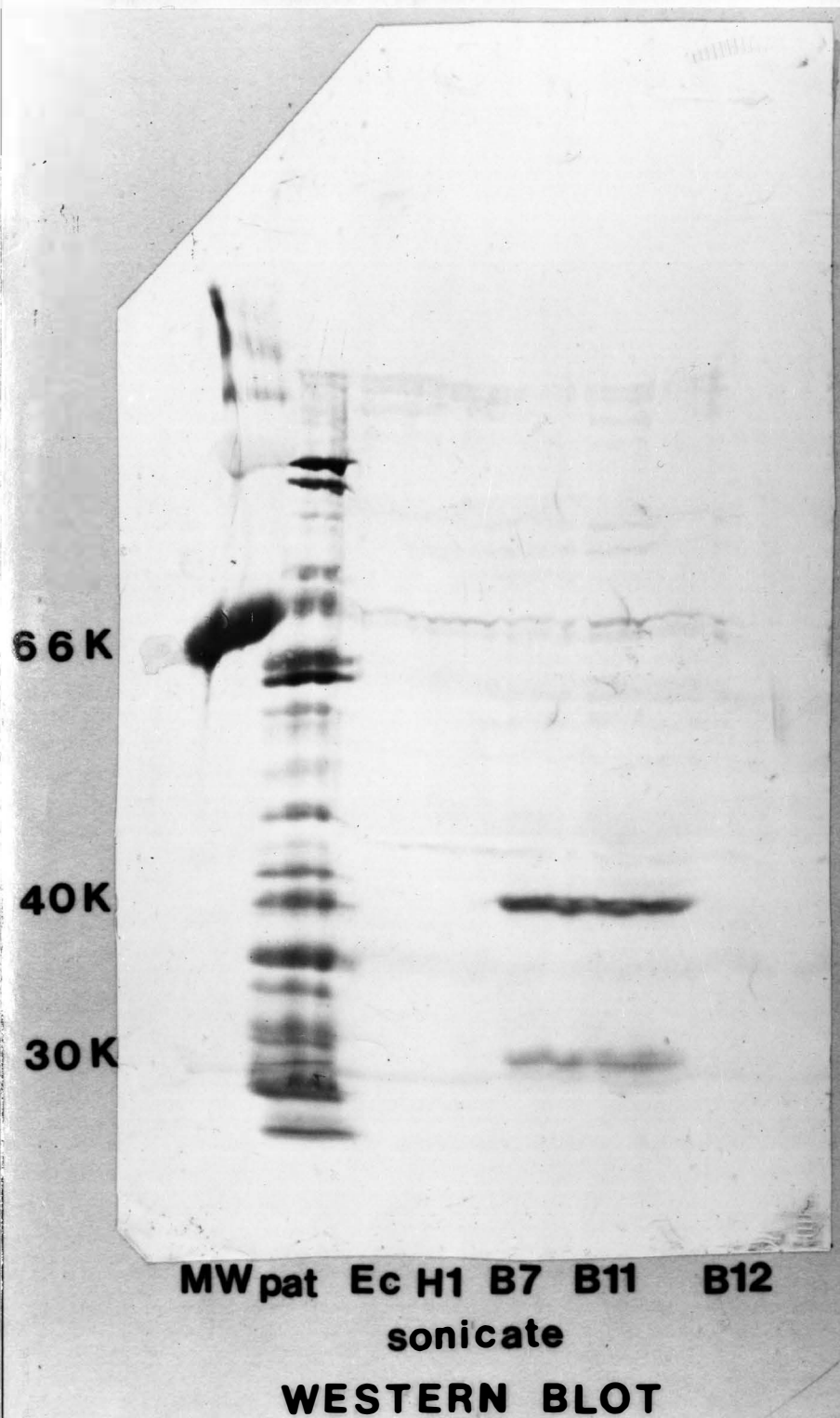


Figure 5. Western or immuno-blot of sonicated preparations of the positive clones, *L. biflexa* serovar *patoc*, and *E. coli* JA221. Lanes: MW-molecular weight markers; pat-serovar *patoc*; Ec-*E. coli* JA221; H1, B7, B11, B12-positive clones.

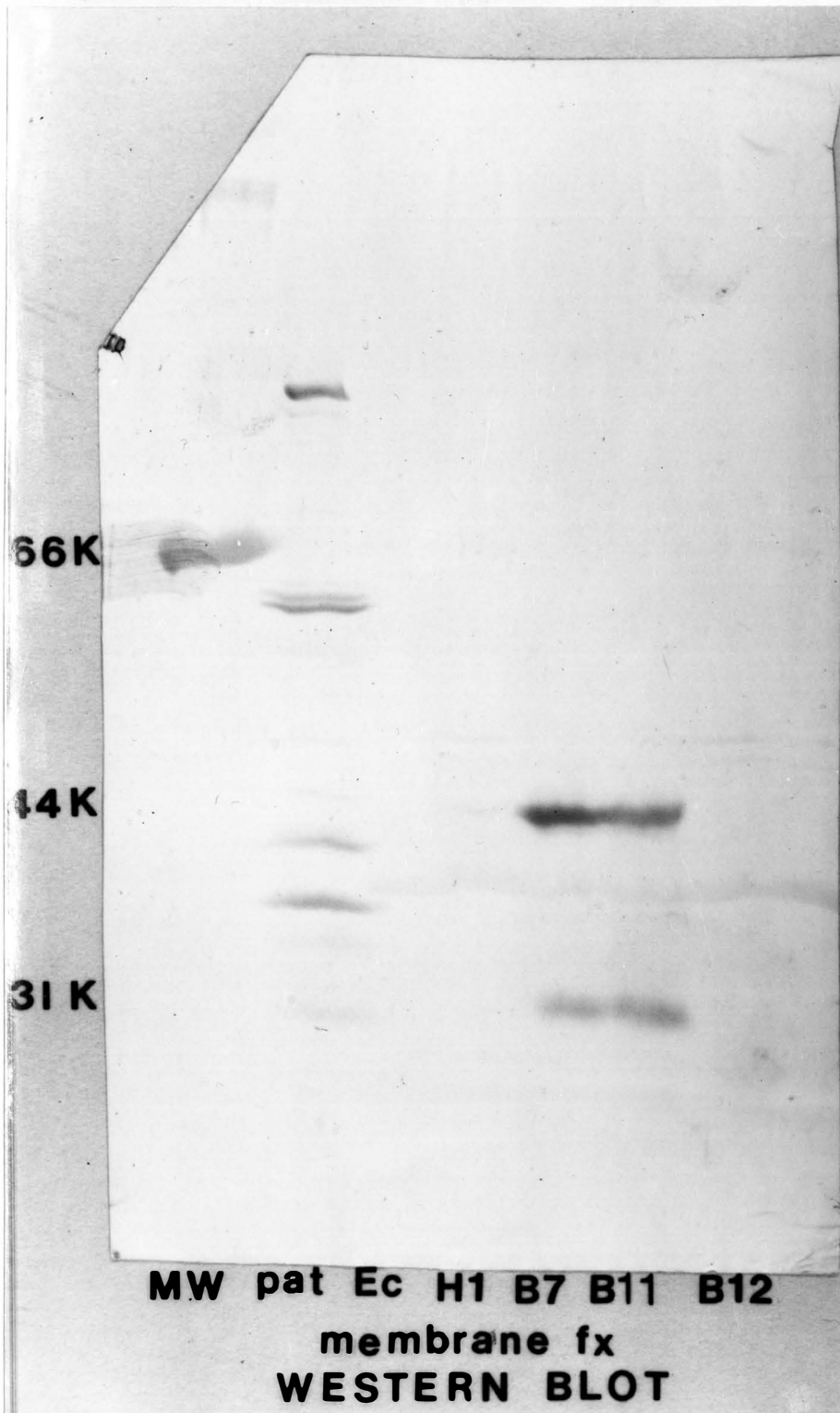
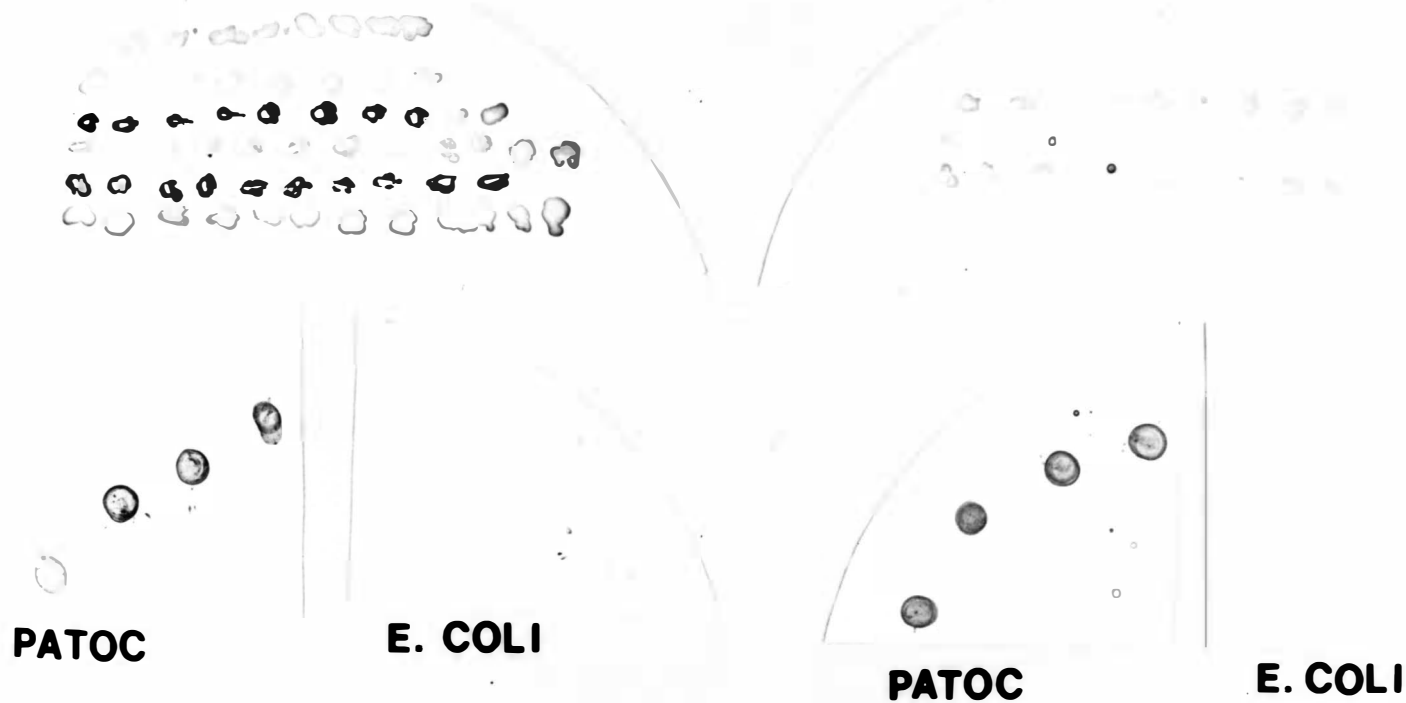


Figure 6. Western or immuno-blot of the membrane fraction of the positive clones, *L. biflexa* serovar patoc, and *E. coli* JA221. Lanes as in Figure 5.

RABBIT ANTI-POMONA ANTISERUM

RABBIT ANTI-AUTUMNALIS ANTISERUM



PEROXIDASE-LABELED ANTI-RABBIT IgG, IgM, & IgA

Figure 7. Genus-specific testing of positive clones by FB-EIA using *L. interrogans* serovars pomona and autumnalis antisera. Rows (top to bottom) H-1, H-2, B-7, B-9, B-11, and B-12. Positive control, PATOC; negative control, E. COLI.

positive clones. Although each serum was absorbed in the same manner that had been successful with the patoc antiserum, completion of this evaluation with 10 other L. interrogans serovars (data not shown) obscured the results somewhat due to unsuccessful removal of E. coli antibodies from certain antisera. Due to insufficient serum quantities, the antisera were not analyzed by ELISA to screen for effective absorption, nor were they reabsorbed. The reaction of B-7 and B-11, however, was consistently much stronger when tested with eleven of the 12 antisera. Serovar tarassovi antiserum, with a reported MAT titer of 1:12,800, reacted weakly in FB-EIA and western blotting, and when retested by MAT was found to have a 1:100 titer to serovar pomona, a 1:400 titer to serovar canicola, and was negative for serovars hardjo, grippotyphosa, icterohaemorrhagiae and bratislava. Tarassovi is not one of the serovars routinely used as an antigen in the MAT (26), thus the suspicion that the serovar tarassovi antiserum was relatively nonreactive could not be substantiated due to lack of a culture of serovar tarassovi for use as the antigen. Unfortunately, ELISA results of serovar- and genus-specific testing (data not shown) using both whole cell and sonicated antigen preparations were negative (OD₄₉₀ values approximately equaled the E. coli JA221 negative control) for all positive clones. For these analyses, plates were coated with 20 ug protein/ml as determined by the Bio-Rad protein determination. In addition, plates coated with whole cells at a protein concentration of 50, 100, and 200 ug /ml were also negative for all positive clones.

Western blotting of sonicated B-7 and B-11 clones from denatured polyacrylamide gels (not shown) failed to demonstrate the genus-specificity of the 44K and 31K protein bands. Identical analyses using antisera from L. biflexa serovars (other than patoc) failed to demonstrate species-specificity also. Western blotting of sonicated samples of all positive clones (data not shown) using nondenatured gels did not demonstrate any genus-, species-, or serovar-specific protein bands using L. interrogans serovar pomona, and L. biflexa serovars codice and patoc antisera, respectively.

DISCUSSION

The presence of interfering anti-E. coli antibodies in the rabbit anti-patoc immune serum became painfully obvious during early attempts to screen the gene libraries for expression of leptospiral antigens. Growth curves were, therefore, used to determine the length of incubation required to yield cells in the stationary phase of growth to be used in absorption of interfering antibodies in the immune serum. Due to the lack of OD₅₂₀ values between 13, and 22.5 hrs of the growth curve of E. coli JA221 (Figure 2), stationary phase was not firmly established until after 22.5 hr of incubation. Although it may have begun prior to that time, harvesting cells after 24 hr incubation assured that they were in stationary phase. Cells harvested in early stationary phase were used for absorption, since the greatest number of mature, antigen-bearing cells are available during this period of growth.

Effective absorption of E. coli antibodies from the rabbit anti-patoc antiserum was achieved, as monitored by the ELISA using nontransformed E. coli JA221 whole and sonicated cells as the antigen. Absorption with sonicated E. coli cells was not done to remove precipitins (antibodies to soluble antigens) as had been done by Engleberg and colleagues (44) in their work with Legionella pneumophila. In spite of the lack of sonicate absorption, the absorbed antiserum worked well in the FB-EIA screening procedure.

Because bovine leptospirosis is quite a problem in terms of diagnosis, it was of interest to evaluate the ability of the positive clones to detect anti-leptospiral antibodies in a clinical sample from a field case of leptospirosis caused by serovar pomona. The bovine serum was absorbed using an identical absorption scheme. However, the same effect was not achieved. Due to the limited amount of bovine serum, the ELISA was not used to evaluate the effectiveness of absorption. Unfortunately, the FB-EIA analysis of the positive clones was inconclusive due to reactivity of the antisera with nontransformed E. coli JA221 (data not shown). Cattle may have either a higher total titer of E. coli antibodies in their serum, or a higher level of antibodies to soluble antigens which were not removed in the whole cell absorption. The probability is high that cattle have a titer of E. coli antibodies higher than that of laboratory rabbits because cattle experience daily exposure to E. coli in feedlots and pastures (Clyde Kirkbride, personal communication).

Comparison of the two gene libraries revealed that the number of Hind III colonies expressing leptospiral antigens is 8 times greater than leptospiral antigen-bearing Bam HI colonies (0.25% of the Hind III colonies versus 0.03% of the Bam HI colonies). Since only a small number of Hind III colonies were screened, this discrepancy could be due to chance alone. Alternatively, the Hind III restriction endonuclease could have cleaved the leptospiral DNA in such a way that the genes coding for leptospiral antigens were

preserved. The latter is possible, because a specific restriction endonuclease yields a unique "family of fragments" (48) from the same DNA molecule. A more comprehensive screening of the Hind III gene library might clarify the difference in expression between the two gene libraries.

The expression rates found in this research are significantly lower than the 3% rate of expression of L. pneumophila antigens reported by Engleberg et al (44), and the 1.8% expression rate of Treponema pallidum antigens reported by Stamm and colleagues (101). Several possibilities may explain this apparent discrepancy: (i) pathogens such as L. pneumophila and T. pallidum may possess multiple copies of genes coding for the same antigen, thus increasing the probability that the recombinant DNA would contain antigen-coding material; (ii) increased cleavage of L. biflexa serovar patoc DNA within the genes coding for leptospiral antigens could result in cloning of genes that can not be transcribed into functional proteins, (iii) the strength of the promoter and/or composition and length of the untranslated leader sequence of the cloned DNA (77) may affect the efficiency of translation, or (iv) leptospiral antigens are expressed on the surface of the E. coli cells while the Legionella and Treponema antigens were not, thus the initial screening with chemical lysis results in the appearance of a decreased expression rate due to denaturation of the leptospiral surface antigens.

The fourth possibility is perhaps the most likely. It is supported in this research by the observation (Figure 3) that unlysed positive clones reacted more strongly than lysed clones. This trend was noted on numerous rescreenings of the positive clones. In addition, the presence of immunogenic protein bands in the membrane fraction of clones B-7 and B-9 also supports evidence published by other investigators indicating surface expression of leptospiral antigens. Charon and colleagues (25) demonstrated the presence of antigens in the outer sheath of leptospires by electron microscopic observation of antibody-coated latex beads attached to the outer surface of L. interrogans cells.

To rule out the presence of soluble antigens adhering to the NC filters and giving the appearance of surface expression of leptospiral antigens, cultures of the positive clones were centrifuged to remove the cells. The supernatant was then filtered to remove any remaining cells using a 0.2 um low-protein binding filter, the filtered supernatant was spotted onto NC filters, and dried filters were analyzed by FB-EIA without chemical lysis (data not shown). None of these samples from the positive clones showed evidence for soluble antigens. In addition, Engleberg et al (44) reported decreased reactivity of unlysed clones, while Stamm (101) found no difference in the reactivity of lysed and unlysed positive clones. Therefore, if leptospiral antigens were expressed on the surface of the E. coli cell and antigens of L. pneumophila and T. pallidum were not, one would expect a decrease in the expression rate

of leptospiral antigens caused by their denaturation in the initial screening procedure.

The presence of leptospiral DNA in transformed *E. coli* JA221 was substantiated by agarose gel electrophoresis of plasmids that had been reisolated from the positive clones. As expected, Figure 4 shows that all plasmids from the positive clones do not migrate as far as pBR322, which indicates an increased molecular weight (77) presumably due to insertion of leptospiral DNA. The ampicillin-resistance, tetracycline-sensitivity of all positive clones corroborates this finding, since native pBR322 without any DNA inserts is both ampicillin- and tetracycline-resistant (15,77).

B-9 showed no evidence for the presence of foreign DNA; it appears to be smaller than native pBR322 (Figure 4). The finding that B-9 is tetracycline-sensitive like the positive clones seemed somewhat puzzling at first. However, deletions within the tetracycline gene that result in the loss of tetracycline resistance are not uncommon (15). Thus, it is possible to isolate a tetracycline-sensitive colony from a gene library containing pBR322 that does not contain foreign DNA. A deletion in the tetracycline gene would explain both the smaller size of the plasmid isolated from B-9 and the tetracycline-sensitivity of B-9.

Characterization of the positive clones by SDS-PAGE and western blotting revealed the existence of two protein bands in clones B-7 and B-11. The molecular weight of the larger band varies depending on the fraction tested by immunoblotting (sonicated

fraction, 40K; membrane fraction, 44K). The molecular weight of the smaller protein varies only by 1K, which is probably an insignificant difference. The molecular weight difference in the larger protein may be due to different solvents: PBS was the solvent used for the sonicated samples, while 0.15 M sodium chloride was used to solubilize the membrane fraction. Differences in the chemical nature of these solvents could have altered the protein slightly, and changed its apparent molecular weight.

In addition to demonstrating identical protein bands on western blotting, plasmids isolated from B-7 and B-11 were of similar size, and both clones reacted similarly during genus-specific testing by FB-EIA. Therefore, these clones may contain the same leptospiral DNA. Determination of the size of the DNA inserts would not only add to the knowledge about the DNA coding for leptospiral antigens, but it might verify that B-7 and B-11 are expressing the same DNA. Restriction mapping of the inserts in these two clones also might validate this hypothesis (77).

Knowledge of the size of the DNA inserts may also help explain the presence of the two antigenic protein bands in clones B-7 and B-11. The DNA insert may be too small to consist of more than one gene [gene size is approximately 1000 base pairs (Carl Westby, South Dakota State University, Brookings, SD, personal communication)], or conceivably, there may be enough DNA to code for more than one protein. The two proteins do not appear to represent a monomer and dimer, since one would expect approximate doubling of the

molecular weight were that the case. They could, however, represent different sized oligomers. It is also possible that a nonprotein moiety is missing from the smaller molecule. Further analysis to test for the presence of lipids and carbohydrates may be valuable in explaining the presence of the two protein bands. Antigens with common antigenic determinant sites but differing molecular weights were found in Leptospira by Niikura et al (83), and by Gulig and associates (53) in Haemophilus influenzae.

Other investigators have found antigens with molecular weights similar to those described in this research. The genus-specific antigen (as determined by complement fixation and ELISA) of Sakamoto et al (96) that was prepared by Triton X-100 treatment of L. interrogans serovar kremastos had a molecular weight of 62K. However, chemical analysis of this antigen indicated a composition of 61% (w/w) protein, 7.5% (w/w) neutral sugar, and 6% (w/w) amino sugar, accounting for only 74.5% of the total dry weight of this antigen (96). The authors suggest that the remainder of the dry weight is Triton X-100 bound to the antigen (96). If this is the case, the actual molecular weight of the glycoprotein antigen would be 46K, and the molecular weight of the protein component would be 38K. Both values are within reasonable range of the higher molecular weight bands found in this study (40K, and 44K in sonicate and membrane fraction, respectively). Niikura and his colleagues (83) demonstrated a thermostable 32K MW antigen in a virulent but not an avirulent clone of L. interrogans serovar copenhageni. This protein

identified by SDS-PAGE failed to react with either virulent or avirulent antiserum in immunoblotting experiments (83). Another antigen extracted from the avirulent clone, had a molecular weight of 41K and reacted with both virulent and avirulent antisera in immunoblotting experiments (83). The report by Chapman and colleagues (24) of antigens in the range of 29-35K MW was the only literature found that indicated detection of genus-specific antigens by immunoblotting. These antigens were shown to contain protein and carbohydrate components (24). The smaller protein band found in this investigation correlates well with this molecular weight range.

The failure to demonstrate leptospiral protein bands in clones H-1, H-2, and B-12 may be due to the loss of antigenic reactivity due to destruction of the tertiary structure of the protein by the denaturing conditions of the gel (93). Screening these positive clones by nondenaturing gels, however, did not reveal immunogenic protein bands. Since resolution of proteins in a 7.5% polyacrylamide gel is limited to the range of 14K to 200K MW (Eric Nelson, South Dakota State University, personal communication), any antigens having a higher molecular weight than 200K would not be resolved on the gels used in this research. It is also possible that the sonication process used in the preparation of the clones for SDS-PAGE caused denaturation of these proteins, making them unreactive on western blots.

It seems unlikely that leptospiral antigens other than proteins would be expressed in E. coli, since expression of lipids,

polysaccharides, or lipopolysaccharides would require the presence of several enzymes for their correct assembly. The probability of successfully cloning antigens requiring multistep assembly is quite remote, because (i) transformation is much less efficient when large pieces of DNA are used, and (ii) the chances are low that a restriction enzyme will cleave the DNA in the correct locations to preserve the multiple genes required for assembly. The possibility of cloning an enzyme capable of converting an E. coli lipid to a leptospiral lipid in a one-step process is possible (Carl Westby, personal communication), however, it is unlikely that such an antigen would be detectable on western blotting. With the exception of Loeb's work (74) with Haemophilis influenzae Type b surface components, lipopolysaccharides have not been successfully detected by western blotting. Notably, the western blot procedure used by Loeb (74) eliminates methanol from the transfer buffer, but in the current research methanol was a component of the transfer buffer. Thus, had successful cloning of an antigenic lipid occurred in this experiment it is highly improbable that it would have been detected by western blotting.

Genus-specific screening of the positive clones by FB-EIA appeared to indicate the presence of genus-specific antigens in clones B-7 and B-11. Immunoblotting and the ELISA screening of these clones failed to confirm this, however. The apparent discrepancy between FB-EIA and immunoblotting may be due to denaturation of the antigens in the latter test (19,106). It seems reasonable that a

single protein could be genus-specific due to tertiary structure in the nondenatured form and serovar-specific in the denatured form. Alternatively, the genus-specific nature of the antigen could reside in a lipid or carbohydrate moiety which is not transferred to the NC paper during western blotting (19,106). Nondenatured gels failed to demonstrate any leptospiral bands, however, which may be due to denaturation in the preparation of the clones by sonication or to the high molecular weight of these proteins preventing separation in a 7.5% polyacrylamide gel.

Failure to detect leptospiral antigens using the ELISA, while the FB-EIA test was successful, may be due to the increased protein-binding capacity of NC filters (14,51). Binding of sonicated E. coli clones to polystyrene at 20 ug protein/ml probably represents approximately 99% E. coli protein, thus leptospiral protein binding may be at an undetectable level (69). Kenny and Dunsmoor (69) reported that, when mixtures are used to coat ELISA plates, unless at least 1% of the total coated protein represents the desired antigen it may be undetectable. Attempts to coat with larger protein quantities were also unsuccessful. This was probably due to the inability of leptospiral protein to compete for the limited number of protein binding sites on the polystyrene. The ELISA as applied here, using a mixture of E. coli and leptospiral antigens with the majority of the protein being of E. coli origin, is therefore subject to decreased sensitivity and specificity (69).

If the genus-specific nature of the antigens expressed by B-7 and B-11 can be verified, it may be possible to isolate these antigens and use them in serologic testing as well as in vaccine production. A number of investigators have reported studies involving genus-specific antigens. Fairbrother (45) has suggested that the genus-specific antigens do not stimulate protective immunity due to stimulation of a solely IgM response in cattle. The genus-specific antigens used in the experiments were the Pat E antigen of Baker and Cox (7,104) and the Pat F antigen of Myers and Coltorti (80). Neither of these antigens have been chemically characterized. Adler and Faine (2) found that the lipopolysaccharide (LPS) of leptospires, unlike other gram negative bacteria, does not stimulate protective antibodies. Isogai and colleagues (60) also found that leptospiral LPS was a much less potent immunogen than E. coli LPS. The partially purified genus-specific protein antigen of Sakamoto and colleagues (96), has not been evaluated for its ability to stimulate protective antibodies. Purified proteins have been successfully used, probably most notably in the diphtheria-pertussis-tetanus (DPT) vaccine (53,115). Since it remains to be proven that genus-specific protein antigens do not stimulate protective immunity, it may be possible to use a purified genus-specific protein antigen in vaccine production.

Currently most vaccines (bacterins) used for control of leptospirosis are merthiolate-inactivated whole cultures containing a minimum of 1×10^9 leptospires from each serovar included in a

multivalent vaccine (10). Virulent leptospires have been used because they are better immunogens than avirulent leptospires (9). While it appears that genus-specific antigens of uncertain chemical composition do not stimulate protective immunity (45), and that nonpathogenic leptospires are not as immunogenic as pathogens, a purified genus-specific protein antigen may be effective because it is a better immunogen than lipids or carbohydrates. An added advantage of a genus-specific vaccine would be elimination of the need to vaccinate for all serovars endemic in a given geographic area which is currently necessary to adequately control leptospirosis. Eventually, a safe, genus-specific vaccine might be developed for the at-risk human population, whereas one is currently not available (5).

Because expression requires that a gene be transcribed as well as translated (48), a promoter must be present for transcription. In the positive clones isolated during this investigation, it appears that either restriction-enzyme cleavage did not separate the leptospiral promoter from the gene, or the gene can be transcribed using an E. coli promoter on the plasmid pBr322 (48).

Considering the two possibilities, the most probable candidate for an E. coli promoter in the Bam HI clones may be the tetracycline promoter of pBR322 (118), since the leptospiral DNA was inserted into the tetracycline gene. This cannot be the case for the Hind III library, because the Hind III cleavage site lies within the tetracycline promoter and insertion inactivates this promoter (103).

Five major promoters were identified in pBR322 by Stuber and Bujard (103), therefore one of these promoters may allow transcription of genes in the Hind III clones. The major advantage of an E. coli promoter is the increased probability of its recognition by E. coli RNA polymerase, resulting in transcription (77). However, if the promoter were of leptospiral origin, the probability of correct spacing of the promoter regions in relation to the transcription start site is increased (77), which would also promote expression of the cloned gene. Yelton and Cohen's (119) analysis of the cloned *trpE* gene (from the same Bam HI library used in this research) indicated that the promoter was most probably of leptospiral origin. Evidence consisted of the findings that leptospiral DNA could be cloned into pBR322 in either orientation without affecting the expression rate, and that transposon mutagenesis of regions on either side of the cloned DNA did not affect the expression of the *trpE* gene (119). Without additional experimentation, the origin of the promoter for the leptospiral antigens remains purely speculative.

CONCLUSION

The research presented here adequately answered the first question posed: leptospiral antigens can be expressed in E. coli JA221. A search of the available literature indicates that this research represents the first published instance of cloning a gene coding for leptospiral antigens and expression of those antigens in E. coli. The utility of these antigens in the serologic diagnosis of leptospirosis, however, remains questionable. Preparation of these antigens for serological testing would be very time consuming and impractical due to (i) the necessity for extensive absorption of E. coli antibodies from animal sera, (ii) the variability in the level of antibodies from species to species and animal to animal, and (iii) the probable need to absorb with both sonicated and whole cells to effectively remove antibodies to soluble as well as particulate antigens. Thus, unless a different host bacterium can be found which would eliminate the need to absorb host antibodies from clinical serum samples, these antigens (in their present form) are not practical for use in routine diagnosis of leptospirosis.

The final question about the nature of the leptospiral antigens expressed in E. coli was partially answered. Clones B-7 and B-11 possess antigenic protein bands (44K and 31K MW) in the membrane fraction of sonicates. Due to apparent conflicting results of FB-EIA and immunoblotting, only the serovar-specific nature of these proteins could be verified.

Continuation of the research could add to the accumulating knowledge about Leptospira by: (i) learning more about the chemical nature of the leptospiral antigen through investigations to determine the presence of lipids or carbohydrates that may be attached to the protein portion; (ii) removal of leptospiral DNA inserts with restriction enzymes followed by isolation of the inserts by agarose gel electrophoresis and base sequence analysis of the DNA; and (iii) exploring the potential that these clones might lead to a genus-specific vaccine for leptospirosis. In addition, purification of the protein bands identified by immunoblotting, and/or selection of an alternative host for the recombinant DNA (eliminating the need for extensive absorption procedures), might result in an alternative antigen source for serologic diagnosis of leptospirosis.

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