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Veerapandian Raja
Bharathidasan University, India

Santhanam Shanmughapriya
Bharathidasan University, India

Murugesan Kanagavel
Bharathidasan University, India

Sergey Artiushin
University of Kentucky

Sridhar Velineni
University of Kentucky, sridharvelineni@uky.edu

See next page for additional authors

Authors
Veerapandian Raja, Santhanam Shanmughapriya, Murugesan Kanagavel, Sergey Artiushin, Sridhar Velineni, John F. Timoney, and Kalimuthusamy Natarajaseenivasan

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Notes/Citation Information
Published in *Clinical and Vaccine Immunology*, v. 23, no. 1, p. 65-72.

Digital Object Identifier (DOI)
http://dx.doi.org/10.1128/CVI.00509-15

AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical and Vaccine Immunology

In Vivo-Expressed Proteins of Virulent *Leptospira interrogans* Serovar Autumnalis N2 Elicit Strong IgM Responses of Value in Conclusive Diagnosis

Veerapandian Raja,[a] Santhanam Shanmughapriya,[a]* Murugesan Kanagavel,[a] Sergey C. Artiushin,[b] Sridhar Velineni,[b] John F. Timoney,[b] Kalimuthusamy Natarajaseenivasan[a]

Medical Microbiology Laboratory, Department of Microbiology, Centre of Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli, India[a]; Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky, USA[b]

Leptospirosis is a serious zoonosis that is underdiagnosed because of limited access to laboratory facilities in Southeast Asia, Central and South America, and Oceania. Timely diagnosis of locally distributed serovars of high virulence is crucial for successful care and outbreak management. Using pooled patient sera, an expression gene library of a virulent *Leptospira interrogans* serovar Autumnalis strain N2 isolated in South India was screened. The identified genes were characterized, and the purified recombinant proteins were used as antigens in IgM enzyme-linked immunosorbent assay (ELISA) either singly or in combination. Sera ($n = 118$) from cases of acute leptospirosis along with sera ($n = 58$) from healthy subjects were tested for reactivity with the identified proteins in an ELISA designed to detect specific IgM responses. We have identified nine immunoreactive proteins, ArgC, RecA, GlpF, FliD, TrmD, RplS, RnhB, Lp28.6, and Lrr44.9, which were found to be highly conserved among pathogenic leptospires. Apparently, the proteins ArgC, RecA, GlpF, FliD, TrmD, and Lrr44.9 are expressed during natural infection of the host and undetectable in *in vitro* cultures. Among all the recombinant proteins used as antigens in IgM ELISA, ArgC had the highest sensitivity and specificity, 89.8% and 95.5%, respectively, for the conclusive diagnosis of leptospirosis. The use of ArgC and RecA in combination for IgM ELISA increased the sensitivity and specificity to 95.7% and 94.9%, respectively. ArgC and RecA thus elicited specific IgM responses and were therefore effective in laboratory confirmation of *Leptospira* infection.

Leptospirosis is a reemerging infectious disease of global importance, caused by pathogenic spirochetes of the genus *Leptospira*. Increased economic and recreational activity in pristine areas as well as flooding associated with climatic change has increased human exposure to leptospires. Indeed, several large outbreaks have been reported in Nicaragua (1, 2), Brazil (3), New Caledonia (4), the Federated States of Micronesia (5), Japan (6), and India (7–10) in recent years. Conventionally, culture positivity, seroconversion, or 4-fold rises in microscopic agglutination test (MAT) titers are demonstrated for confirmation of leptospirosis. Per WHO recommendations, the standard reference method for diagnosis of leptospirosis is MAT (11). Enzyme-linked immunosorbent assay (ELISA) offers reasonable sensitivity and the possibility of handling many samples at a time. ELISA has been developed with a number of modifications (12).

Immune responses include antibodies to numerous leptospiral proteins constitutively expressed or upregulated during infection (13). Antibodies of the IgM isotype appear about 4 days after onset of fever and headache and are responsible for clearance of leptospires from the bloodstream. Hypothetically, proteins specifically induced during *in vivo* growth but not expressed by *in vitro* cultures have enhanced value because of their potential involvement in pathogenesis and participation in acquired immune protective responses (14). Examples of such proteins that have gained prominence as diagnostic reagents and vaccine candidates are LigA and LigB immunoglobulin-like proteins and HbpA expressed strongly *in vivo* and found only on freshly isolated pathogenic *Leptospira* species (15–18). The diagnostic and vaccine efficacy of LigA protein and its immunogenic epitopes has been studied extensively (15, 19–23). Thus, studies of proteins increased in abundance *in vivo* not only contribute to understanding of host-pathogen interactions but also are helpful in design of novel diagnostics and vaccines.

In the present study, we have identified *in vivo*-expressed proteins of virulent *Leptospira interrogans* serovar Autumnalis strain N2 through an expression gene library screening. The library was screened with pooled patient sera, and IgM ELISA incorporating the identified proteins was highly sensitive and specific for the conclusive diagnosis of human leptospirosis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Leptospira interrogans* serovar Autumnalis strain N2 was used for the genomic DNA isolation and library

Received 8 September 2015 **Returned for modification** 28 September 2015
Accepted 11 November 2015

Accepted manuscript posted online 25 November 2015

Citation Raja V, Shanmughapriya S, Kanagavel M, Artiushin SC, Velineni S, Timoney JF, Natarajaseenivasan K. 2016. *In vivo*-expressed proteins of virulent *Leptospira interrogans* serovar Autumnalis N2 elicit strong IgM responses of value in conclusive diagnosis. Clin Vaccine Immunol 23:65–72. doi:10.1128/CVI.00509-15.

Editor: D. W. Pascual

Address correspondence to Kalimuthusamy Natarajaseenivasan, natarajaseenivasan@rediffmail.com.

* Present address: Santhanam Shanmughapriya, Center for Translational Medicine, School of Medicine, Temple University, Philadelphia, Pennsylvania, USA.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/CVI.00509-15.

construction. The MAT was performed to evaluate serological evidence of leptospiral infection (11). A panel of 12 reference strains was used which included the following serogroups: Australis (serovar Australis, strain Ballico), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Sejroe (serovar Hardjo, strain Hardjoprajitno), and Pyrogenes (serovar Pyrogenes, strain Salinem). The strains were obtained from the WHO Reference Centre for Leptospirosis, Indian Council of Medical Research (ICMR), Regional Medical Research Centre (RMRC), Port Blair, India, and maintained by regular subculturing in Ellinghausen-McCullough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) at the Medical Microbiology Laboratory, Bharathidasan University, Tiruchirappalli, India.

Escherichia coli SOLR and XL1-Blue MRF′ (Stratagene, La Jolla, CA) were hosts for phage manipulation and plasmid excision. *E. coli* NovaBlue, BL21(DE3), and BL21(DE3)pLysS (Novagen, Madison, WI) were used for cloning and expression of recombinant proteins and were routinely grown in Luria-Bertani (LB) broth or on LB agar.

Study site, patients, case definition, and ethics. The samples for this study were obtained through our routine hospital-based surveillance at Annal Mahatma Gandhi Memorial General Hospital, Tiruchirappalli, Tamilnadu, India. Tiruchirappalli is a municipal corporation of urban agglomeration surrounded by villages actively involved in agriculture (especially cultivation of rice, sugarcane, and groundnut) and cattle rearing. In Tiruchirappalli, the temperature ranges from 28°C to 38°C, and the geographical position of this area is 10.81°N latitude and 78.69°E longitude.

In total, 118 serum samples (see Table S1 in the supplemental material) with a MAT titer of ≥1:160 were selected from a bank of 754 laboratory-confirmed samples (a positive IgM ELISA, isolation of leptospires from the blood, seroconversion, or 4-fold rise in titer by MAT) collected during the early phase of illness (0 to 10 days after the onset of disease). The age and sex distributions of the confirmed cases included in the study are given in Table S2 in the supplemental material. A total of 58 seronegative healthy controls selected from a group of cases matched with respect to age (±5 years) and sex and 98 patients who were hospitalized with a clinical suspicion of leptospirosis and subsequently diagnosed as having other illness based on laboratory evidence (MAT and ELISA negative for leptospirosis) were also included to study the efficiency of the ELISA developed in the present study. Informed written consent was obtained from both cases and controls before sampling, and the study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (DM/2007/101/373/Project No.2) as well as permitted by the Directorate of Health Services (reference no. 5796/TV 1/07), Tamilnadu. The obtained sera were stored at −80°C until use.

Preparation of heat-extracted antigen. Leptospiral heat-extracted antigen was prepared from 7-day-old leptospiral culture (1×10^8 to 2×10^8/ml) of *L. interrogans* serovar Autumnalis. The culture was killed by formalin (final concentration, 0.5% [vol/vol]), heated in a boiling water bath for 30 min, and centrifuged for 30 min at 10,000 × *g*. The supernatant was used as heat-extracted antigen. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Sigma, St. Louis, MO).

Polyclonal antisera. Polyclonal antisera were raised in New Zealand White rabbits by subcutaneous administration of 1 μl of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma, St. Louis, MO) and 100 μg of heat-extracted antigen prepared from laboratory-grown *L. interrogans* serovar Autumnalis strain N2 or purified recombinant proteins adsorbed to aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, NY). Booster injections containing 100 μg (subcutaneous) of the antigen were administered 14 and 28 days after the primary immunization. Serum was obtained 35 days after the primary immunization (23). The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Bharathidasan University (BDU/IAEC/2011/29/29-03-2011).

Genomic DNA library construction and screening. A λZAPII library containing 3- to 5-kb random fragments of *L. interrogans* serovar Autumnalis strain N2 was screened to identify phage that expressed gene products reactive with pooled patient sera (see Table S3 in the supplemental material) as described elsewhere (24) with some modifications. The secondary antibody was horseradish peroxidase (HRP)-labeled antihuman IgG (Sigma, St. Louis, MO) diluted 1:4,000. Secondary screening was performed with hyperimmune serum (HIS) raised in rabbit against heat-extracted antigen to identify plaques reactive with patient sera but not HIS. Negative clones that did not react with HIS were considered potential producers of *in vivo* antigens.

DNA sequencing and expression of recombinant proteins. Suspected plaques on agar plugs were transferred to 500 μl of SM buffer and allowed to elute overnight. Plasmids containing the inserts were rescued from selective reactive phages by using ExAssist helper phage and *E. coli* SOLR according to the manufacturer's instructions. Plasmid DNA was isolated from reactive clones using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) and sequenced in a commercial sequencing facility (Macrogen, South Korea) using T3, T7, and custom-designed primers. Sequences were edited with Chromas 1.61 (Technelysium Pty. Ltd., Queensland, Australia) and aligned and connected with DNASIS (Hitachi Software Engineering Co., Ltd., San Francisco, CA). DNA sequences thus obtained were compared with the whole-genome sequences of *L. interrogans* serovar Lai strain 56601 and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, and homologies were identified by a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Primer sequences used for the specific amplification of the identified genes are given in Table S4 in the supplemental material. Following PCR amplification (see Fig. S1A in the supplemental material), the amplicons were cloned into predigested pET15b (Novagen, Madison, WI) (*argC*, *glfP*, *fliD*, *trmD*, *rplS*, *rnhB*, *lp28.6*, and *lrr44.9*) or pRSETA (Invitrogen, USA) (*recA*). Recombinant plasmids were transformed into *E. coli* BL21(DE3) for pET15b and *E. coli* BL21(DE3)pLysS for pRSETA. The presence of the correct inserts was confirmed by colony PCR, restriction endonuclease analysis (REA), and DNA sequencing (see Fig. S1B in the supplemental material).

Expression of His_6 proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when cultures reached an optical density at 600 nm (OD_{600}) of 0.6, and cells were harvested after 2 to 3 h. Recombinant His-tagged proteins were isolated using Talon metal affinity resin (Clontech Laboratories, Inc.) in buffer containing 8 M urea according to the manufacturer's recommendations. The purified recombinant proteins were dialyzed against 10 mM Tris (pH 7.5) containing 50 mM NaCl, and their immunoreactivity was assessed by immunoblotting.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Proteins were separated on 10% acrylamide gels by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. After blocking, membranes were incubated with pooled patient sera or rabbit HIS followed by incubation with anti-human or anti-rabbit IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO). Membranes were developed and visualized using 4-chloro-1-naphthol (Sigma, St. Louis, MO).

Enzyme-linked immunosorbent assay. A checkerboard titration was performed in 96-well flat-bottom polystyrene Microtest III assay plates (Falcon; Becton, Dickinson, Oxnard, CA) with patient and control sera in doubling dilutions and tested against 0.1 to 5.0 μg/well of antigen to determine the optimum concentration of the recombinant proteins in diagnosis. Wells were coated with 0.2 μg of recombinant proteins (optimized concentration in single) using carbonate buffer (pH 9.6) overnight at 4°C followed by blocking with 4% nonfat dry milk. All sera were added in a dilution of 1:200 in triplicate and incubated for 1 h at 37°C. Bound

TABLE 1 Conservation of identified proteins among different species of *Leptospira*

Protein	GenBank accession no.	Identity (%)[a]									Molecular mass (kDa)
		L. interrogans	*L. borgpetersenii*	*L. kirschneri*	*L. weilii*	*L. noguchii*	*L. alexanderi*	*L. santarosai*	*L. kmetyi*	*L. biflexa*	
ArgC	AGW25359	98	84	97	83	92	83	84	84	59	37.8
RecA	AGW25358	99	95	99	95	99	95	96	95	78	39
GlpF	AGW25365	99	87	97	85	96	85	88	91	67	25.4
FliD	AGW25366	99	87	98	94	96	89	87	92	53	69.9
TrmD	AGW25364	99	91	98	91	96	90	NA	90	60	25.6
RplS	AGW25363	99	90	96	92	96	87	88	93	68	15.5
RnhB	AGW25362	99	81	92	83	92	82	92	81	41	26.5
Lp28.6	AGW25361	99	63	94	69	87	65	93	66	NA	28.6
Lrr44.9	AGW25360	100	93	98	93	98	93	94	91	44	44.96

[a] *L. interrogans*, YP_001698, WP_017861766, WP_000535417.1, NP_713634.1, NP_712569.1, NP_712568.1, NP_712567.2, WP_000778152.1, NP_712564.1; *L. borgpetersenii*, WP_002761303, WP_002761315, WP_002752499.1, WP_002752524.1, WP_004280339.1, YP_798039.1, YP_798038.1, WP_002751302.1, WP_002751417.1; *L. kirschneri*, WP_004779134, WP_004752583, WP_025183617.1, WP_004781580.1, WP_004760731.1, WP_004780226.1, WP_004780231.1, WP_020779461.1, WP_004751784.1; *L. weilii*, WP_004504782, WP_002996988, WP_004506831.1, WP_003010986.1, WP_004507221.1, WP_002996170.1, WP_002995931.1, WP_002996364.1, WP_002996221.1; *L. noguchii*, WP_004454314, WP_004424072, WP_004426432, WP_004426445.1, WP_004430719.1, WP_004453264.1, WP_004423647.1, WP_004430727.1, WP_004424196.1; *L. alexanderi*, WP_010578142, WP_010578143, WP_020985028.1, WP_010576705.1, WP_010579003.1, WP_010579002.1, WP_010579001.1, WP_010579000.1, WP_010578998.1; *L. santarosai*, WP_016754218, WP_004468644, WP_016755555.1, WP_004494481.1, WP_004487102.1, WP_020767000.1, WP_020766986.1, WP_004470165.1; *L. kmetyi*, WP_020985623, WP_010574861, WP_010573697.1, WP_020985940.1, WP_010574774.1, WP_010574773.1, WP_010574772.1, WP_010574771.1, WP_010574769.1; *L. biflexa*, YP_001962522, AAC43586, WP_012387177.1, YP_001837994.1, YP_001839046.1, YP_001839047.1, YP_001962701.1, YP_001839051.1. NA, not available.

IgM was detected using peroxidase-conjugated anti-human IgM (Sigma), followed by development using 0.08% (wt/vol) 5-aminosalicylic acid. Optical densities (ODs) were read at 490 nm. For combination ELISA, checkerboard titration was carried out to determine the best working concentration of the recombinant proteins in combination. Combinations were made by mixing ArgC (0.2 μg) and RecA (0.1 μg) (combo 1); GlpF (0.1 μg) and FliD (0.2 μg) (combo 2); and TrmD (0.1 μg), RplS (0.1 μg), RnhB (0.2 μg), Lp28.6 (0.1 μg), and Lrr44.9 (0.1 μg) (combo 3).

Statistical analysis. Data were analyzed using version 6.0 of the Statistica software package (StatSoft, Tulsa, OK). Cutoff values for each recombinant antigen ELISA were defined as the corresponding mean plus 2 standard deviations (SD) from the sera of healthy controls. Sensitivity was defined as the percentage of the laboratory-confirmed cases of leptospirosis whose serum samples gave mean ODs greater than the relevant cutoff value. Specificity was calculated as the percentage of the control individuals whose samples gave mean ODs below the relevant cutoff value. The positive and negative predictive values (PPV and NPV, respectively) are the proportions of true positives and true negatives, respectively. The percent agreement between the results of the recombinant protein-based ELISA and the results of the MAT, and the corresponding kappa coefficients, were determined using Epi Info version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA). The LigA-C peptides identified in our previous study (23) were used as positive controls in ELISA in order to compare the diagnosis efficacy of the newly identified proteins with that of a well-defined *in vivo*-expressed protein.

Nucleotide sequence accession numbers. The sequences obtained were submitted to GenBank with the accession numbers KF240786, KF240787, KF240788, KF240789, KF270743, KF270744, KF270745, KF270746, and KF270747 for *recA*, *argC*, *lrr44.9*, *lp28.6*, *rnhB*, *rplS*, *trmD*, *glpF*, and *fliD*, respectively.

RESULTS

Prevalence of leptospirosis among samples. Among the 118 cases included in the study, circulating antileptospiral antibodies were detected by MAT and titers were reported to be in the range between 1:160 and 1:1,280. The most frequently observed circulating serovar was Autumnalis (25.4%), followed by Australis (17.8%) and Canicola (15.3%) (see Table S5 in the supplemental material). Of the 118, 7 cases were found to be culture positive, with 4 isolates identified as serovar Canicola and 3 as Autumnalis.

Identification of immunoreactive proteins by DNA library screening. Screening of approximately 10^5 plaques of the lambda library revealed 18 reactive plaques, and final screening identified highly expressive immunoreactive clones N2 + λ 4-3 III, N2 + λ 16-1 III, and N2 + λ 17-3 III. Sequences of plasmids rescued from the selected lambda phages were compared with genome sequences of *L. interrogans* serovar Lai strain 56601 and serovar Copenhageni strain Fiocruz L1-130. Sequence analysis confirmed two open reading frames in clone N2 + λ 4-3 III (*argC* and *recA*), two in N2 + λ 16-1 III (*glpF* and *fliD*), and five in N2 + λ 17-3 III (*trmD*, *rplS*, *rnhB*, *lp28.6*, and *lrr44.9*). The conservation of the proteins among various leptospiral species was determined by multiple-sequence alignment. The corresponding protein sequences (as given in Table 1) were retrieved from GenBank and analyzed with the BioEdit sequence alignment editor (version 7.1.3.0). The proteins were found to be highly conserved (~80 to 100% sequence identity) among pathogenic leptospires but shared ~23 to 78% identity with their counterparts in nonpathogenic *Leptospira biflexa* and other pathogenic bacteria (Table 1; see also Table S6 in the supplemental material).

Production of recombinant proteins and their immunoreactivity. The purity of complete His_6 tag fusion proteins expressed in either *E. coli* BL21(DE3) or *E. coli* BL21(DE3)pLysS was confirmed by SDS-PAGE and Coomassie brilliant blue staining (see Fig. S2 in the supplemental material). The determined molecular masses of the purified proteins were consistent with those predicted from their amino acid compositions (Table 1). The purified proteins were immunoblotted with pooled patient sera, and the immunoreactivity of the purified proteins was assessed (Fig. 1A).

***In vivo* expression of the identified proteins during natural infection.** Western blotting assays of the purified proteins developed with HIS (against laboratory culture N2) did not reveal reactivity of the identified proteins except for RplS, RnhB, and Lp28.6 (Fig. 1B). This made us hypothesize that the newly identified and characterized proteins ArgC, RecA, FliD, TrmD, and Lrr44.9 could possibly be expressed during natural infection of the host and not *in vitro*.

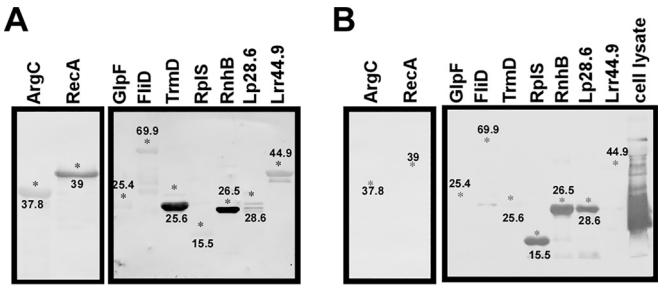

FIG 1 Immunoreactivity of the purified proteins to patient sera or hyperimmune rabbit serum raised against laboratory-grown N2 strain. (A) Western blot (WB) of the purified proteins probed with pooled patient sera. Purified proteins: ArgC (37.8 kDa), RecA (39 kDa), GlpF (25.4 kDa), FliD (69.9 kDa), TrmD (25.6 kDa), RplS (15.5 kDa), RnhB (26.5 kDa), Lp28.6 (28.6 kDa), and Lrr44.9 (44.9 kDa). (B) Western blot of the purified proteins probed with rabbit HIS. Purified proteins: ArgC (37.8 kDa), RecA (39 kDa), GlpF (25.4 kDa), FliD (69.9 kDa), TrmD (25.6 kDa), RplS (15.5 kDa), RnhB (26.5 kDa), Lp28.6 (28.6 kDa), and Lrr44.9 (44.9 kDa).

To further prove the proteins ArgC and RecA to be expressed *in vivo*, *L. interrogans* serovar Autumnalis strain N2-MACS (mouse-adapted challenge strain) was selected from its parent strain N2 (laboratory strain), by passaging the isolate in BALB/c mice (cyclophosphamide treated) for ~15 times. *L. interrogans* strain N2-MACS was passaged *in vitro* (N2-MACS1, -2, -3, and -4) four times. Heat-extracted antigens were prepared from N2-MACS, N2-MACS1, N2-MACS2, N2-MACS3, N2-MACS4, and N2 and immunoblotted with hyperimmune rabbit sera for ArgC/RecA/LipL32. The levels of ArgC and RecA expression were significantly higher in N2-MACS and decreased as the isolates were passaged *in vitro*. No differential expression was observed for LipL32 (Fig. 2).

Further to confirm that ArgC and RecA are not temperature regulated, heat-extracted proteins of *L. interrogans* serovar Autumnalis strain N2 grown at 30, 37, and 42°C were immunoblotted with ArgC/RecA-specific polyclonal rabbit serum. The immunoblots did not show any detectable level of ArgC/RecA (Fig. 3; see also Fig. S3 in the supplemental material). In contrast, LipL32 was expressed by laboratory-grown cultures at different temperatures. LigA-C was used as a positive control. This confirms that ArgC and RecA are expressed *in vivo* and are not temperature regulated.

Evaluation of the recombinant proteins in serological diagnosis. The optimum antigen concentration of the recombinant protein to be utilized in IgM ELISA for serological diagnosis as determined by checkerboard assay was 0.2 μg/well. The mean plus 2 SD of the absorbance values from healthy individuals was defined as the cutoff value to achieve diagnostic specificity. The cutoff values for the recombinant proteins were in the range between 0.101 and 0.186 (Fig. 4A to I). ELISA with purified recombinant proteins demonstrated a sensitivity in the range between 44.9 and 92.3% and a specificity between 46.9 and 96.9%. Among all the recombinant proteins analyzed, ArgC had the highest sensitivity and specificity for the diagnosis of leptospirosis (Table 2). A positive correlation was demonstrated between MAT titer and ELISA OD values (see Fig. S4 in the supplemental material). Then, we asked whether the combination of recombinant proteins in ELISA format could increase the sensitivity and specificity of the test. To test that, we used three different combinations based on the immunoreactive clones obtained (N2 + λ 4-3 III, N2 + λ 16-1 III, and N2 + λ 17-3 III): combo 1 (ArgC and RecA), combo 2 (GlpF and FliD), and combo 3 (TrmD, RplS, RnhB, Lp28.6, and Lrr44.9). The purified recombinant proteins were mixed per the proportions mentioned above and used in ELISA. As expected, the combination of recombinant proteins increased the sensitivity.

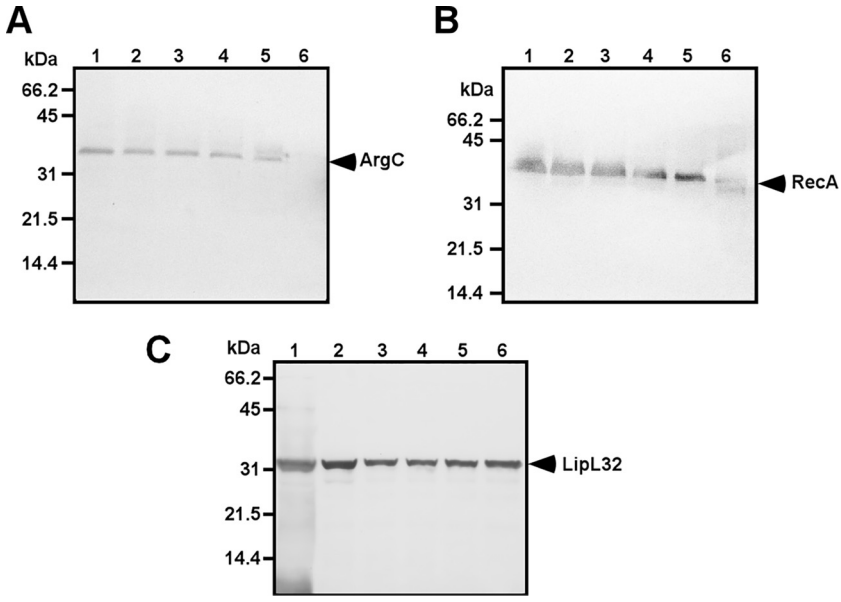

FIG 2 ArgC and RecA are proteins expressed only during natural infection *in vivo*. Heat-extracted proteins from *L. interrogans* serovar Autumnalis strains N2-MACS (lanes 1), N2-MACS1 (lanes 2), N2-MACS2 (lanes 3), N2-MACS3 (lanes 4), N2-MACS4 (lanes 5), and N2 (lanes 6) were immunoblotted with hyperimmune rabbit sera for ArgC (A), RecA (B), and LipL32 (C). The levels of ArgC and RecA expression were significantly higher in N2-MACS and decreased as the isolates were passaged *in vitro*. No differential expression was observed for LipL32.

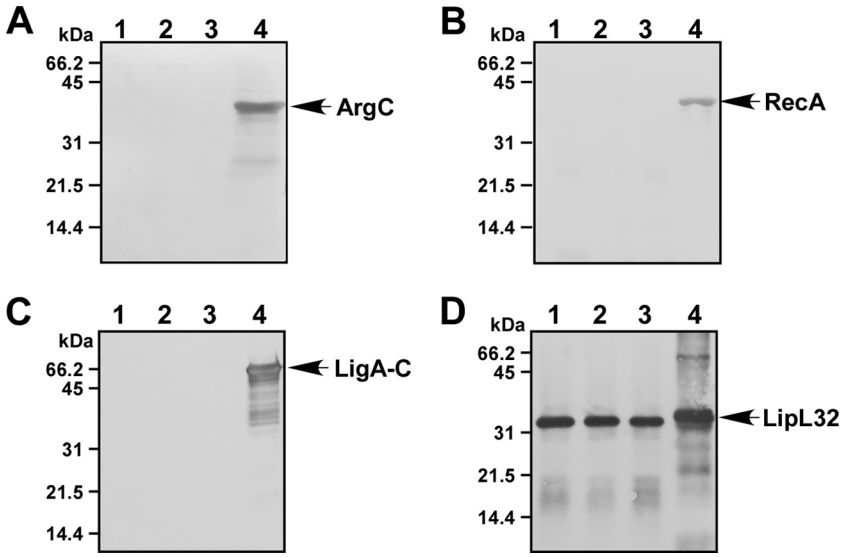

FIG 3 ArgC and RecA expression levels are not temperature regulated. Whole-cell lysate of *L. interrogans* serovar Autumnalis strain N2 grown at 30°C (lanes 1), 37°C (lanes 2), and 42°C (lanes 3) and purified recombinant protein (lanes 4), immunoblotted with hyperimmune rabbit sera for ArgC (A), RecA (B), LigA-C (C), and LipL32 (D).

Combo 1 had a sensitivity of 95.7% and a specificity of 94.9% (Table 2). The sensitivity of combo 1 ELISA was ~5 to 10% greater than that of the ELISA using ArgC or RecA separately, and it was comparable to the sensitivity and specificity (Table 2; see also Fig. S5 in the supplemental material) of LigA-C peptides identified in our previous study (23).

DISCUSSION

Leptospira interrogans has caused serious fatal illness worldwide. The early clinical presentation of fever, headache, and myalgia may be difficult to distinguish from febrile illness associated with diseases such as dengue, viral hemorrhagic infections, and rickettsiosis. Thus, an objective test that promptly detects the causative *Leptospira*, its DNA, or a specific antibody would be extremely valuable in guiding treatment and providing awareness of outbreaks, thereby facilitating provisional diagnosis of patients with clinical presentations/histories. In the present study, the use of purified recombinant ArgC and RecA in combination proved to be an effective antigen to provide conclusive diagnosis of leptospirosis. Percent sensitivity and specificity were >90% based on comparisons with sera of healthy individuals and of patients with other febrile infectious diseases.

Humoral immune responses to pathogenic bacteria are mainly directed at surface-exposed and secreted antigens accessible to antibody in plasma and mucosal secretions. Thus, a screen of an expression gene library with immune serum identifies a disproportionately large number of proteins with sequences predictive of surface exposure (25–27). A consequence of this approach has been failure to consider cytoplasmic proteins. The proteins ArgC and RecA used as effective antigens are cytoplasmic proteins of *L. interrogans* that participate in host immune responses. Neither of these proteins was identified in a recent high-density protein microarray of serovar Copenhageni for which the selection criteria included potential biological importance and antigenic features (13).

The majority of studies of putative virulence factors important in microbial pathogenesis have focused on nonmetabolic gene products. This is due, in part, to the belief that housekeeping and other genes common to pathogens and nonpathogens are unimportant for pathogenesis. In fact, during the crucial early phase of microbial proliferation *in vivo*, the ability of the pathogen to synthesize or acquire scarce/unavailable nutrients becomes critical. For example, iron acquisition factors are well accepted as important for leptospiral pathogenesis (17, 18). Acquisition or synthesis of other nutritional factors is less well appreciated but supported by a variety of reports (28–30). The frequent identification of biosynthesis genes following library screening of random gene fusions or randomly generated transposon mutants argues for their importance in pathogenesis (28, 29, 31).

The findings to date suggest that decreased levels of arginine in human urine induce *argC*. Disruption of the *carAB* gene involved with arginine and pyrimidine biosynthesis significantly reduced the ability of *Salmonella* to cause systemic infection (29). These studies also showed that *in vivo* arginine biosynthesis is required for optimal renal infection but not for optimal infection of bladder and urine. RecA is a ubiquitous bacterial recombination protein essential both for DNA transformation (32, 33) and for mediation of bacterial SOS responses (34). Colonization and infection of host organs are potentially stressful for invading pathogens because they include the effects of antibacterial factors such as pH, elevated temperature, osmolarity, and factors released by other microorganisms on the mucosal surface. Some stresses result in DNA damage, the repair of which involves RecA, an enzyme critical for pairing of single-strand DNA with complementary regions of double-stranded DNA. Single-strand DNA-RecA produced following DNA injury activates self-catalysis of LexA protein responsible for blocking expression of SOS genes for proteins that repair DNA. Thus, RecA also functions as a LexA coprotease. A significant decrease in intracellular pH has been shown to result both in DNA damage via depurination in *Salmonella enterica* serovar Typhimurium and in induction of the DNA

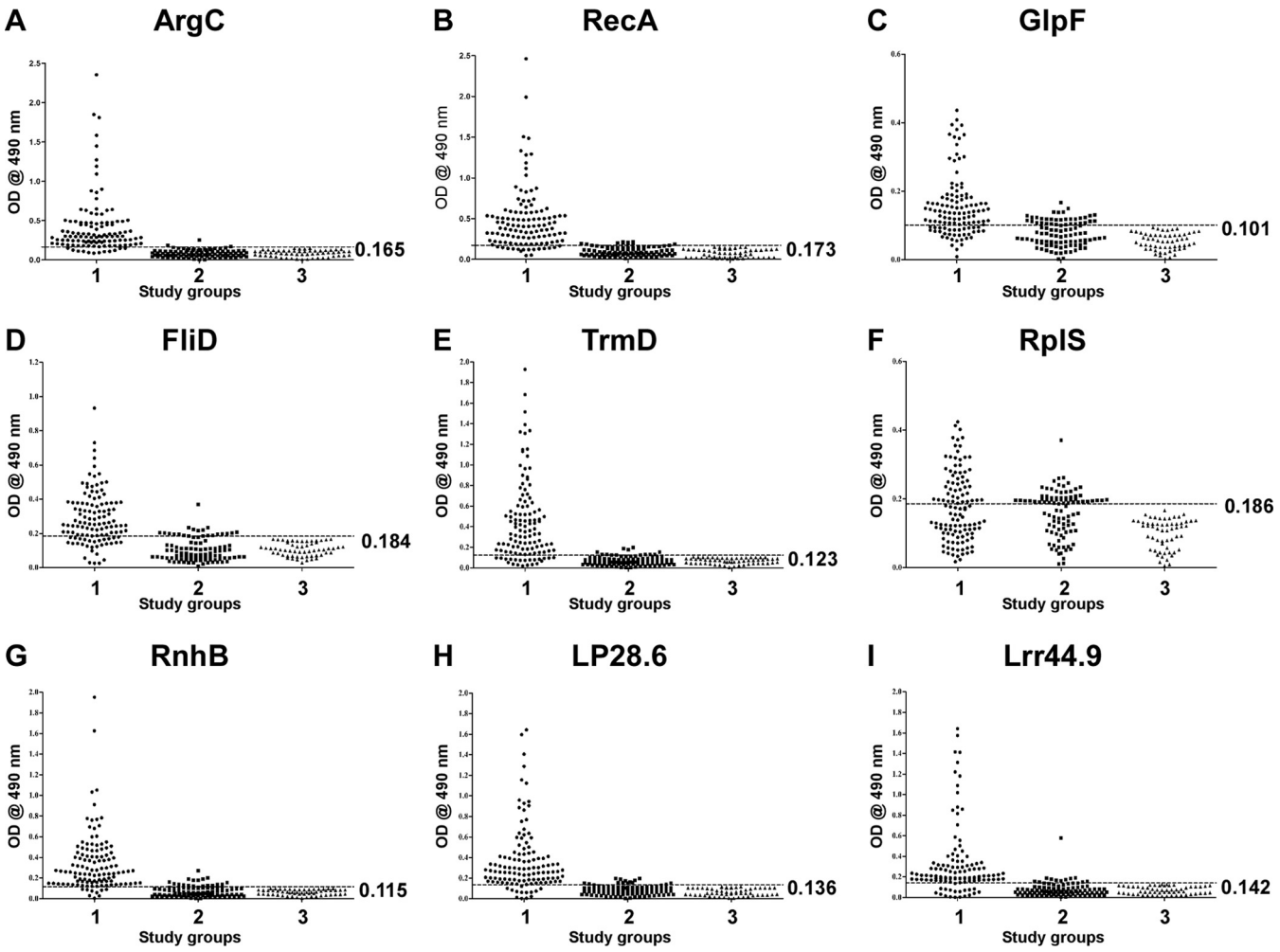

FIG 4 Evaluation of purified recombinant antigens in IgM ELISAs. Study groups are indicated on the *x* axis, and the optical density (OD) at 490 nm is indicated on the *y* axis. IgM responses to purified recombinant proteins (A to I) are shown. Study groups: group 1, laboratory-confirmed cases of leptospirosis; group 2, confirmed for other infectious diseases; group 3, seronegative healthy controls. The dashed lines represent the cutoff values for each antigen with the absolute cutoff values on the right.

repair protein PolA (35). RecA may enhance resistance to oxidative killing by phagocytes based on evidence that a Δ*recA* mutant of *Salmonella* Typhimurium is sensitive to the killing effect of the oxidative burst of macrophages (36). Furthermore, *recA* mutation diminishes adherence to and colonization of rabbit intestine by *Vibrio cholerae* (37). Infection by serovar Autumnalis and other *Leptospira* serovars is clearly associated with consistent expression of this immunogenic protein in the early stages of invasion/bacteremia, raising the possibility that it has a role in pathogenesis in addition to that of DNA repair.

The identified *in vivo*-expressed proteins would have been involved in infection and stress responses of *L. interrogans* and thus are upregulated during infection and elicited specific IgM responses in patients during the acute phase. Further studies focusing on the importance of the identified proteins will help determine whether these proteins represent site-specific or more global virulence factors.

TABLE 2 Sensitivity, specificity, positive predictive value, and negative predictive value for IgM ELISA using identified proteins separately and in combination

Antigen	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
ArgC	89.8	96.9	97.2	88.7
RecA	86.4	89.8	91	84.6
GlpF	71.1	65.3	71.1	65.3
FliD	74.5	78.5	80.7	71.9
TrmD	82.2	86.7	88.1	80.1
RplS	44.9	46.9	50.4	41.4
RnhB	92.3	81.6	85.8	89.8
Lp28.6	88.9	85.7	88.2	86.6
Lrr44.9	83.9	87.7	89.1	81.9
Combo 1 (ArgC and RecA)	95.7	94.9	95.7	94.9
Combo 2 (GlpF and FliD)	96.6	74.4	82	94.8
Combo 3 (TrmD, RplS, RnhB, Lp28.6, and Lrr44.9)	93.2	78.5	83.9	90.5
LigA-C peptides				
Peptide 1 (VVIENTPGK)	97.4	97.9	98.2	96.9
Peptide 2 (TALSVGSSK)	98.3	98.9	99.1	97.9

ACKNOWLEDGMENTS

We kindly acknowledge the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, for the

Research and Development grant (BT/PR13502/MED/15/36/2010) provided to carry out this study. K.N. is thankful to the Department of Health Research (DHR), Indian Council of Medical Research (ICMR), for a long-term overseas training fellowship [SEC/DHR/HRD-FELLOW/1 (2)/2011].

FUNDING INFORMATION

Department of Biotechnology, Ministry of Science and Technology (DBT) provided funding to Kalimuthusamy Natarajaseenivasan under grant number BT/PR13502/MED/15/36/2010. Indian Council of Medical Research (ICMR) provided funding to Kalimuthusamy Natarajaseenivasan under grant number SEC/DHR/HRD-FELLOW/1(2)/2011.

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