

## Obtaining and characterization of a recombinant LipL32 protein for detection of leptospirosis

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**Abstract.** Leptospirosis is a disease which affects domestic and wild animals as well as humans. A highly conserved outer membrane lipoprotein LipL32 is considered to be an important antigen for detection of leptospirosis. In this study the recombinant protein LipL32 was extracted and characterized by immunochemical methods. The recombinant protein LipL32 was produced in *Escherichia coli*. We have optimized a *LipL32* gene for expression in Rosetta (DE3) strain. 0.5% sodium lauryl sulfate was used to solubilize the protein and expressed into inclusion bodies. Immunochemical studies demonstrated a high reactivity of the recombinant LipL32 with antibodies elicited during infection of pathogenic *Leptospira* serovars. This study tested the diagnostic performance of the recombinant LipL32 in an in-house ELISA. The indirect ELISA was developed and its specificity was compared to that of a traditional microagglutination test (MAT).

### INTRODUCTION

Leptospirosis a zoonotic and human infection is caused by spirochete bacteria from gen *Leptospira* (McBride, 2005). Leptospirosis is a natural disease found in wild and domesticated animals and can also affect humans. The disease is found in various biomass and geographical areas of the world. The distribution of leptospirosis globally is due to the abundance of reservoir hosts to the pathogenic *Leptospira*, as well as the high degree of antigenic, ecological and genetic biodiversity of the causative agent (Baryshnikov, 2014). The disease is commonly spread by rodents (Faisal, 2012) and farm animals that can also serve as reservoirs hosts Human infections predominantly occur in farmers and people working closely with domesticated mammals. Rodents, wild and domesticated animals are the main source of the infection

while humans are sporadic hosts of the disease (Manjila, 2012).

According to statistics provided by the Ministry of Agriculture of Kazakhstan the pathogen causes sporadic disease. Murzabaev (2010) showed that 3.5% of cattle, 3.8% of sheep, 6.2% of pigs, and 1.3% of horses in Kazakhstan show evidence of previous infection although no symptomatic cases were found in the studied herds (Murzabayev, 2010).

Modern serological diagnosis of leptospirosis utilizes recombinant antigens from the bacterium. LipL32 is an outer membrane lipoprotein of the pathogenic *Leptospira* which is expressed in high levels during replication of the pathogen. Haake *et al.* (2001) isolated and cloned a gene of *Leptospira* encoding a 32 kDa (LipL32) protein and expressed it in *E. coli*. An amino acid sequence of the LipL32 antigen resides in the NCBI database

(GenBank: AAF60225.1). The protein is highly evolutionary and is conserved among various serovars and serotypes of pathogenic *Leptospira*. LipL32 that is produced by *Leptospira* during all stages of its life cycle and the antigen elicits efficient immune response in humans and animals. This protein is an immunologically dominant antigen during infection and high-titer production of antibodies is observed in almost all cases. It has been shown that the antibodies against LipL32 can be detected in sera of leptospirosis patients during all stages of the disease and are highly specific with no detectable cross-reactivity occurring to unrelated antigens (Vaganova, 2011; Ahmed, 2012; Gebriel, 2006).

Since LipL32 is a highly conserved antigen specific to *Leptospira*, it has great diagnostic potential for development in an enzyme-linked immunosorbent assay (ELISA) for the detection of leptospirosis.

Existing veterinary diagnostics tests in Kazakhstan do not provide sufficient levels of convenience and safety for epidemiologic studies. This may be a reason why the results of the disease vary among regions of Kazakhstan. Considering international cattle trade in which Kazakhstan intensively participates, it is important to utilize a convenient and robust test to detect the infection in imported cattle. The imported animals may show no signs of the disease yet can serve as vehicle for a cross-border transmission and a source of the infection. This paper describes an effective ELISA to suit the diagnostic needs for detection of leptospirosis in farm animals.

## MATERIALS AND METHODS

### *Generation of a genetic construct pET28c/LipL32 and a producer strain*

Sequences of a gene encoding *LipL32* were downloaded from the Genbank database. A sequence was codon-optimized to increase efficiency of expression in *E. coli*. The *LipL32* gene was synthesized *de novo* using a PCR-based method (Xiong, 2006). Oligonucleotides, 60 nt-long were paired and are capable of annealing to form nearly a full-

length sequence. A product of a first round PCR was used as a template to conduct a second round PCR with primers flanking the artificial sequence. Amplified products of the PCR were cloned into a pET28c(+) plasmid (Novagen) using NdeI and HindIII sites. A selection of cloning was performed using competent cells of *E. coli* Rosetta (DE3) strain. The selection of transformed clones were carried out on a solid medium containing 0.05 mg/ml of kanamycin. The plasmid pET28c(+) has sites complementary to a T7 forward (5'-TAATACGACTCAC TATAGGG-3') primer and a T7 reverse (5'-GCTAGTTATTGCTCAGCGG-3') primer which were utilized to conduct bidirectional sequencing.

A plasmid pET28/LipL32 was electroporated into Rosetta (DE3) cells. Transformed cells were selected on solid medium with kanamycin. Resulting colonies were inoculated into LB medium supplemented with 0.05 mg/ml kanamycin. Cultures were grown at 37°C to OD600 = 0.6 concentration. At this point an expression of the recombinant protein was induced by an addition of isopropyl β-D-1-thiogalactoside (IPTG) to 0.2 mM. After the addition of IPTG, incubation temperatures were lowered to 30°C and 10-ml samples of the culture were collected after 4 hours, 6 hours, 8 hours and overnight incubation. Bacteria were pelleted by centrifugation (6000g, 7 min) and disrupted using an Omni Raptor 4000 sonicator: 6 times with 30 sec pulse at 40% amplitude with 60 sec pause. Obtained lysates were spun down (18,000g, 30 min) and subjected to an SDS-PAGE.

### *Preparation of a detergent-soluble fraction of a Leptospira antigen*

LipL32 recombinant protein from *E. coli* cells were solubilized using a modified approach by Auran *et al.* (Auran, 1972; Nunes-Edwards, 1985; Brown, 1991). Bacterial cells containing pET28/LipL32 plasmid were collected and resuspended in a buffer (20 mM Tris pH 7.5, 20 mM NaCl) containing 40 mg/ml lysozyme and incubated for 30 minutes at room temperature (RT). A lysed cell suspension was treated with ultrasonic waves as previously described. Lysates

were centrifuged at 18000g for 30 minutes. Pellets were washed twice with 1% Triton X-100 in PBS, then spun again at 18000g and resuspended in a buffer containing 8M Urea, 1.5M NaCl, 2% Triton X-100, 20mM Tris pH 8.0, 0.25% SDS. Finally the pellets were resuspended in 20 mM Tris-HCl pH 8.0, 0.5% SDS/20 mM imidazole and quickly sonicated. The precipitate was used to extract the recombinant protein by dissolving it in a solution of 20 mM Tris-HCl pH 8.0 and 1% sodium lauryl sulfate. A solution of the recombinant protein was dialyzed against PBS pH 7.4 overnight.

#### *Sera*

Sera were obtained from blood of immunized rabbits. The rabbits were hyper-immunized with *Leptospira*. A commercial kit was used for ELISA which provides control sera against following *Leptospira* serogroups: 1- *Pomona*, 2- *Grippotyphosa*, 3- *Icterohaemorrhagiae*, 4- *Canicola*, 5- *Australis*, 6- *Javanica*, 7- *Hebdomadis*, 8- *Tarassovi*, 9- *Autumnalis*, 10- *Pyrogenes*, 11- *Cynopteri*. The kit was purchased from the FKP "Armavir Bio-factory" (Russia). These sera tested positive in a microagglutination test (MAT).

#### *Indirect ELISA*

All 96-well flat-bottom plates (Nunc) were coated with the recombinant LipL32 antigen. A checkerboard titration was used to determine the best antigen concentration in wells (400 ng/wel). The antigen was dissolved in 10 mM PBS with an addition of 0.1 M NaCl and the plates were incubated at 4°C overnight. The plates were blocked with 2% (w/v) bovine serum albumin in PBS for 1 hr at 37°C. The plates were then rinsed four times with 0.05% Tween 20 in PBS and 0.1 ml of sera with antibodies against corresponding *Leptospira* serogroups and positive or negative control sera (1:100 in PBST) were added to wells. The plates were incubated for 1 hr at 37°C. After this step the PBST-washed plates were incubated with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Sigma, USA, Cat#A6154) diluted 1:5000 in PBST. The incubation continued for 1 hr at 37°C. TMB reagent (3,3',5,5'-tetramethylbenzidine 0.4

mM) (Sigma Co., USA) in 40 mM sodium citrate buffer (pH 4.0) with 3 mM peroxidase substrate was used to detect results of the ELISA. The substrate was incubated for 15 minutes in the dark and the reaction was stopped by adding of 0.05 ml of 1M sulfuric acid. Optical absorption of the reaction products was measured at a 450 nm.

#### *Immune blotting*

Samples of the recombinant LipL32 antigen in quantities of 500 ng were loaded into wells of a 10% polyacrylamide gel for electrophoresis in a presence of sodium dodecyl sulfate (SDS-PAGE). The protein after electrophoresis was transferred to a nitrocellulose membrane for 2 hrs at 60 V and 250 mA current. The membrane was then blocked with 5% nonfat dry milk in PBST overnight at 4°C and the membrane was rinsed three times with PBST and incubated for 2 hrs at 37°C in a solution containing varying concentrations of a positive control serum diluted 1:500 in PBST. Various serogroups of pathogenic *Leptospira* were used to test reactions in the immune blotting. Sera from non-immunized rabbits were used as negative control sera. Membranes were then rinsed three times in PBST and kept for 1 hr at 37°C in a solution of a peroxidase-labeled goat anti-rabbit antibody (Sigma Cat#A6154) diluted 1:5000. After proper washing the reaction was visualized with 4-chloro-1-naphthol (Thermo Fisher, USA, Cat#34010) substrate.

#### *Microagglutination test (MAT)*

A MAT test was performed and interpreted as indicated per manufacturer's instruction. Agglutination profiles were determined classically by using *Leptospira* cultures in a microplate. The reaction was performed in a company diagnostic laboratory.

## RESULTS

### **Amplification of a LipL32 gene**

An outer membrane protein LipL32 is expressed in all major *Leptospira* serovars was cloned and expressed in *E. coli*. An amplicon was constructed *de novo* from

oligonucleotides. As shown in Figure 1a PCR product (765 bp) was obtained after two-rounds PCR. Flanking forward primer 5'-ggaattccatatggcctatctccgttgactctttgc-3' (site NdeI underlined) and reverse primer 5'-cccaagcttgggttacagcgggctcacacctgg-3' (site HindIII underlined) were used to introduce a 6×His tag to the N-terminus. Flanking forward primer 5'-ccacggatccccatgggcgtatctccgttgactctttg-3' (NcoI underlined) and reverse primer 5'-actaaagcttctcgagttaatgatgatgatgatgcagcgggctcacacctg-3' (HindIII underlined) were used to introduce a 6×His tag to the C-terminus. The resulting PCR product was cloned into a pET28c(+) (Novagen) plasmid using the flanking restriction sites. Bidirectional sequencing confirmed the presence of an insertion of the *LipL32* gene in a generated expression construct pET28c/*LipL32*. Haake *et al.* (2000) demonstrated that an amino acid sequence of the major outer membrane protein LipL32 is highly conserved in many *Leptospira* serovars. The obtained plasmid was designed to drive an expression of the

antigen conserved among majority of pathogenic *Leptospira* serovars. The recombinant protein has 238 amino acid residues as shown below:

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MAISVALFASITACGAFGGLPSLKSSFVLSE
DTIPGTNETVKTLLPYGVSVINYYGYVKPGQ
APDGLVDGNKKAYLYVWIPAVIAEMGVRM
ISPTGEIGEPGDGDLVSDAFKAATPEEKS
MPHWFDTWIRVERMSAIMPDQIVKAAKA
KPVQKLDDDDDDGDDTYKEERHNKYNLSLR
IKIPNPPKSFDDLKNIDTKKLLVRGLYRISF
TTYKPGEVKGSFVASVGLLFFPGIPGVSP

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### Production of a detergent-soluble fraction of the recombinant LipL32 antigen

The purified recombinant protein shows high sensitivity to salts and detergents. Solubilization of the recombinant protein using a Triton X-114 was described in detail by Haake (2001). Herein we describe a method for precipitation of the antigen using a combination of Triton X-100, urea and SDS. Treatment of bacterial cell with chaotropic agents brought on an additional problem with protein separation on a polyacrylamide gel (Figure 2). We observed two proteins in the stained SDS-PAGE gel. A 28 kDa band most likely represents the LipL32 and an 18 kDa band was recognized as a proteolytic degradation product. Interestingly, when we probed the recombinant antigen in an immune blotting using various hyperimmune sera we obtained dissimilar results for various *Leptospira* serovars (Figure 3B).

### Immunochemical determination of a specificity of the recombinant LipL32 antigen

An immunochemical experimentation with the recombinant antigen was done using immune blotting with sera from immunized rabbits. The immune blotting confirmed that the 28 kDa protein was indeed an isoform of the expected antigen LipL32. A specificity of the recombinant LipL32 antigen was confirmed by a lack of interaction with serum from a non-immunized rabbit. Also a second band (18 kDa) was evident in immune blotting when sera from rabbits sensitized against

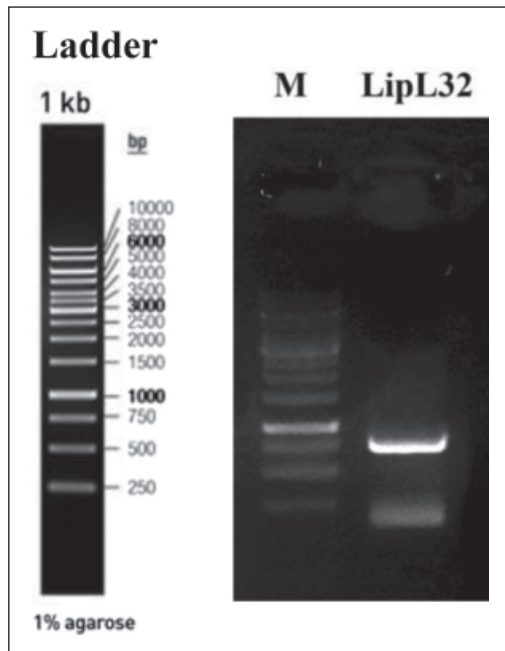


Figure 1. Results of amplification of a *LipL32* gene.

Left panel presents a DNA ladder used for a comparison of sizes of fragments. M – DNA ladder; *LipL32* – gene, expected size 765 bp.

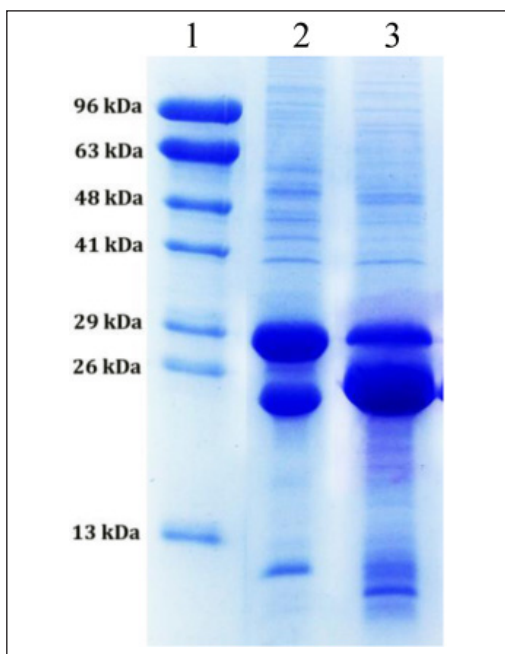


Figure 2. Analysis of a detergent-soluble recombinant protein in a SDS-PAGE. Lane 1 – Protein marker; Lane 2 – Pellet of a C-terminally 6xHis-tagged recombinant protein LipL32 after purification; Lane 3 – Pellet of an N-terminally 6xHis-tagged protein LipL32.

*Leptospira Javanica*, *Pyrogenes*, *Tarassovi* serotypes were used. Negative control sera did not show reactivity with the LipL32 (Figure 3).

According to manufacturer's guidelines the used hyperimmune sera had following titers in MAT test: *Pomona* – 1:64000; *Grippotyphosa* – 1:32000; *Icterohaemorrhagiae* – 1:64000; *Canicola* – 1:32000; *Australis* – 1:32000; *Javanica* – 1:32000; *Hebdomadis* – 1:64000; *Tarassovi* – 1:32000; *Autumnalis* – 1:64000; *Pyrogenes* – 1:64000; *Cynopteri* – 1:64000. The recombinant LipL32 antigen showed titers in ELISA as follows: *Pomona* – 1:51200; *Grippotyphosa* – 1:51200; *Icterohaemorrhagiae* – 1:51200; *Canicola* – 1:25600; *Australis* – 1:51200; *Javanica* – 1:25600; *Hebdomadis* – 1:51200; *Tarassovi* – 1:25600; *Autumnalis* – 1:12800; *Pyrogenes* – 1:25600; *Cynopteri* – 1:51200.

We transformed results of the dilution series (Figure 4) to a logarithmic scale by calculating them using an equation “dilution number= $\log_2$  titer” as suggested by Kirckwood (2003).

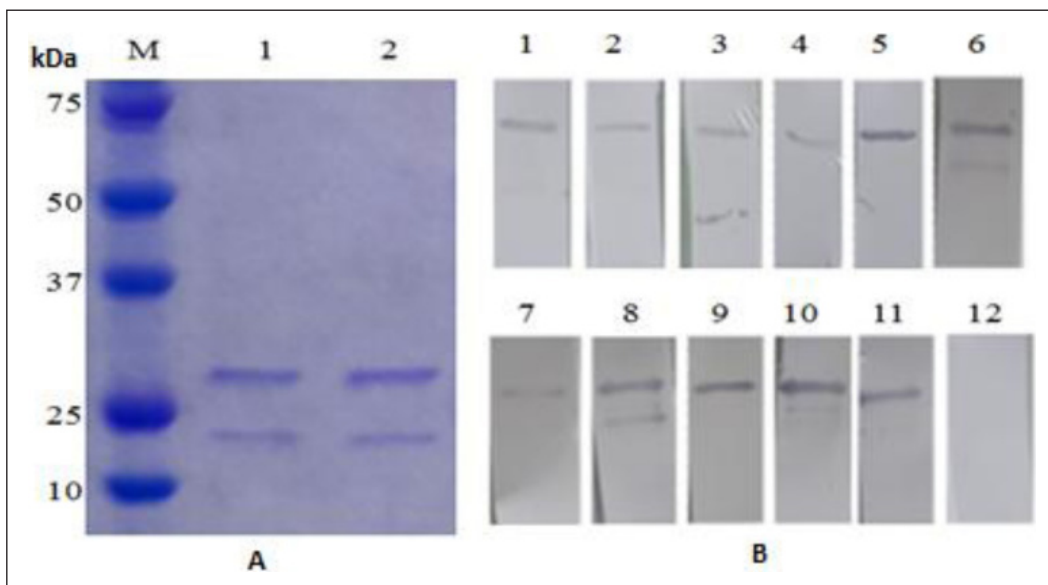


Figure 3. Coomassie staining of an SDS-PAGE gel and immune blotting of a recombinant antigen LipL32 reacted with sera from rabbit immunized with *Leptospira*. The LipL32 antisera detect a 28 kDa band in all experiments; also a 18 kDa band is detected in three experiments.

A – Coomassie brilliant blue staining of the recombinant protein LipL32 in a 10% polyacrylamide gel. M-protein marker, 1 and 2 lane represents the LipL32 antigen, 500 ng per well; B – immune blotting with positive control sera, each strip was reacted with a serum raised against a particular serovar: 1- *Pomona*; 2- *Grippotyphosa*; 3- *Icterohaemorrhagiae*; 4- *Canicola*; 5- *Australis*; 6- *Javanica*; 7- *Hebdomadis*; 8- *Tarassovi*; 9- *Autumnalis*; 10- *Pyrogenes*; 11- *Cynopteri*; 12- immune blotting with a negative control serum.



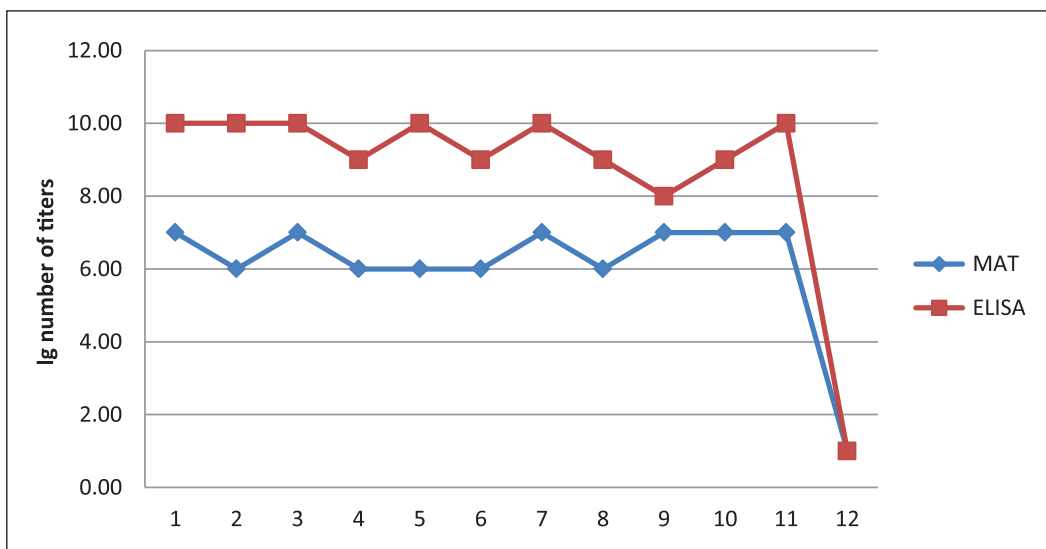


Figure 4. A relationship between titers in an ELISA and MAT test. Titers of antibodies raised against various serovars are shown: 1- *Pomona*; 2- *Grippityphosa*; 3- *Icterohaemorrhagiae*; 4- *Canicola*; 5- *Australis*; 6- *Javanica*; 7-*Hebdomadis*; 8- *Tarassovi*; 9- *Autumnalis*; 10- *Pyrogenes*; 11- *Cynopteri*; 12- negative control serum.

## DISCUSSION

Detection of leptospirosis during early stages of infection is very important for undertaking preventive measures and eliminating the disease (Gebriel, 2006). Currently the commonly used diagnostic methods utilizes the microbiological methods, although serological methods are considered to be more sensitive (Prasad, 2015). In this study we have synthesized the recombinant major outer membrane protein (LipL32) in *E. coli* which is conserved among majority of pathogenic *Leptospira* serovars, that includes the following serovars: *Australis*, *Autumnalis*, *Canicola*, *Grippityphosa*, *Hebdomadis*, *Icterohaemorrhagiae*, *Javanica*, *Pomona*, *Tarassovi* were found in Kazakhstan (Murzabayev, 2010).

This study demonstrates that amplification and isolation of the LipL32 recombinant protein is possible using a combination of Triton X-100 and SDS/Urea. In an SDS-PAGE two bands with a reactivity to the leptospiral antigen LipL32 (Figure 3A) were identified. The formation of two bands from recombinant LipL32 antigen is because the extracted protein was not subjected to a

suitable chromatographic purification. A metal affinity chromatography was used in an isolation process and found that the recombinant LipL32 resisted solubilization in common conditions. It was decided to elute the recombinant protein by dissolving inclusion bodies in a detergent solution. Two bands were observed in the SDS-PAGE. The upper band in the gel represented the full-length LipL32 antigen and the lower band is probably a byproduct of proteolytic degradation. The LipL32 protein is found in a wide variety of isoforms having molecular masses from 29 to 14 kDa (Cullen, 2002; Vaganova, 2011). Haake *et al.* (2000) observed a discrepancy between an observed mobility of the antigen and a calculated molecular mass of the protein. He found slower mobility of this protein in the SDS-PAGE which was explained by a highly acidic nature of the protein. This study detected the upper band (28 kDa) and lower band (18 kDa), although the lower band was not detected during an immune blotting with all sera (Figure 3B). A variability in detection of the lower band can be explained by an existence of a pathogen-specific characteristics in the detected isoforms.

Titers of anti-leptospiral antibodies in hyperimmune rabbit sera were found to be very high (Figure 4). A high analytical sensitivity of the recombinant LipL32 antigen was determined via ELISA. The specific antibodies in all seropositive sera were bound to the recombinant LipL32 antigen in ELISA. The tested sera indicated high specificity of the used antigen in a classical MAT test. Levels of a specific serological activity in the MAT test were 1:16000-1:64000 and the titers against the recombinant LipL32 antigen in indirect ELISA were 1:12800-1:51200. A logarithmic equation was used to convert the ELISA antibody titers and the MAT dilution titers. A comparison of data showed similar patterns of a reactivity in the indirect ELISA and MAT tests.

#### CONCLUSION

In summary, we expressed the recombinant LipL32 antigen and studied its suitability for detection of leptospirosis in ELISA. Immunochemical characterization showed high specificity for the *Leptospira* serovars found in Kazakhstan. The recombinant protein LipL32 has potential for use as a rapid screening test which can serve as a valuable diagnostic tool.

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#### DISCLOSURE

No conflict of interest exists in this work.

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