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Immunodiagnostics and Immunotherapy of Leptospirosis

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Abstract

The article summarizes the research and development of modern manufacturing technology of biological preparations for immunodiagnostics and immunotherapy of animals and humans. In particular, for the first time in the Republic of Kazakhstan, we designed and successfully tested a highly sensitive, antigenic erythrocyte diagnosticum for express-diagnostic of humans and animals. IHT (indirect hemagglutination test) with antigenic erythrocyte diagnosticum, developed by us, is sensitive and simple and gives results very fast with a minimum expenditure of money and labor, so it can be used for instant analysis of leptospirosis on humans and animals. We developed a selective culture medium from rabbit and sheep serum and optimal modes of extraction of bacterial antigens for animals' hyperimmunization. We also developed the scheme of immunization of animals—producers, depending on the dose, the multiplicity, and the injection site, and produced a multivalent antileptospirosis hyperimmune serum for immunotherapy of animals.

Keywords

Erythrocyte; Sensitization; Addition of tannin; Synanthropic mammals; Antigens; Indirect hemagglutation test; Immunodiagnosis; Laser therapy; the skin form of leptospirosis; SES (Sanitary-Epidemiological Station); RMAL (reaction of microagglutination lysis)

Introduction

Leptospirosis is one of the most common zoonoses. Pathogens of leptospirosis circulate in wildlife in different geographic areas and landscapes. Leptospires also infect livestock and pets, which are the main sources of leptospirosis for humans because of the permanent and close contact between the two, and has a massive and long period of infectious beginning [1]. Pathogens of leptospirosis have a complex antigenic structure, which is expressed by the existence of a great number of serotypes among them. However, immunological diagnosis and immunotherapy of leptospirosis in practice still don't have optimal and rational solutions. Solution methods are usually based on the use of a large number of living *Leptospira*. But in case of large-scale researches, it makes the process more complicated. In the research laboratories of many countries, immunological tests for leptospirosis diagnostic are characterized by a wide arsenal of reactions, including new ones. The most widely observed reaction is erythrocyte diagnosticum. In the scientific literature, there is sufficient comparative data showing no absolute diagnostic immunoreagents [2]. Interaction of insoluble carrier of immunologically active components is often called "sensibilization," and components used for this process are "sensitins" by analogy with the term "hemosensibilization," first used to refer the processes of erythrocyte loading [3]. Nowadays, we have developed numerous variations of fixing erythrocytes, getting sensitin, and employing methods of erythrocyte loading to construct a specific immunoreagent [4,5].

According to the analysis of the latest materials on the diagnosis, treatment, and prevention of leptospirosis, nowadays the study of immunotherapy and immunodiagnostics of animals is a relevant topic.

Materials and Methods

For serology research in RMAL (reaction of microagglutination lysis) and in IHT (indirect hemagglutination test), blood was taken on the 5th-7th day of the animal disease and again 7-10 days later.

For RMAL, live cultures of *Leptospira* were used. We also used strains of *Leptospira* recommended by the WHO (World Health Organization) Scientific Group on Research in Leptospirosis. For the experiments, biomass and antigen were obtained for hyperimmunization and production of diagnosticum strains of many serogroups: Icterohaernorrhagiae, Javanica, Grippotyphosa, Canicola, Pomona, Tarassovi, Hebdomadis, Bataviae, Autumnalis, Ballum, Pyrogenes, Australis, Cynopteri.

Analysis of the effectiveness of nutrient media was carried out by a slurry density of *Leptospira*, estimating the number of cells using a microscope (ocular 10, lens 10) and dry yield of Boivin antigen per unit of biomass of *Leptospira*. The dry antigen was prepared by lyophilization. For industrial manufacturing of antileptospirosis, polyvalent serum hyperimmunization of producer animals was carried out with a suspension of *Leptospira* 5×10^8 m.k. in 1 ml, according to a previously developed scheme.

IHT with *Leptospira* antigenic erythrocyte diagnosticum was conducted by the method previously developed by our team, which is described as follows: serum is diluted to 1:25; for this we need to mix 0.1 ml of whole serum with 2.5 ml of 0.85% sodium chloride solution. In a vial with diagnosticum, we add 19 ml of 0.85% sodium chloride solution and mix until a homogenous slurry is formed.

For IHT, in 6 wells of U-shaped microtiter plate or round-bottomed microplate, we instill 0.05 ml of serum at a dilution of 1:25 and mix. Then, by moving from one well to another, 5-10 inclusive, 0.05 ml of serum is titrated. The 6th well is important. Next we instill 1 drop (0.025 ml) of diagnosticum in all wells, shake gently and leave on a flat surface at (22 ± 3)°C for 2-3 h, and record the results.

In case of absence of specific antibodies in the tested serum, we will see the sediment in the form of narrow ring in the center of well. In the presence of serum antibodies to *Leptospira*, erythrocytes cover the bottom of well as an "umbrella." This result is considered

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as a positive result for 4 crosses. Intermediate results are possible for 2-3 crosses. Diagnostic titer is done in the ratio 1:150-200. In order to obtain reliable results, the survey should be carried out twice and the dynamics of increase in titer should be considered (at least two to three times).

Results and Discussion

Leptospiral erythrocyte diagnosticum production was composed of the following stages:

- The cultivation and preparation of the biomass of *Leptospira*;
- Preparation and verification of *Leptospiral* antigens;
- Design and study of *Leptospira* antigenic erythrocyte diagnosticum.

To obtain *Leptospiral* antigens, we needed to find cheaper and more efficient alternatives of rabbit serum, intended as the basis of the nutrient medium for serial biomass of *Leptospira*. Since the biomass of *Leptospira* interested us in terms of the subsequent extraction of the active antigen, for the production of erythrocyte diagnosticum, we studied the growth quality of culture medium using a sheep serum, which is a waste product in the production of erythrocyte diagnosticum.

Boivin antigen yield from 100 ml of biomass (*Leptospira* sediment, obtained by centrifugation), grown on sheep and rabbit sera, was substantially similar. Some differences were found in the series of each serum, depending on their concentration and depending on the serogroup of *Leptospira*. Experiment results have established the possibility of using cheap sheep serum as a basis for the liquid medium without loss of yield of the final product.

It was found that the output of dry biomass of *Leptospira* in 50 ml of nutrient medium strains of Ballum, Bataviae, Tarassovi, Grippotyphosa, Australis, and Pomona serogroups did not differ with growth in 2.5-10.0% sheep serum, and other *Leptospira* serogroups dramatically (about 25%) increased biomass yield with growth in 5-10% serum media, compared with 2.5% serum media. Finally, we used the 3-5% nutrient medium.

We prepared four serum culture media:

- as usual, with autoclaving of salt medium and adding sheep serum immediately before sowing *Leptospira*;
- with separate autoclaving of salt medium and 6-10% sheep serum, which were mixed in equal volumes before sowing;
- with autoclaving of ready 3-5% medium poured in vials, which were sowed by *Leptospira*;
- with separate autoclaving of salt medium and 6-10% sheep serum, followed by separate filtration, mixing in equal volumes, and repeated autoclaving.

Next we studied the possibility of obtaining the most active antigens in terms of their specificity and hemosensitive characteristics based on water-soluble fractions of *Leptospira*. Antigenic properties of fractions were evaluated with IHT by the degree of neutralization of homologous and heterologous sera and by sensitivity of erythrocyte diagnosticum in IHT. Hemosensitive properties were evaluated in terms of sensitivity in IHT and sensitin consumption per unit of ready product with a single method of sensitization of erythrocytes.

Method of extracting antigens	Yield of antigen, mg	Yield of diagnosticum, ml
Boivin	5-6	450
Westphal	7-8	320
Fuller	2-3	40
Ultrasound	8	160
Boiling	11	220

Table 1: Yield of antigen and erythrocyte diagnosticum from 70 mg of *Leptospiral* biomass

Serum		The geometric average titer of serum for methods				
		MRAL		IHT with ED (erythrocyte diagnosticum)		ELISA
Animal group	Number	Serogroup of *Leptospira*	Titer	Protein	Polysaccharide	With conjugate on the bases of K°—diagnostics and leptospirosis serum
1	2	3	4	5	6	7
Vaccinated horses	8	Pomona Tarassovi Grippotyph Icterohaem Canicola	230.0 ± 1.11 210.0 ± 1.11 1:200 1:400 1:400	910.0 ± 1.14	560.0 ± 1.12	8,600.0 ± 1.49
Sick horses	8 5 3	Canicola Pomona Hebdomad	1,120.0 ± 156 460.0 ± 1.21 101.0 ± 1.48	2,480.0 ± 1.69 1,840.0 ± 1.54 2,679.0 ± 1.68	2,120.0 ± 1.67 1,260.0 ± 1.47 1,707.0 ± 1.51	19,100.0 ± 1.86 10,760.0 ± 1.83 27,750.0 ± 1.96
Healthy horses, not vaccinated	12	Canicola Pomona	1:100 1:100	1:50	1:50	1:100
Vaccinated pigs	46	Hebdomad Pomona Tarassovi Grippotyph Icterohaem Canicola	1:100 1:100 1:100 1:100 1:100 1:100	140.0 ± 1.12	160.0 ± 1.12	3,720.0 ± 1.6
Sick pigs	24 15	Pomona Tarassovi	680.0 ± 1.284 460.0 ± 1.23	1,210.0 ± 1.46 1,600.0 ± 1.51	820.0 ± 1.16 760.0 ± 1.19	9,150 ± 1.0 14,160.0 ± 1.82
Healthy pigs, not vaccinated	16	Pomona Tarassovi Canicola	1:100 1:100 1:100	1:50	1:50	1:100

Table 2: Effectiveness of serological methods in the evaluation of patients and vaccinated horses and pigs

We studied the antigenic activity of water-soluble fractions obtained from a mixture of biomass from 11 serogroups of *Leptospira* processed by:

- Ultrasound;
- Heating in a boiling water bath for 30 min;
- Formamide, method of A.T. Fuller (1938);
- Phenol, method of Westphal *et al.* (1952);
- Trichloroacetic acid, method of A. Boivin *et al.* (1933).

In terms of IHT titer and hemosensitive activity, the most effective were diagnosticums derived from antigens Boivin and Westphal. The least active were diagnosticums based on antigens derived by heating *Leptospira* in a boiling water bath. These antigens also have the highest optimal dose of hemosensibilization (ODH) (0.5-1.0 mg/ml), which puts it among the least suitable antigens for the preparation of erythrocyte diagnosticum.

It should be noted that when agglutinating with *Leptospiral* sera, the IHT titer with diagnosticums based on Boivin antigen was two times higher than with diagnosticums based on Westphal antigen, except antisera for serogroups Canicola, Pyrogenes, Autumnalis, and Grippotyphosa. With these antisera, IHT titer was two to four times higher with diagnosticum based on antigen of Westphal [6,7].

Considering the ODH of antigens and yield of dry mass of the antigen from 100 ml of *Leptospiral* biomass, we calculated the yield of 0.5% *Leptospira* antigenic diagnosticum per unit of *Leptospiral* biomass.

Comparative evaluation of the sensitivity of diagnosticums in IHT, based on antigens of Westphal and Boivin, was carried out on the basis of research of 18 positive *Leptospiral* sera of horses and pigs at a titer of 1:800-1:1,000. The most active and almost equal ($p > 0.5$) were diagnosticums prepared using tannin and rivanol based on the antigen of Boivin. All preparations with the antigen of Westphal were ($p > 0.01$) less effective than preparations based on antigen of Boivin, except the method of sensibilization at with high temperature, where inactivation of Boivin antigen, apparently, was more significant [8,9].

So, we have developed an efficient method of preparation of *Leptospira* antigenic erythrocyte diagnosticum, which has signs of specific activity and is used to detect antibodies to *Leptospira* [10-13]

Research of 62 blood sera of workers from slaughterhouse and livestock complexes and farms (pig farm, hippodrome, *etc.*) revealed 19 to 32 positively reacting sera [14-17].

Using IHT and MRAL, we detected 19.3% sera containing antibodies to serogroups of leptospirosis, Pomona, Grippotyphosa, Canicola, and Tarassovi (91%), in a titer from 1:100 to 1:1,600.

Among the majority of pig-farm workers, we marked antibodies to serogroups of Pomona and Tarassovi (83%), among the racecourse workers—antibodies to serogroups of Grippotyphosa and Canicola (75%), among slaughterhouse workers—antibodies to Pomona, Grippotyphosa, Canicola, and Tarassovi (91.5%). Results of RMAL completely coincided with the results of IHT.

Further we made a survey of adult horses and pigs with a separate history depending on the immunization of clinical signs of leptospirosis and others. Examination was carried out in 30 days after the vaccination of horses and in 3 months after immunization of pigs.

In general we can confirm the recommendations of instructions about conditional diagnostic titer for these animals. For antibodies to serogroups of Pomona and Canicola, *Leptospiral* IHT titer with erythrocyte diagnosticum based on polysaccharide antigen approached and even exceeded the titer with a protein antigen. The titer determined by ELISA (enzyme-linked immunosorbent assay) is more than nine times higher than by IHT.

We also took into consideration the value of geometric mean titer of infected and vaccinated animals and individual values of IHT titer in vaccinated (horses—from 1:500 to 1:1,600 and pigs—from 1:100 to 1:200 with erythrocyte diagnosticum (ED) based on a protein antigen) and infected animals (horses—from 1:1,600 to 1:4,000 and pigs—from 1:800 to 1:3,200). According to these results, we can take an indicative conditional diagnostic titer of IHT for the production series in the study of adult horses' serum—1:400 for vaccinated and 1:100 for unvaccinated. We also need to consider epizootic situation and try to repeat researches 8-10 days later to monitor the dynamics of antibody activity [18,19].

We also conducted examination at some hog enterprises in the area of Almaty. Results of IHT were taken as positive if the value of determined titer exceeded shareware diagnostic titer considering vaccination.

In general, using the IHT and MRAL, we revealed 30.9% of animals reacting positively for leptospirosis. Using ELISA conjugate based on *Leptospiral* serum and catalase, we identified 42.8% of positively reacting animals. The range for MRAL is from 1:100 to 1:1,600, for IHT—from 1:1,000 to 1:6,400, and for ELISA—from 1:2,000 to 1:24,800.

In the serum with positive response, in 53.8% of cases, antibodies belonged to serogroups of Pomona (according to MRAL), 34.6%—Tarassovi, and 11.5%—Grippotyphosa.

Leptospira antigenic erythrocyte diagnosticum is used to detect the antibodies in the sera of vaccinated, infected, and recovered animals and humans.

Method of preparation, developed in our laboratory, and application of *leptospira* antigenic erythrocyte diagnosticum in practice can greatly simplify and make cheaper the process of preliminary selection of sera positive for leptospirosis, with following transcript by MRAL that is the basis for the introduction of new tactics in immunological studies on leptospirosis. Further, we have implemented a production release of erythrocyte antigen *Leptospira* diagnostics in order to use in veterinary practice.

The drug is manufactured according to FS 42, by the order of the Ministry of Health, and approved by the Committee of Veterinary of the Republic of Kazakhstan for the purpose of its application in veterinary practice.

In the preparation of antigens for the production of diagnosticum series, we used three *Leptospira* strains, which are a part of the standard diagnostic kit for IHT. Technological parameters of manufacturing are performed at the following biological scheme.

Biological Scheme

Sheep blood bacterial mass of *Leptospira* culture	
0.9% sodium chloride Centrifugation – Solution of 2,000 rev/min (10 ± 1) min (6 ± 2)°C	Acetone (−10 to 15)°C
Sheep erythrocytes 3% formaldehyde	Bacterial weight of *Leptospira* microbe, killed by acetone Phenol 70% (4 ± 2)°C
Formalinized erythrocytes	Polysaccharide antigen of *Leptospira* microbe
Tannin (37 ± 1)°C	
Erythrocytes with addition of tannin Liquid diagnosticum Lyophilization Dry diagnosticum	

For each series of formalinized erythrocytes, we matched the proper titer of optimal dose with a specific series of antigen. Then, the series of antigen dilutions was calculated at 40, 80, and 160 mg per 1 ml of 0.5% erythrocyte suspension. Preformalized and tannin-added 0.5% erythrocytes were poured into test tubes in a volume of 4 ml. After adding various concentrations of antigen in a volume of 4 ml, the mixture was placed in a refrigerator at (4 ± 3)°C for 18-20 h. After sensibilization, sensitin was fixed by 1% of formalin and incubated for 2 h at a temperature (4 ± 3)°C or 30 min at a temperature (45 ± 1)°C. After fixing, the erythrocytes were washed by centrifugation with a solution of Tween 80 four times in a dilution of 1:50,000 at 2,000 rev/min for 5 min.

The obtained erythrocytes, sensibilized with various concentrations of antigen, were checked in IHT with *Leptospiral* agglutinating serum. Cultivation of antigen, which identifies antibodies to *Leptospira* bacteria in a dilution of 1:2,000, was taken as an optimal dose.

The required amount of 10% formalinized erythrocytes was poured into a beaker, precipitated, and washed by formaldehyde in 0.85% sodium chloride solution, pH (6.8 ± 0.14) for 10 min at 2,000 rev/min. The washed formalinized erythrocytes were diluted to 5% suspension by 0.85% sodium chloride solution and heated up to (37 ± 1)°C.

To a 5% suspension of washed formalinized erythrocytes, we added an equal volume of tannin solution at a dilution of 1:20,000 and stirred constantly at (37 ± 1)°C for 15 min. Then we washed the suspension of erythrocytes three times in centrifuge with 0.85% sodium chloride solution, pH (6.8 ± 0.14) for 10 min at 2,000 rev/min. The sediment was diluted with the same sodium chloride solution until a 5% suspension of erythrocytes with tannin was obtained, and while stirring we added an equal volume of the antigen solution, then put it in the refrigerator for sensibilization at the temperature of (4 ± 3)°C for 17-18 h or for 1 h at (45 ± 1)°C in water bath. We also added 1% formalin to the mixture of formalinized erythrocytes with sensitin to fix antigen to erythrocytes receptor. During sensibilization at the temperature of (4 ± 2)°C, fixation took 2 h; at (45 ± 1)°C, it takes 30 min. To remove residues of sensitin, the erythrocytes were washed four times for 10 min at 2,000 rev/min by a solution of Tween 80 at a dilution of 1:50,000 for 25-fold volume of erythrocyte sediment. After washing, the sediment of sensibilized erythrocytes was diluted by 0.85% sodium chloride solution of pH (6.8 ± 0.1) to 2.5% concentration of erythrocytes [20].

At the end, we obtained the preparation, formalinized and tannin-added sheep erythrocytes sensibilized with the antigen of *Leptospira*. The diagnosticum was released in liquid and dry form in vials of 2.0 ml containing a 5% suspension of erythrocytes. The preparation was an amorphous mass in form of brown tablets, rehydrated in 19 ml of 0.85% sodium chloride solution for 1 min. The shelf life of dry diagnosticum is 2 years, and the diluted drug – If necessary, the diagnosticum control is provided appended serum.

As a result, multiple commission audits found that the tested leptospira diagnosticum is specific, highly sensitive, and cost-effective, and a convenient biologic preparation for IHT.

All experiments, needed to prepare antileptospirosis serum, were made in the M.Auezov South Kazakhstan State University. Experience has been enabled by five local breed horses (mare) at the age from 5 to 7 years. For industrial manufacture of the drug, hyperimmunization of horses was carried out by a suspension of *Leptospira* 5×10^8 m.k. in 1 ml, by a previously developed scheme.

Obtained by hyperimmunization of horses, biological products contain antibodies to *Leptospira* of *L. qrippotyphosa*, *L. icterohaemozzhaqiae*, *L. pomona*, *L. tarassovi*, and *L. sanicola* serogroups, which were used as immunogens for hyperimmunization of producer animals and influence on other serogroups of *Leptospira*. Rabbit agglutinating serum is the part of the set of *Leptospira* antigenic erythrocyte diagnosticum, and is used to control IHT.

Manufactured polyvalent therapeutic antileptospirosis serum is an immunospecific drug. Obtained from producer animals, multivalent *Leptospira* serum, after checking for the bacterial contamination and specificity, is packaged in vials of 2 ml, and then lyophilized. After lyophilization, the preparation looks like an amorphous mass in the form of brown tablets. Lyophilization was carried out at the Kazakh Scientific Center of Quarantine and Zoonotic Infections named after M. Aykimbaev.

The hyperimmune serum specificity was tested by antigenic erythrocyte diagnosticums: plague, pasteurellosis, pseudotuberculosis, *Leptospira*. Only *Leptospira* antigenic diagnosticum gave positive result at a titer of 1:2,560. It shows the high specificity of this preparation.

The activity of producer animal's serum was tested in the experiment on outbreeds of white mice. It was found that the serum has maximum activity in dilutions of 1:10 and 1:20 (100% activity), and in dilutions of 1:40 and 1:80, it has, respectively, 53.3 and 26.6% of activity. All infected animals died.

#	Producer	The way of injection	Injection dose of antigen (in ml)					
				In 4 days	In 11 days	In 18 days	In 27 days	In 35 days
			1-injection	2-injection	3-injection	4-injection	5-injection	6-injection
1	Mare 3 years	Intravenously	6	9	15	18	25	28
		Subcutaneously	3	–	–	–	–	–
2	Mare 6 years	Intravenously	7	10	16	19	26	29
		Subcutaneously	3	–	–	–	–	–
3	Mare 7 years	Intravenously	7	11	17	20	27	30
		Subcutaneously	3	–	–	–	–	–
4	Mare 5 years	Intravenously	7	12	18	21	28	31
		Subcutaneously	4	–	–	–	–	–
5	Mare 6 years	Intravenously	8	13	19	22	29	32
		Subcutaneously	4	–	–	–	–	–

Note: For hyperimmunization, local-breed horses aged of 5-7 years were used.

Table 3: Scheme of hyperimmunization of animals – producers for manufacturing industrial series of polyvalent serum antileptospirosis

After receiving a positive result in the experience of mice protection, hyperimmune polyvalent serum was tested in the treatment of dogs and cattle. As a result, 10 out of 10 dogs, treated by 3-5 ml of the preparation injected subcutaneously, recovered. The same amount of controlled animals, injected by saline, stayed with the pathological process. In experiments, carried out with cattle, antileptospirosis serum was injected at a dose of 20 ml subcutaneously in combination with symptomatic treatment. Final recovery from *Leptospira* infection was observed between the sixth and eleventh day. Similar frequentative experiments testing antileptospirosis serum (40 dogs and 12 head of cattle) also gave positive results.

Conclusions

1. For the first time in Kazakhstan, highly sensitive *Leptospira* antigenic erythrocyte diagnosticum was developed. After a successful test of biological product, its industrial production was started.
2. IHT with erythrocyte antigenic diagnosticum, developed by us, is sensitive and simple and gives results very fast with a minimum expenditure of money and labor, so it can be used for instant analysis of leptospirosis on humans and animals.
3. We developed a selective culture medium from rabbit and sheep serum and optimal modes of extraction of bacterial antigens for animals' hyperimmunization.
4. We developed the scheme of immunization of animal – producers, depending on the dose, the multiplicity, and the injection site and produced a multivalent antileptospirosis hyperimmune serum for immunotherapy of animals.

Explanation of veterinary terms

1. IHT—indirect hemagglutation test
2. RMAL—reaction of microagglutination lysis
3. SES—sanitary-epidemiological station
4. Immunotherapy—treatment of patients with the immune serum of animals
5. Immunoreagent—a diagnostic biological product
6. ED—erythrocyte diagnosticum
7. ELISA—enzyme-linked immunosorbent assay
8. ODH—optimal dose of hemosensibilization
9. Hyperimmunization—multiple management antigen for therapeutic serum

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