Latex Test System for Rapid Diagnosis of Leptospira Infection

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A diagnostic test system based on polymer latex carriers sensitized by IgG to *Canicola* and *Icterohaemorrhagiae* serogroup Leptospira was developed and tried.

Key Words: *leptospirosis; Leptospira; latex test system; test system*

Leptospirosis is a highly prevalent infection of wild, domestic, and agricultural mammalians infecting humans. It is detected in animals belonging to the *Canis* genus. The agents of canine leptospirosis are most often represented by Leptospira belonging to *Canicola* (86.5%) and *Icterohaemorrhagiae* (12.3%) serogroups [2,6].

Clinical polymorphism and specific features of biology and antigenic structure of Leptospira presented by more than 200 serological variants united into 25 serological groups create certain difficulties in the diagnosis of Leptospira infection. This disease has no pathognomonic clinical signs either in humans or animals and can be asymptomatic, which often leads to diagnostic errors.

Improvement of the existing and creation of new methods for the diagnosis of leptospirosis is a pressing problem. These methods should ensure rapid diagnosis of the disease, reliable detection of patients and carriers, and be available for wide practical use.

One of such methods is latex agglutination. Diagnostic preparations based on polymeric latex carriers (PLC) are widely used in the diagnosis of various diseases for the detection of antibodies or antigens in substrates. This study was aimed at the development of a diagnostic test system based on antibody latex diagnostic test systems (ALDTS) for rapid detection of Leptospira antigens and simultaneous identification of their serogroup.

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MATERIALS AND METHODS

The study was carried out on live Leptospira cultures, experimentally infected albino mice and dogs, and dogs with suspected spontaneous canine fever.

We used 19 Leptospira strains belonging to the following serological groups: *Australis*, *Autumnalis*, *Ballum*, *Bataviae*, *Canicola* (strains Kashirskii, Hond Utrecht IV), *Cynopteri*, *Grippotyphosa*, *Hebdomadis*, *Icterohaemorrhagiae* (strain M-20 serovar *copenhageni*, strain BGNKI-2 serovar *icterohaemorrhagiae*), *Javanica*, *Mini*, *Pomona*, *Pyrogenes*, *Sejroe*, *Tarassovi*, and *Semaranga*.

Leptospira were cultured in Fletcher's liquid medium [8]. The content of Leptospira per ml medium was estimated by the formula presented previously [1].

Antisera to Kashirskii strain (*Canicola* serogroup) and M-20 strain (*Icterohaemorrhagiae*) with the maximum IgG content were obtained by immunization of rabbits according to a previously described protocol [11].

Antiserum activity was controlled in the microagglutination test [8]. Specificity control was carried out by immunoelectrophoresis.

Three methods for Ig isolation and purification were used [7]: precipitation by adding 12% water-soluble polymer (polyethylene glycol (PEG; Sigma) of different molecular weight (PEG-4000 and PEG-6000) to hyperimmune sera; precipitation with 40% ammonium sulfate solution with subsequent purification of the resultant preparations and separation of Ig into isotypes by ion-exchange chromatography on a column with DEAE-Sepharose CL-6B anion exchanger (Pharmacia) with discontinuous NaCl gradient.

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Activity of the preparations obtained by precipitation and purification was evaluated by the microagglutination test; protein concentration was evaluated by the method of Lowry [9]. Ig preparations were identified by immunoelectrophoresis [7] and later used for PLC sensitization.

Activity of prepared ALDTS series was studied by the standard latex agglutination test on the glass with Leptospira cultures and specimens of biological material from infected animals. The test was carried out routinely [8].

Threshold sensitivity of the diagnostic test systems was determined in experiments with cultures of homologous microorganism serogroups at concentrations of 10^8-10^5 bacterial cells/ml.

Serogroup specificity was verified in the latex agglutination test with live Leptospira of 16 serological groups, ALDTS stability was evaluated by the same test after 1-, 6-, 12-, and 15 month storage at 4°C.

The potentialities of utilization of latex test system with technological purposes for quantitative analysis and identification of serogroup and antigens of Leptospira during vaccine making and for standardization of vaccine preparations, latex agglutination test was carried out with Dipentavak vaccine (Vetzverotsentr Firm) containing the Leptospira component.

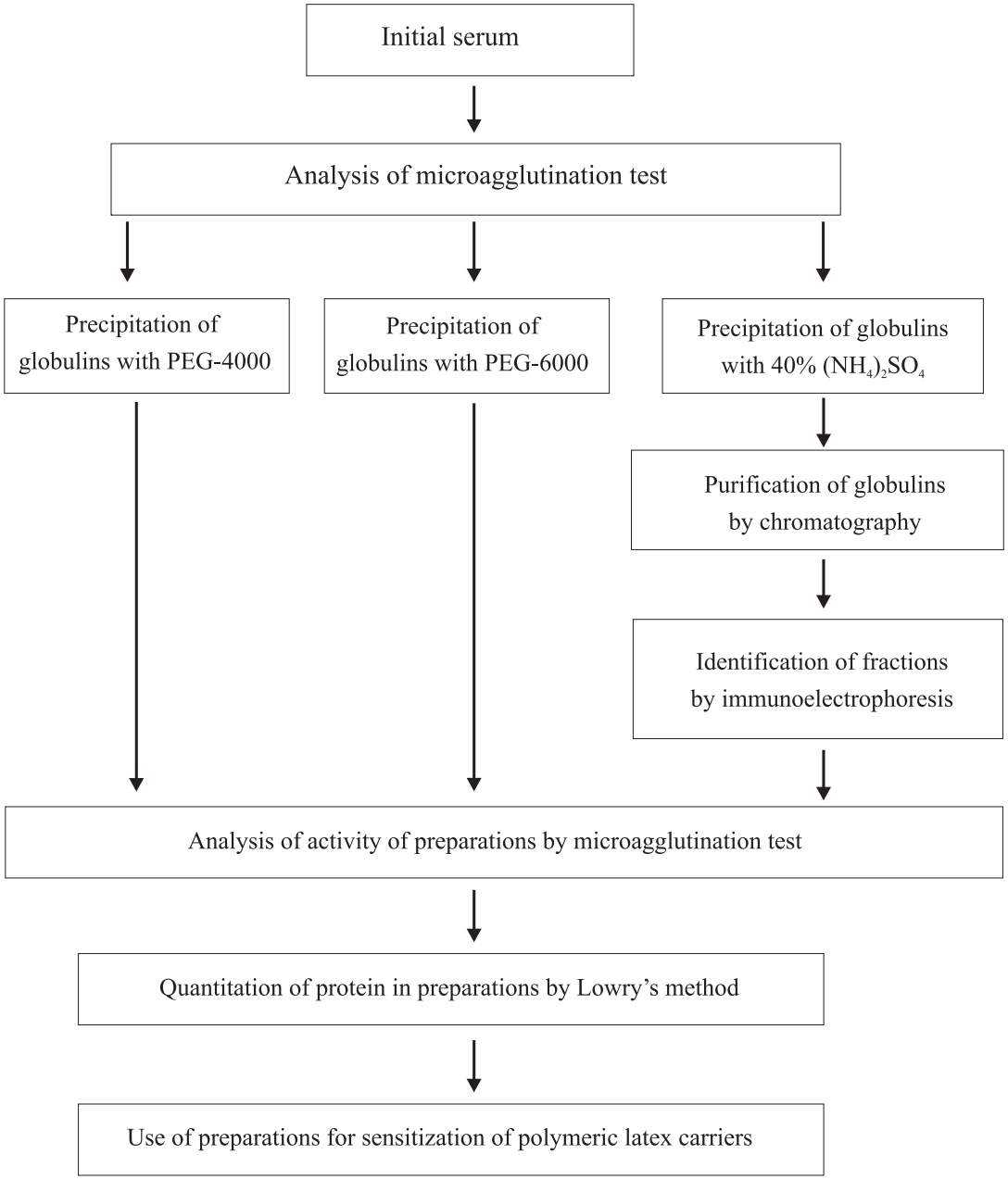

Fig. 1. Scheme of isolation and identification of anti-Leptospira Ig from rabbit immune serum.

TABLE 1. Results of Tests in Experimentally Infected Animals

Days after infection	No.	Latex agglutination test		Isolation of hemoculture	Antibody titer in microagglutination test	
		Icterohaemorrhagiae	*Canicola*		*Icterohaemorrhagiae*	*Canicola*
0	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
4	1	+	—	+	—	—
	2	—	+	+	—	—
	3	+	+	+	—	—
6	1	+	—	+	—	—
	2	—	+	+	—	—
	3	+	+	+	1:50	1:100
8	1	+	—	+	—	—
	2	—	+	+	—	1:200
	3	—	—	—	1:1 600	1:6 400
11	2	—	—	—	—	1:1 600
	3	—	—	—	1:1 600	1:6 400
14	2	—	—	—	—	1:1 600
	3	—	—	—	1:1 600	1:6 400
21	2	—	—	—	—	1:6 400
	3	—	—	—	1:800	1:6 400
28	2	—	—	—	—	1:3 200
	3	—	—	—	1:800	1:6 400
35	2	—	—	—	—	1:800
	3	—	—	—	1:50	1:800

Note. + and – mean the presence and absence of Leptospira

The possibility of using ALDTS for the detection of Leptospira in biological substrates, latex agglutination test was carried out with blood and urine of dogs experimentally infected with live Leptospira cultures.

The diagnostic test system was tested with specimens from animals (25 mice and 3 dogs) experimentally infected with Kashirskii and M-20 strains. For infection of dogs the Leptospira cultures were passaged in golden hamsters as described previously [5]. BALB/c mice (150-200 g) were intraperitoneally injected with cultures (2-5 ml) containing 1.2×10^7 Leptospira cells/ml.

TABLE 2. Clinical Testing of ALDTS

Parameter	Presence of Leptospira (reference method)	
	present (infected, *n*=35)	absent (healthy, *n*=52)
Latex agglutination test		
positive	33	3
negative	2	49

After infection blood was repeatedly tested for Leptospira by the cultural method and in the latex agglutination test over the course of infectious process. Changes in hematological parameters and concentrations of Ig of different isotypes in dog blood were evaluated by immunodiffusion test as described previously [7].

The final clinical trials of the test system were carried out in veterinarian stations and private veterinarian clinics of Moscow and Podolsk district of the Moscow region. A total of 87 dogs with suspected leptospirosis and clinically healthy animals (brought for vaccination) were examined in 2002-2003. The incidence of Leptospira infection at the territory of the Russian Federation reaches 27.9% [2], which is fraught with infection of humans.

The test system includes: anti-*Canicola* and anti-*Icterohaemorrhagiae* ALDTS, inactivated Leptospira culture (Kashirskii strain) as the positive control for *Canicola* serogroup Leptospira, inactivated Leptospira culture (M-20 strain) as the positive control for *Icterohaemorrhagiae* serogroup Leptospira, and water-serum medium for Leptospira culturing as the negative control.

RESULTS

Proteins of the rabbit hyperimmune sera (Fig. 1) were separated by ion-exchange chromatography into fractions, which were analyzed by immunoelectrophoresis. The results of immunoelectrophoresis were evaluated by the presence and localization of precipitation lines. Fractions giving precipitation lines with antisera to rabbit serum proteins in the migration area characteristic of IgG and IgM were selected (Fig. 2).

Immunochemically pure preparations of anti-Leptospira IgG and IgM and preparations containing a mixture of these Ig isotypes precipitated using PEG were obtained. All preparations were specific to homologous Leptospira in the standard microagglutination test [4]. Ig preparations were stored at -20°C before use.

The aggregation stability of PLC in electrolyte salines was evaluated in five buffer systems. The maximum PLC stability was observed in normal saline (pH 6.0), and hence, PLC suspensions in this saline were used in subsequent experiments.

PLC sensitization with Ig was carried out by covalent binding of Ig amino groups to carboxyl functional groups on the surface of PLC particles activated using water-soluble carbodiimides.

Optimal concentration of water-soluble carbodiimides was determined: 0.1-0.4% of PLC volume; maximum precipitation of Ig on PLC surface was attained at this concentration.

At the next stage the isotype and optimal sensitizing dose of Ig ensuring the maximum sensitivity of diagnostic test systems were determined. A total of 40 series of latex diagnostic test systems differing by the concentration of sensitizing protein were prepared.

Testing of the resultant ALDTS series with live Leptospira cultures (10^8-10^5 bacterial cell/ml) showed maximum activity and sensitivity of PLC sensitized by IgG in a concentration of 1.4 mg/ml 4% latex suspension. They reacted in the latex agglutination test with homologous cultures containing at least 10^6 Leptospira/ml within 1-3 min.

The serogroup specificity of ALDTS was tested in the latex agglutination test with Leptospira of 16 serogroups. The developed ALDTS reacted only with the strains of homologous serogroups (*Canicola* and *Icterohaemorrhagiae*, respectively), but not with Leptospira of other serological groups, which attests to high specificity of the preposed test system. ALDTS activity was retained at the initial level during 12-15 months and only later decreased.

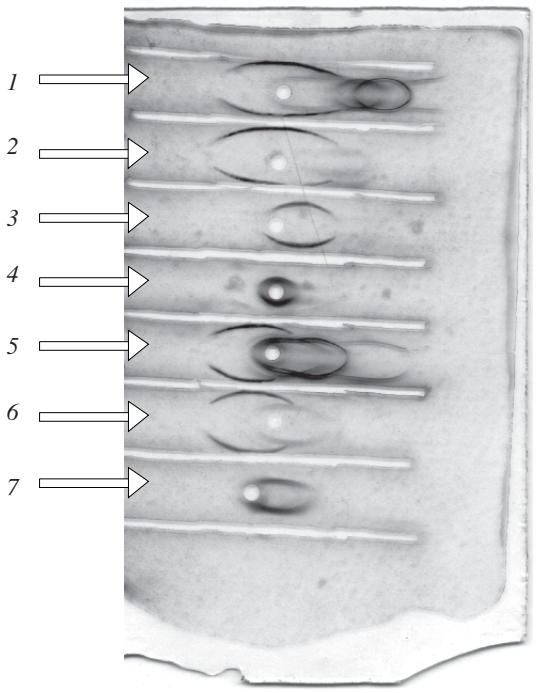

Fig. 2. Comparative immunoelectrophoretic analysis of IgM and IgG fractions obtained by chromatographic purification of anti-Leptospira Ig from the rabbit hyperimmune serum. *1*) anti-*Canicola* hyperimmune serum; *2*) "0" fraction (purified IgG); *3*) fraction "2" (purified IgM); *4*) control; *5*) anti-*Icterohaemorrhagiae* hyperimmune serum; *6*) fraction "0" (purified IgG); *7*) fraction "2" (purified IgM).

Trials of ALDTS with Dipentavak vaccine containing Leptospira of *Canicola* and *Icterohaemorrhagiae* serogroups showed that the proposed diagnostic test system can be used with technological purposes for quantitative evaluation of the antigen and standardization of vaccines containing the Leptospira component.

Experiments with the blood and urine of dogs experimentally infected with live Leptospira showed that canine blood components had no negative effect on the course of latex agglutination test. However, canine urine often gives an acid reaction (pH 5.0-6.5), therefore spontaneous agglutination of ALDTS is possible. Therefore infected urine (5-10 ml) was centrifuged (1500 rpm, 30 min), supernatant was discarded, and 1 ml Fletcher's liquid medium was added to the precipitate for Leptospira culturing (pH 7.2-7.4), after which the sample was tested in the latex agglutination test.

Infected mice were narcotized 30, 90, 120, and 150 min after infection. The blood was collected from the heart by direct puncture with a Pasteur pipette and aseptically inoculated into Fletcher's liquid medium

for Leptospira culturing. A portion of blood was treated with heparin and studied in the latex agglutination test; the results showed the presence of Leptospira in the blood of experimentally infected mice and allowed to determine the serogroup of Leptospira 90, 120, and 150 min after infection. These results were confirmed by isolation of the hemoculture [10].

In order to evaluate the efficiency of ALDTS in the diagnosis of leptospirosis in dogs, 3 animals were experimentally infected with virulent Leptospira strains (Table 1). Acute leptospirosis with typical clinical manifestations developed in 2 of 3 animals. The blood of experimentally infected dogs was repeatedly analyzed over the course of infectious process.

Neutrophilic leukocytosis with a shift of the leukocytic formula to the left and characteristic changes in the quantitative levels of IgM and IgG in immunodiffusion test were observed starting from the first days after infection in all animals. The maximum IgM concentrations were observed on days 6-11 postinoculation, after which they decreased. The maximum accumulation of IgG was observed on days 11-21 after infection.

Typical primary immune response to challenge with live virulent Leptospira was simulated under experimental conditions.

Antigens of Leptospira of homologous serogroups were detected in the latex agglutination test on day 4 postinfection (Table 1). The duration of antigenemia was 4 days. Serogroup of Leptospira was determined simultaneously with the detection of their antigens in the latex agglutination test.

The peak of antigenemia in the blood of dogs was observed on days 4-8 postinfection, which corresponded to the results of hematological studies. The presence of Leptospira in the blood of animals was also confirmed by isolation of hemoculture. Starting from day 6 we observed the appearance of anti-Leptospira antibodies and increase in their titers in the microagglutination test.

Clinical trials of the test system at consultations in veterinarian clinics were carried out in 87 dogs (Table 2).

Of 35 animal blood samples tested in the latex agglutination test with the new test system, 33 were positive (truly positive) and 2 negative (false-negative).

Of 52 blood samples from animals without Leptospira, 49 were negative in the latex agglutination test with the new test system (truly negative) and 3 were positive (false-positive).

Hence, the diagnostic sensitivity of the developed test was 94.28% and diagnostic specificity 94.23%.

Our results confirmed high efficiency of the ALDTS-based diagnostic test system intended for detection of Leptospira of *Canicola* and *Icterohaemorrhagiae* serogroups in the latex agglutination test, this recommending it as a rapid method for detection and identification of Leptospira of these groups in cultures of microorganisms and biological fluids of warm-blooded animals. Presumably, this diagnostic test system will be effective in detection of antigenemia in humans with Leptospira infection.

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