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COMPARATIVE INVESTIGATION OF DIFFERENT METHODS FOR THE DETECTION OF INFECTION IN RABBITS CHALLENGED WITH *L. interrogans* SEROTYPE *hardjo*

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Abstract

Keeping up-to-date with modern diagnostic techniques for leptospirosis as well as continuous improvement of laboratory diagnostic methods resulted in abundant knowledge on the nature and consequences of this infection and its importance in both human and veterinary medicine. In that respect, development and introduction of novel diagnostic tests and procedures have become the paramount issue in the diagnostics of leptospirosis and related infectious diseases. Thus, the goal of this research was to investigate the application of diverse laboratory methods and to evaluate their validity in the diagnostics of leptospirosis. Eleven rabbits were artificially infected with live cultures of *L. interrogans* serovar *hardjo* by the method of skin scarification. Blood and blood serum samples of challenged animals were collected every other day throughout the 3-week period (i.e. until day 21), and then once weekly during following five weeks. Blood sera were tested for the presence of *L. interrogans* serovar *hardjo* specific antibodies applying the methods of microscopic agglutination (MA) test and ELISA. Blood samples were examined using the method of cultivation in liquid medium by *Johnson* supplemented with 200*μg*/1*ml* 5- fluorouracil (5-*FU*). Presence/absence of *L.interrogans* serovar *hardjo* was confirmed by polymerase chain reaction (*PCR*) method. In this reaction, a pair of primers separated from the basic structure of the *Leptospira interrogans rrs* (16S) gene. In MA test, the presence of specific antibodies against *L. hardjo* in rabbits was confirmed in 67 (36.61%) of 183 investigated sera. Initial positive specific antibody finding was recorded on day 9 post challenge, and it persisted until day 17. In ELISA test, positive and suspect findings

were confirmed in 67 and 18 samples, respectively. Initial ELISA-positive finding was observed on day 15, showing increasing tendency throughout the monitoring period and reaching its maximum value on day 42. Method of blood sample cultivation resulted in isolation of *L. interrogans* serovar *hardjo* in 33 (18.03%) on day 3 at the earliest, whilst highest isolation rate was observed on day 17 post challenge. Applying polymerase chain reaction (PCR) method, genome or genome sequences of *L. interrogans* serovar *hardjo* were detected in 67 (56.30%) out of 119 blood serum samples. PCR method revealed positive finding as early as on day 1 post challenge, whereas the highest rate of positive findings was recorded on day 19. Comparison of the results obtained by methods of cultivation and PCR during the period from experimental day 1 to 21, i.e. period prior to administration of chemotherapeutic agents, demonstrated high level of linear correlation of $r = 0.8105$ at the 0.01 significance level. After dihydrostreptomycin therapy administered from day 21 post infection, *L. interrogans* serovar *hardjo* could not be isolated using the method of blood sample cultivation. Contrary to that, PCR method revealed the presence of *L. interrogans* serovar *hardjo* genome in 23 samples.

Key words: Leptospirosis, diagnostic methods, *L. interrogans* serovar *hardjo*

UPOREDNO ISPITIVANJE RAZLIČITIH METODA ZA DOKAZIVANJE INFEKCIJE VEŠTAČKI INFICIRANIH KUNIĆA SA *L. interrogans* SEROTIP *hardjo*

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Kratak sadržaj

Praćenjem savremenih metoda dijagnostike leptospiroze, a posebno usavršavanjem laboratorijskih dijagnostičkih metoda, došlo se do brojnih saznanja o prirodi i posledicama ove infekcije, o njenom zdravstvenom značaju kako u veterinarskoj tako i humanoj medicini. Sve ovo ima za posledicu nastojanje da se u dijagnostici zaraznih bolesti kakva je i leptospiroza, razvijaju i uvedu novi dijagnostički testovi i procedure. Imajući u vidu sve navedeno, kao cilj ispitivanja postavljena je potreba da se ispita primena ra-

zličitih laboratorijskih metoda i oceni njihova valjanosti u dijagnostikovanju leptospiroza. U ogledu je veštački inficirano postupkom skarifikacijom kože, 11 kunića živom kulturom *L. interrogans* serovar *hardjo*. Kunićima su uzeti uzorci krvi i krvnog seruma, svakog drugog dana do 21. dan, a zatim 1 nedeljno narednih 5 nedelja. Krvni serumi su ispitani na prisustvo specifičnih antitela protiv *L. interrogans* serovar *hardjo* metodima mikroskopske aglutinacije (MA) i *ELISA*. Uzorci krvi su ispitani metodom kultivacije na tečnoj hranljivoj podlozi po *Johnson*-u sa dodatkom 200*μg*/1*ml* 5- fluorouracila (5-*FU*), a prisustvo/odsustvo genoma *L.interrogans* serovar *hardjo* metodom lančane rakcije polimeraze (*PCR*). U reakciji je korišćen par prajmera izdvojen iz osnovne strukture *Leptospira interrogans rrs* (16S) gena. Specifičnih antitela protiv *L. hardjo* kod kunića primenom MA utvrđena su kod 67 uzoraka seruma ili 36,61% od ukupno ispitanih183, pozitivan nalaz specifičnih antitela najranije je zabeležen 9. dana od inficiranja i održavao se do 17. dana. Primenom *ELISA* testa pozitivni nalazi utvrđeni su kod 67, a sumnjivi kod 18 uzoraka. Najraniji pozitivan nalaz *ELISA* testom zabeležen je petnaestog dana, a zatim broj pozitivnih nalaza raste, dostiže maksimum 42. dana. Metodom kultivacije uzoraka krvi, *L. interrogans* serovar *hardjo* je izolovana kod 33 (18,03%) uzorka, najranije trećeg dana, a najveći procenat izolata zabeležen je 17. dana od inficiranja. Primenom metoda lančane reakcije polimeraze (*PCR*) genom ili delovi genoma *L. interrogans* serovar *hardjo* dokazan je u krvnom serumu kod 67 (56,30%) od 119 uzoraka. Primenom metoda lančane reakcije polimeraze (*PCR*) pozitivan nalaz zabeležen je već prvog dana, a najveći procenat pozitivnih nalaza zabeležen 19. dana od inficiranja. Poredeći rezultate dobijene metodom kultivacije i *PCR* počev od 1. do 21. dana eksperimenta odnosno za period pre prmene hemioterapeutika, ustanovljen je visok nivo linearne korelacije od $r = 0,8105$ na nivou značajnosti od 0,01. Nakon primene dihidrostreptomycine od 21. dana nakon inficiranja, u uzorcima krvi kunića metodom kultivacije nije izolovana *L. interrogans* serovar *hardjo*. Za razliku od metoda kultivacije, primenom *PCR* metoda, dokazana je prisustvo genoma *L. interrogans* serovar *hardjo* kod 23 uzorka.

Ključne reči: Leptospiroza, metodi dijagnostike, *L. interrogans* serovar *hardjo*

INTRODUCTION

Most cases of *L. interrogans* serotype *hardjo* infection in animals are asymptomatic, yet associated with shedding of leptospires in the environment via the urine. Identification of infected animals, particularly carriers, is a multifaceted problem in the view of validity of applied serological tests as well as highly complex and time-consuming isolation procedure. Keeping up-to-date with modern diagnostic techniques for leptospirosis as well as continuous improvement of laboratory diagnostic methods resulted in abundant knowledge on the nature and consequences of this infection and its importance in both human and veterinary medicine. In that respect, development and introduction of novel diagnostic tests and procedures have become the paramount issue in the diagnostics of leptospirosis and related infectious diseases. Thus, the goal of this research was to investigate the application of diverse laboratory methods and to evaluate their validity in the diagnostics of leptospirosis. Immunological assays are highly applicable in the diagnostics of leptospirosis and thus increasingly used in laboratory practice. Reaction of microscopic agglutination (MA), being an eligible diagnostic method, has been the most widely used serological test so far. In the recent years, ELISA has become widely used. However, all the aforementioned tests demonstrated some limitations and drawbacks regarding sensitivity and specificity in the detection of subclinical infections in animals. While the method of isolation is the most accurate and reliable diagnostic tool yet highly demanding, time-consuming and expensive, the polymerase chain reaction (PCR) is sufficiently sensitive, specific and rapid method enabling detection of even small number of leptospires. Capacity of polymerase chain reaction (PCR) to detect presence of 5-10 bacteria per *ml* of the sample indicates its high sensitivity, which makes it more appropriate and applicable as compared to the method of cultivation *Smith et al.* (1994).

MATERIAL AND METHODS

Population of 11 rabbits were challenged with live culture of *L. interrogans* serovar *hardjo* by skin scarification procedure. Blood and blood serum samples of challenged animals were collected every other day throughout the 3-week period (i.e. until day 21), and then once weekly during following five weeks. Blood sera were tested for the presence of *L. interrogans* serovar *hardjo* specific antibodies applying the method of microscopic agglutination (MA) test with live seven-day-old cultures of reference leptospiral serotypes (*Royal Tropical Institute Amsterdam, The Nederland*) as well as modified *ELISA* for

detection of *L. hardjo*-specific antibodies in blood serum samples of infected rabbits. In this assay, basic components of the commercial bovine kit were used (*IgG* and *IgM*, producer: *Central Diagnostic, Lelystad, the Netherlands A*). The modification has implicated replacement of the conjugate with anti-rabbit immunoglobulins (*IgG, IgA, IgM*) produced in pig and conjugated with peroxidase (*MP Biomedicals, Inc; Aurora, Ohio* SAD, *cat. No 1135)*. The titre of used immunoglobulins was determined by chessboard titration. The working titre of conjugated immunoglobulins was 1:200. Detection of *L. hardjo* in blood serum was performed by the method of cultivation in liquid medium (by *Johnson*) supplemented with bovine albumin and 200 *μg/ml* 5 fluorouracil (*5FU*) and by polymerase chain reaction (*PCR*). The reaction was done according to adapted protocol for isolation and diagnostics of leptospiral DNA described by *Merien et al.* (1992). Isolation of DNA from urine samples was performed according to *QIamp® DNA Mini and Blood Mini Kit* (*Quiagen®*, Germany) protocol for DNA purification from blood and body fluids (spin-protocol). PCR reaction was accomplished using two oligonucleotide primers described by *Mrerien et al.* (1992), which correspond to nucleotides 38-57 *A Lepto (5'-GGC GGC GCG TCT TAA ACA TG-3')* and *B Lepto (5'-TTC CCC CCA TTG AGC AAG ATT-3')* from the primary structure of *L. interrogans* serotype *canicola* strain *Moulton* 16S *rRNA* gene. The resulting specific product was 331 *bp* in size. DNA amplification was performed in a 25 μl reaction, using a thermal cycler "*MultiGene Thermal Cycler, TC9600-G*" manufactured by *LabnetInternational, Inc (NY., USA*). Temperature regimen encompassed 15-minute *Taq* polymerase activation period at 95 ^{0}C with simultaneous phase of initial denaturation of DNA template from the sample, followed by 35 cycles encompassing 1.5-minute denaturation at 94 °C, 1-minute hybridization (*annealing*) at 63 °C, 2-minute elongation at 72 °C and final elongation (extension) at 72 °C during 10 minutes. The resulting amplification product was applied onto the 1.5% gel supplemented with *ethidiumbromid* and subjected to electrophoresis. The results were captured and interpreted using the illuminator.

RESULTS AND DISCUSSION

The results of the detection of specific antibodies against *L. hardjo* in rabbits using dark field microscopic agglutination (MA) are displayed in Table 1 and Chart 1. The results pertain to the period from the start of the experiment until day 56, i.e. the experimental period is divided into two phases. The first phase encompassed sampling from day 1 to day 21, whereas second phase encompasses weakly sampling throughout 5-week period after administration of chemotherapeutic agents.

Table 1. Finding of antibodies against *L. hardjo* in blood serum of rabbits challenged by skin scarification (microscopic agglutination method, MA)

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	-	-	-	-	1:100	1:100	1:100	1:100	1:250	1:100	1:50	1:25	-	-	-	-	-
2	-	-	-	1:25	1:50	1:100	1:100	1:100	1:.250	1:100	1:25	1:25	1:25	-	-	-	-
3	-	-	-	-	1:25	1:50	1:50	1:100	1:100	1:100	1:25	1:25	-	-	1:50	1:25	1:25
4	-	-	1:50	1:100	1:250	1:500	1:250	1:500	1:500	1:100	1:100	1:100	1:100	1:75	1:75	1:50	1:25
5	-	-	-	1:100	1:500	1:100	1:250	1:100	1:250	1:100	1:50	1:50	1:50	1:25	1:25	1:25	1:25
21	-	-	-	-	1:50	1:100	1:250	1:250	1:250	1:250	1:100	1:100	1:100	1:250	1:250	1:100	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø
23	-	-	-	-	1:50	1:100	1:100	1:100	1:50	-	-	-	-	-	-	-	-
24	-	-	-	1:100	1:100	1:100	1:100	1:100	1:50	-	1:25	1:25	1:25	1:50	1:100	-	-
25	-	-	-	1:50	1:100	1:250	1:250	1:250	1:100	1:100	1:50	1:25	1:25	1:100	1:25	-	-
26	-	-	-	1:100	1:250	1:500	1:250	1:250	1:100	1:100	1:100	1:100	1:50	1:50	1:25	1:25	-

Legend: (-) negative (+) positive Ø – died rabbits

The results of examination of 183 blood sera of rabbits infected via skin scarification and rubbing of live cultures of *L. hardjo* (Table 1) revealed seropositive finding in 67 (36.61%) samples. Monitoring of the increase of geometric mean titre (GMT) of *L. hardjo* specific antibodies, positive titre result was diagnosed on day 9 post infection (Chart 1). Monitoring of antibody titre values of rabbits during the further course of infection revealed that they persisted at the weakly-positive level (1:100) until day 17 post challenge, when the titre values dropped below the lower limits of positivity of MA method (titre <1:100).

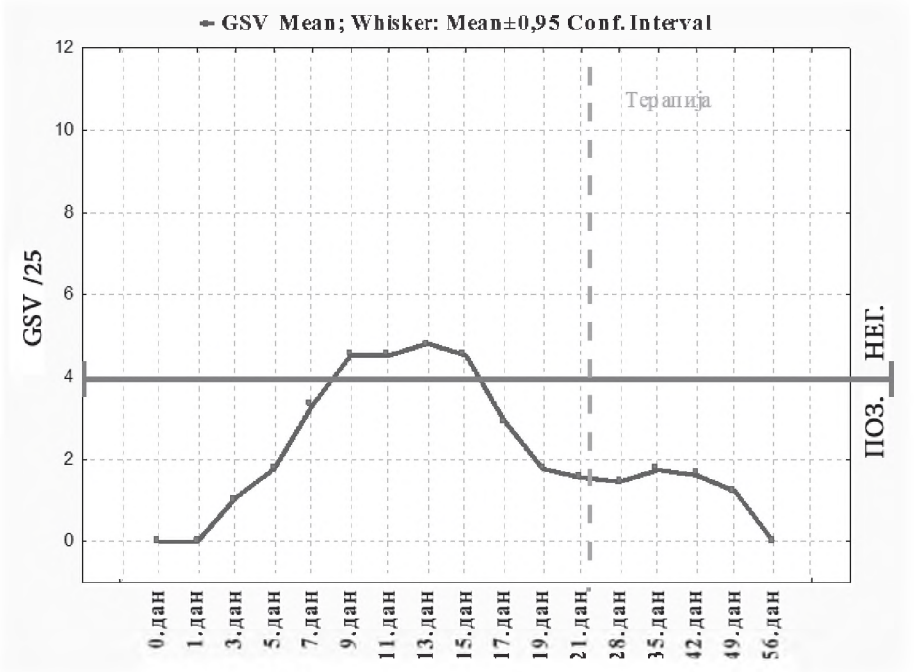


Chart 1. Distribution of *GMT* antibodies against *L. hardjo* in blood sera of rabbits challenged by skin scarification (microscopic agglutination method, MA).

Collected rabbit serum samples were also examined in an immunoenzyme assay, *ELISA*. *L. hardjo* antibody finding obtained by ELISA revealed positive result in 67 (36.61%) samples (rate > 45%), and suspect result (rate 20-45%) in 18 (9.84%) samples. In this test, the earliest diagnosis of seropositive result was accomplished on day 7 (Table 2). Monitoring of distribution of geometric mean titre values revealed that titre of L. *hardjo* specific antibodies exceeds the boundary positivity level on day 15 post challenge. The highest GMT value was recorded on 42nd day of investigation period, showing decreasing tendency in the following days (Chart 2).

Table 2. Finding of antibodies against *L. hardjo* in blood serum of rabbits challenged by skin scarification – ELISA (expressed in %)

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	13%	5%	10%	5%	16%	13%	24%	16%	21%	2%	13%	19%	25%	63%	124%	40%	129%
2	2%	4%	6%	7%	4%	2%	2%	3%	7%	0%	13%	4%	22%	47%	94%	77%	33%
3	12%	3%	13%	4%	4%	-2%	1%	0%	5%	0%	6%	3%	29%	66%	128%	72%	98%
4	4%	6%	4%	13%	19%	8%	8%	9%	13%	13%	25%	28%	31%	57%	145%	124%	129%
5	1%	4%	1%	13%	3%	9%	9%	15%	17%	-3%	36%	39%	44%	69%	103%	90%	89%
21	1%	-6%	-5%	3%	5%	6%	8%	18%	94%	116%	114%	127%	110%	93%	112%	91%	85%
22	-3%	-2%	-7%	25%	4%	6%	3%	1%	48%	19%	39%	27%	31%	W	W	W	W
23	0%	-1%	-1%	-1%	3%	2%	1%	3%	92%	65%	81%	90%	106%	130%	118%	123%	91%
24	-4%	7%	5%	3%	5%	21%	29%	32%	72%	46%	127%	107%	115%	124%	149%	125%	135%
25	14%	-2%	13%	16%	76%	52%	43%	50%	124%	101%	109%	77%	99%	114%	108%	102%	97%
26	1%	-3%	-5%	3%	0%	5%	4%	11%	54%	88%	109%	98%	82%	113%	174%	120%	109%

Legend: (-) negative (+) positive Ø – died rabbits

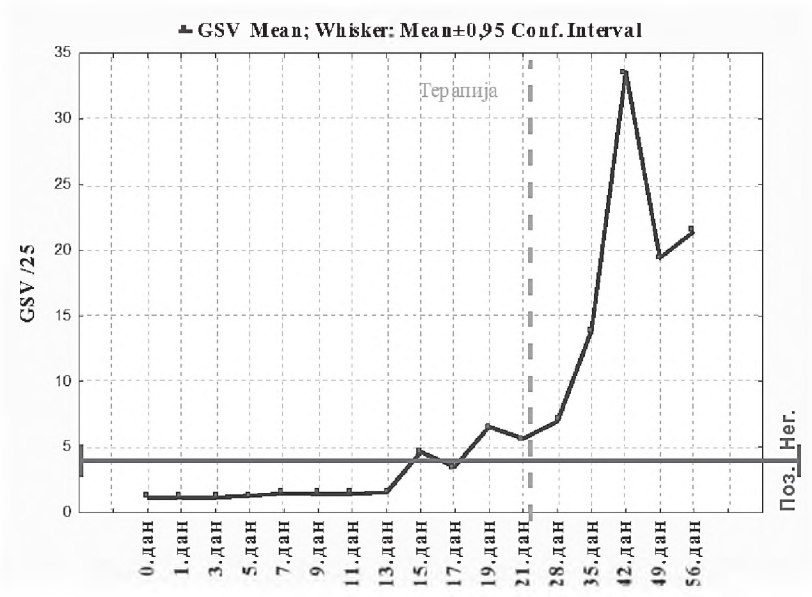
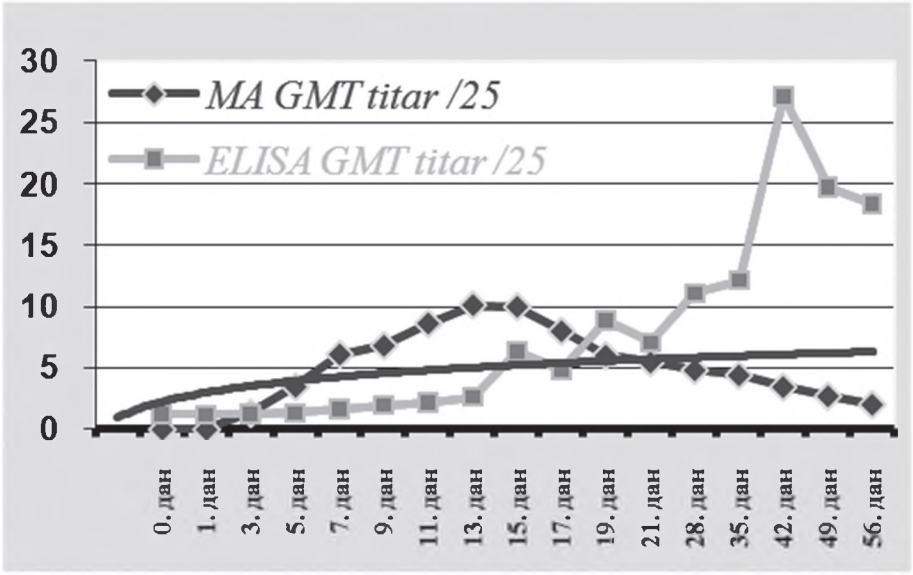


Chart 2. Distribution of *GMT* antibodies against *L. hardjo* in blood sera of rabbits challenged by skin scarification – diagnosed by ELISA

Comparison of GMT values obtained by MT and ELISA tests revealed the following: samples examined by MA test manifested increased levels of specific antibodies, which exceeded the boundary level of 1:100 (*GMT* titre 4 $log2/_{25}$) as early as on day 7. The level of *L. hardjo* specific antibodies diagnosed by MA method increases until reaching the maximum level on days 13 and 15, and then shows decreasing tendency and drops below the boundary positivity level on day 42 (*Cole J.R.Jr,et al.1973*). Contrary to MA method, *ELISA* demonstrated considerably longer period (15 days) between the infection and exceeding the boundary positivity levels being 45% (*PP*) or (*GMT* titre 4 $log2/_{25}$). During the further course of the experiment, GMT values manifested permanent increasing tendency until day 42, when they reached the maximum levels, and then started to drop down gradually. It is to be emphasized that MA method enables seropositivity diagnosis from day 7 to day 42 post-infection. In ELISA, the time span of diagnosis extends from day 15 to day 56, i.e. to the end of the investigation period (Chart 3).

Chart 3. Distribution of *GMT* values for antibodies against *L. hardjo* in blood sera of rabbits challenged by skin scarification – diagnosed by MA test and ELISA



Testing of correlation levels of GMT values for the applied methods (i.e. MA and ELISA) in the population of rabbits challenged by skin scarification revealed weak inverse linear correlation of r = -0.3277 with 0.05 level of

significance. Furthermore, the parity of arithmetic means of GMT values for MA test and ELISA was tested and *t*-value (t = -2.288) and *P* value 0.029 (P <0.05) were calculated, indicating mutual difference between the arithmetic means of tested sets with a risk of 0.05 (*Merien F.et al.1995;Márcia Costa Ooteman et al.2006;* Grgić Ž *et al.* 2007).

Analysis of the results obtained by the method of isolation on culture media, *L. hardjo* was isolated from 33 (18.03%) out of 183 examined blood sera of rabbits challenged via skin scarification. Using the isolation method, *L. hardjo* was detected as early as the day 3 post-challenge, and the highest rate of positive findings was recorded on day 17 post-infection. The results are presented in Table 3 (*Schonberg A.,et al. 199.;Johnson R. C. Et al.1973*).

Table 3. Results of reisolation of *L. hardjo* from blood of rabbits challenged via skin scarification using cultivation in liquid and semisolid medium acc. to *Johnson* supplemented with 200*μg*/1*ml* 5 *FU.*

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
4	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
5	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
21	-	-	+	-	-	-	+	+	-	+	-	+	-	-	-	-	-
22	-	-	-	-	-	-	-	+	+	+	-	-	-	W	W	W	W
23	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
24	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
Σ	0	0	1 9,09%	0	0	0	2 18,18%	6 54,54%	8 72,73%	9 81,82%	4 36,36%	3 27,27%	0	0	0	0	0

Legend: liquid medium o Semisolid o, liquid + semisolid o dead rabbit W positive (+) negative (-)

The presence of *L. hardjo* in blood of infected rabbits was also examined using the method of polymerase chain reaction (PCR). *L. hardjo* was detected in 67 (56.30%) out of 119 examined samples. By the use of PCR, *L. hardjo* was detected as early as on day 1, and the highest rate of positive findings was observed on day 19 post-challenge (Table 4). The method of polymerase chain reaction enabled detection of *L. hardjo* also in the second stage of the experiment, i.e. after administration of antimicrobial therapy, which is an important point for the interpretation of the results obtained in this assay. Polymerase chain reaction method demonstrated high sensitivity, that is, ability of detecting 5-10 bacteria per 1ml of the sample as well as short time of examination, which makes it more appropriate than the method of cultivation (*Gerritsen M. J. et al.1991; Grgić Ž. et al. 2012*).

Table 4. Finding of *L. hardjo* in the sera of rabbits infected via skin scarification using the *PCR* method

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+
2	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+
3	-	-	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+
4	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	-	-
5	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-
21	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
24	-	-	-	-	-	+	-	+	-	+	+	+	-	+	+	-	-
Σ	0	2 28,57%	1 14,29%	2 28,57%	3 42,85%	3 42,85%	3 42,85%	6 85,71%	5 71,43%	6 85,71%	7 100%	6 85,71%	4 57,14%	6 85,71%	5 71,43%	4 57,14%	4 57,14%

Legend: *PCR* positive (+) *PCR* negative (-).

Numerous authors (*Maria Rosa et al. 2005; Cousins D.V., 1991*) reported on the capacities of ELISA and its advantages over the MA method (*Brown P.D, 1995* and *Cousins D.V., et al., 1991*). In that respect, our results should contribute to better understanding of this diseases as well as evaluation of the validity of the applied diagnostic methods. In our research, microscopic agglutination method (*agglutination-lysis test*) was the basic assay, which in recent

period underwent certain modifications aimed at improving the reliability of ELISA method (*Cole et al., 197;, Mårcia Costa Ooteman et al.,2006*). The articles of *Bercovich Z. et al.* (1990) and *Levett P.N.* (2002) confirmed the ability of this method to detect specific antibodies in the serum of infected animals at the earliest between the day 5 and day 10 post-challenge, which corresponds with our results. The method of cultivation of leptospires is highly reliable; however, it manifests some considerable limitations such as poor stability of leptospires and their rapid decay in the environment, time-consuming isolation procedure as well as high level of biohazard for the personnel. The method of polymerase chain reaction (PCR) enables detection of leptospiral infection at a very early stage thus increasing the chance for prompt and successful therapy. This is of particular importance in latent human and animal infections and their suppression and prevention. Data from the available literature pertaining to comparison of the results obtained by PCR and other methods strongly indicated its high sensitivity as compared to other methods used so far in the diagnostics of leptospirosis (*Gravekamp et al., 1993; Grgić Ž., 2011, Doctoral Dissertation*).

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