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Cross-Protection Among Unrelated Leptospira Pathogens Serovars: An Unfinished Story

Odporność krzyżowa u niepowiązanych serotypów *Leptospira* – niedokończona opowieść

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The World Health Organization considers leptospirosis the most neglected zoonotic disease in the last decades. One of the major obstacles in the developing of vaccines for the prevention of leptospirosis is the absence of cross-protection among unrelated serovars. It is accepted that cross-protection among related serovars is due to antibodies generated against lipopolysaccharides (LPs), whereas a cross-protection among unrelated serovars is rarely observed.
Objectives. The objective of the study was to ascertain the existence of cross-protection among vaccine strains of different serovars.
Results. The results of this research demonstrated that a cross-protection among unrelated *Leptospira* serovars strain is possible. The Canicola strain is able to induce protection against homologous, Ballum and Copenhageni strains. The Mozdok strain induced protection only against a homologous challenge. Other strains showed a moderate cross-protection against a heterologous challenge.
Conclusions. These findings suggest that the Canicola and Mozdok strains are ideal candidates for developing a new vaccine formulation for use in Cuba (**Adv Clin Exp Med 2012, 21, 5, 581–589**).

Key words: *Leptospira*, leptospirosis, cross-protection, vaccine, *Mesocricetus auratus.*

Streszczenie

Wprowadzenie. Światowa Organizacja Zdrowia uważa leptospirozę za najbardziej lekceważoną chorobę odzwierzęcą w ostatnich dziesięcioleciach. Jedną z głównych przeszkód w rozwoju szczepionek zapobiegających leptospirozie jest brak odporności krzyżowej wśród niepowiązanych serotypów. Przyjmuje się, że odporność krzyżowa wśród powiązanych serotypów wynika z przeciwciał wytworzonych przeciwko lipopolisacharydom (LPs), a wśród niepowiązanych serotypów odporność krzyżowa występuje rzadko.
Cel pracy. Ustalenie, czy odporność krzyżowa występuje między szczepionkami opartymi na szczepach różnych serotypów.
Wyniki. Badania wykazały, że odporność krzyżowa wśród niepowiązanych szczepów serotypów Leptospira jest możliwa. Szczep *L. canicola* jest w stanie wywołać odporność przed szczepami homologicznymi, *Ballum* i *Copenhageni*. Szczep *L. mozdok* wywołał odporność tylko przed homologicznym szczepem. Inne szczepy wykazały umiarkowaną odporność krzyżową przed heterologicznym wyzwaniem.
Wnioski. Wyniki badań sugerują, że szczepy *L. canicola* i *L. mozdok* są idealnymi kandydatami na składnik nowej szczepionki do użytku na Kubie (**Adv Clin Exp Med 2012, 21, 5, 581–589**).

Słowa kluczowe: *Leptospira*, leptospiroza, odporność krzyżowa, szczepionka, *Mesocricetus auratus.*

Leptospirosis is a widespread disease initially described as Weil's syndrome [1]. It is mainly an occupational disease which affects humans and domestic animals, particularly dogs, cattle and swine [2, 3]. Susceptible animals acquire the infection by direct or indirect contact with the urine or tissues of infected animals [3]. Leptospiras enter the body through small cuts or abrasions, via mucous membranes such as the conjunctiva or through wet skin. They circulate in the blood stream, with the bacteremic phase lasting for up to 7 days [4]. The primary lesion is damage to the endothelium of small blood vessels, leading to localized ischemia in organs, resulting in renal tubular necrosis, hepatocellular and pulmonary damage, meningitis, myositis and placentitis [5].

Leptospiras are spirochetes, about 0.1 μm in diameter by 6–20 μm in length [6]. These include both saprophytic and pathogenic species comprising the genus *Leptospira*, which belongs to the family Leptospiraceae, order Spirochaetales. It has over 200 pathogenic serovars, and is divided into 25 serogroups; many different strains with small antigenic differences can be found in some serovars [5, 7]. According to the genetic classification, 13 pathogenic and 6 saprophytic species have been described [5]. The phenotypic and genetic classification do not necessarily coincide. Various molecular methods can be used to differentiate pathogenic and saprophytic leptospira. The most widely used is polymerase chain reaction (PCR) with primers recognizing the gene sequence *lip*L32 (the lipoprotein component of the outer membrane), a typical marker present only in pathogenic *Leptospira* [8].

Immunity to leptospirosis is predominantly humoral in humans and most animal species, including dogs, pigs, guinea pigs and hamsters. Antibodies against leptospira lipopolysaccharide (LP) molecules can transfer passive immunity between animals in some models of leptospirosis [9, 10]. Pathogenic *Leptospira* are resistant to the bactericidal activity of normal serum, and in absence of specific antibodies they are neither phagocytized nor destroyed by macrophages [11]. The immune response is also implied in the pathogenesis of leptospirosis through the formation of the immunological complex, the liberation of cytokines and the generation of autoimmune vasculitis [11, 12]. Signs and symptoms of lung, renal and hepatic commitment thus appear in the immune phase when the specific antibodies begin to be detected [12]. Until recently, cross-protection was thought to be restricted to antigen related serovars, since immunity against leptospira LPs following infection is generally highly serovar specific (but not exclusively so) [5, 13]. Some studies carried out in last decade have demonstrated in an indirect way that cross-protection can exist among serovars of different serogroups [5, 13, 14]. The objective of the current study was to ascertain the existence of cross-protection among vaccine strains of different serovars, all of them of epidemiologic interest for Cuba.

Material and Methods

Bacterial Strains

The *Leptospira spp*. serovars used in this study (Table 1) were cultivated at 28°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% inactivated rabbit serum. The strains and samples were supplied by the Quality Laboratories of the Finlay Institute, Havana, Cuba. To determine the specificity of the primers, a number of microorganisms were tested, including *L. interrogans* serovar Canicola, *L. biflexa* serovar Patoc (American Type Culture Collection [ATCC] strain) and *Treponema pallidum* spp (ATCC strain).

Table 1. Phenotype classification and LD_{50} of *Leptospira spp* strains

Tabela 1. Klasyfikacja fenotypowa szczepów LD50 *Leptospira* spp.

Strain (Szczep)	Phenotypic complex/ serovar (Kompleks fenotypowy/ serotyp)	LD50 [UFC/ mL]
FoBa	Leptospira interrogans Ballum	7
CE1	Leptospira interrogans Canicola	4
CE2	Leptospira interrogans Mozdok	6
CE3	Leptospira interrogans Copenhageni	8

The Source and Care of the Hamsters Used in the Study

Golden Syrian hamsters (*Mesocricetus auratus*) were obtained from CENPALAB, Havana, Cuba. They were housed and bred at the Animal House facilities of the Finlay Institute. Ethical approval for the study was obtained from the Ethics Committee of the Finlay Institute.

PCR for Confirming Leptospira Pathogenicity

The PCR method as described by Gravekamp et al. [8] for the amplification of *lip*L32 was used, with minor variations, to confirm the pathogenicity of the strains used in the study. The vaccine strain *L. interrogans* serovar Canicola Hond Utrecht IV, *L. biflexa* serovar Patoc (ATCC) and *Treponema pallidum* spp (ATCC) were used as controls. For the DNA extraction the cultures were diluted 1-fold in 1 mL of phosphate-buffered saline (PBS: 0.02 mol/L Na_2HPO_4; 0.15 mol/L NaCl, pH 7.2) and centrifuged at 14,000 *g* for 10 minutes. The pellet was resuspend in 50 µL of PBS and boiled for 10 minutes at 100°C. After processing, the samples were stored at –20°C until they were used for PCR. The *Leptospira interrogans lip*L32 sequence was obtained from GenBank, and PCR primers were designed using vector NTI 5.0 (Informax Inc., Bethesda, Maryland, USA). The PCR primers *lip*L32 [F] 5′–CGCTGAAATGGGAGTTCGTATGATT-3′ and [R] 5′-CCAACAGATGCAACGAAAGATCC TTT-3′ were selected, resulting in a 423 bp amplicon. The PCR products (8 µL) were revealed by means of agarose electrophoresis in 2% gel colored with ethidium bromide (0.05 µg/ µL) in buffer TBE 1X.

LD_{50} Determination

The hamsters (9 weeks old) were divided into 7 groups, each containing 8 hamsters. Six of the groups were given different concentrations of inocula (10^0, 10^1, 10^2, 10^3, 10^4 and 10^5 leptospiras) intraperitoneally in 1 ml quantities, as described by Silva et al. [15]. The control animals were administered with the EMJH media used to grow the leptospiras, while the inoculated groups were given the leptospiras suspended in their respective growth media. The animals were monitored twice daily for up to 21 days post-challenge, but were euthanized whenever signs of terminal disease appeared (severe dehydration, anorexia and immobilization). From the results of the LD_{50} testing, the concentrations required for the vaccines were obtained using the Reed-Muench method [16].

Vaccine Preparation

To generate the monovalent Ballum vaccines the production methodology developed for vax-SPIRAL® [17] was used with slight modifications. The selected isolates were propagated for three weeks in EMJH medium until dense cultures were observed, using dark-field microscopy (an Olympus BX51 microscope with a dark-field condenser). The leptospiras were washed three times by centrifugation at 10 000 *g* for 30 minutes and resuspended in 40 ml of phosphate buffered saline (PBS). After the final wash 0.5% neutral buffered formalin was added for 20 minutes, and the pellet was then similarly washed in PBS four times. The suspension was then diluted to a concentration of 6×10^8 cells/ml. Aluminium hydroxide (1.0 mg/ml) was added as adjuvant and tiomersal (0.05 mg/ml) was added as preservative [18]. The vaccine produced was inoculated onto semi-solid EMJH medium to confirm that there were no viable leptospiras, and a loopful was inoculated in a blood agar and broth tioglicolate medium that was incubated at 37°C for 24 hours to confirm the absence of aerobic and anaerobic microorganisms. Monoclonal antibody testing (MAT) was then used to verify that the strains were pure and of their original identities [19]. The vaccine preparations that were found to have satisfactory results in all the tests were packed aseptically in volumes of 5.2 mL, using 10 mL glass bulbs with rubber covers and protective metallic shields and were stored at 4°C until they were used.

The Experimental Challenge and Post-Mortem Examinations

For the vaccine trial, a total of four groups, each composed of 50 hamsters (aged 4 weeks) were used (Table 2). The groups inoculated with a monovalent formulations were each intramuscularly administered a series of two inoculations, six weeks apart. The challenge trial was carried out as described in Table 2. The animals were challenged intraperitoneally with 100 LD_{50} of each of the isolates.

The animals were monitored twice daily for up to 21 days post-challenge for any sign of illness such as external hemorrhage, dehydration, ruffled hair, decreased activity and isolation from other hamsters in the cages. The clinical signs were scored, using the following scoring system: 0 = no clinical signs; 1 = mild signs such as decreased activity; 2 = moderate signs, including anorexia and depression; 3 = severe clinical signs, including the inability to move or death. Post-mortem examinations were performed and the liver and kidneys were harvested to prove Koch's postulates [20].

Antibody Measurements

Blood was collected by venipuncture of the lateral saphenous vein [21] using microcapillary tubes before each inoculation, prior to the challenge, 2 weeks after inoculation, and then at death or 28 days post-inoculation at euthanasia. Blood collected from hamster was allowed to clot over-

Table 2. The groups of hamsters used in the experiment and the results of tissue-culture and mortality observed in each group

Tabela 2. Grupy chomików użytych w eksperymencie, wyniki hodowli tkankowej i zaobserwowana śmiertelność w każdej grupie

Group name (Nazwa grupy)	Description (Opis)	Number in group pre-challenge (Liczba osobników w grupie przed badaniem)	Mortality (Śmiertelność) %	# culture positive/ # livers evaluated (Pozytywna hodowla/ liczba ocenionych preparatów wątroby)	# culture positive/ # kidneys evaluated (Pozytywna hodowla/ liczba ocenionych preparatów nerek)	Category of pathological findings (Rodzaj patologicznych zmian
A	immunized with Canicola	40				
	challenge with Canicola	10 each	0	0/10	0/10	0
	challenge with Mozdok		100	10/10	10/10	2
	challenge with Copenhageni		0	0/10	0/10	0
	challenge with Ballum		0	0/10	0/10	0
B	immunized with Mozdok	40				
	challenge with Canicola	10 each	100	10/10	10/10	2
	challenge with Mozdok		0	0/10	0/10	0
	challenge with Copenhageni		100	10/10	10/10	2
	challenge with Ballum		100	10/10	10/10	1
C	immunized with Copenhageni	40				
	challenge with Canicola	10 each	60	3/10	5/10	1
	challenge with Mozdok		100	10/10	10/10	2
	challenge with Copenhageni		0	0/10	0/10	0
	challenge with Ballum		20	2/10	2/10	1
D	immunized with Ballum	40				
	challenge with Canicola	10 each	0	2/10	3/10	1
	challenge with Mozdok		100	8/10	10/10	2
	challenge with Copenhageni		30	2/10	3/10	1
	challenge with Ballum		0	0/10	0/10	0

Category 0: no pathology. Category 1: subcutaneous jaundice, bleeding of the nail beds, hemorrhages of the kidney, liver is friable with discolorations and smooth edges. Histologically there is moderate nephritis, hepatocyte necrosis and cellular discohesion. Category 2: gross pitting on the surface of the kidney, with capsular adhesions. Histologically moderate nephritis with acute renal damage to epithelial cells and tubular necrosis. With respect to the liver, there is increased binucleation of hepatocytes, and an increase in the number of cells with foamy cytoplasm.

Kategoria 0: bez patologii. Kategoria 1: żółtaczka podskórna, krwawienie z macierzy pazura, krwotoki z nerki, krucha wątroba z przebarwieniami i gładkimi zakończeniami. Histologicznie umiarkowane zapalenie nerek, martwica hepatocytów i dekohezja komórek. Kategoria 2: wyrwy na powierzchni nerki, zrosty otoczkowe. Histologicznie umiarkowane zapalenie nerek z ostrą uszkodzeniem nabłonka i martwicą kanalików. W wątrobię, istnieje zwiększona liczba jąder w hepatocytach i zwiększenie liczby komórek z „piankowatą" cytoplazmą.

night and centrifuged at 700 *g* for 20 minutes; the serum removed and stored at −20°C until analysis. For the ELISA test, 96-well ELISA plates (MaxiSorp, Nunc, Denmark) were coated using 50 µl per well of 5 µg/ml of each serovar sonicate diluted in 0.1M Na_2HPO_4 (pH 9.0). The plates were incubated overnight at 4°C, washed with PBS containing 0.05% Tween 20 (PBST), blocked at room temperature for 3 hours with 200 µl of 1% BSA in PBS, and again washed with PBST. Serum samples

were thawed and diluted 1:100 and 1:1000 with 1% BSA in PBST, then added to the plates at 100 µl per well and incubated at room temperature for 2 hours. These concentrations were found to give readings in the linear part of the assay without increasing background readings in pilot experiments that evaluated a large range of serum dilutions from 1:10 to $1:10^6$ and included known negative and known positive sera. After washing, 100 µl of mouse anti-bovine IgG conjugated to horseradish peroxidase (1 µg/ml, Serotec) diluted with 1% BSA in PBST was added to the plates and incubated at room temperature for 45 minutes. Following 10 µl of 30% H_2O_2 was added to 11 ml of freshly thawed 0.3 mg/ml 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.1M citric acid (pH 4.35) and 100 µl per well was added to the plates. The plates were incubated with the substrate at room temperature for 25 minutes, the color reaction was stopped by adding 50 µl per well of 1% SDS, and optical density at 405 nm was determined using a Dynex plate reader. The microscopic agglutination test was conducted as described by Cole et al. [22] and the sera were serially diluted until an endpoint titer was obtained, as is conventional. Antibody levels were therefore expressed as IgG U/ mL.

Statistical Issues and Analyses

The data was analyzed at a 5% significance level using STATISTICA software version 6.1 (USA). Kaplan-Mayer tests were performed to determine if there were significant differences in survival rates.

Results

PCR Leptospira Pathogenicity

The four strains of *Leptospira spp*. used for the study tested positive by PCR. Figure 1 shows an ethidium bromide stained agarose gel after electrophoresis in which a 423 base pair amplicon represents the *lip*L32 sequence, which is a feature of pathogenic *Leptospira* genus. Besides the control strain *Leptospira interrogans* Canicola Hond Utrecht IV produced a 423 base pair amplicon, which was absent from PCR products obtained from *L. biflexa* serovar Patoc (ATCC) and *Treponema pallidum* spp (ATCC).

LD_{50} Determination

All the *lip*L32 positive isolates showed a high virulence. These strains caused death within 6–14 days of inoculation. The Canicola strain tested caused death within 8–10 days of inoculation. Mozdok and Copenhageni caused death within 7–10 days, while Ballum caused death within 6–16 days. The LD_{50} value of Canicola, Mozdok, Copenhageni and Ballum were 11, 9, 8 and 10 organisms respectively. The statistical comparison of these results did not show a significant difference among them.

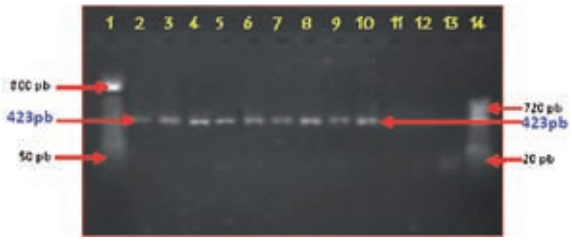

Fig. 1. *lipl*32 polimerase chain reaction. Line 1: Weight marker [4521 Promega], Lines 2–3: *L. interrogans* Canicola serovar, Lines 4–5: *L. interrogans* Mozdok serovar, Lines 6–7: *L. interrogans* Copenhageni serovar, Lines 8–9: *L. interrogans* Ballum serovar, Line 10: *L. interrogans* Canicola (ATCC) serovar, Lines 11–12: *L. biflexa* Patoc (ATCC) serovar, Line 13: *Treponema pallidum spp* (ATCC), Line 14: Weight marker 720-20 pb.

Ryc. 1. lipl32 reakcja łańcuchowa polimerazy. Linia 1: oznaczenie masy [4521 Promega]. Linie 2–3: *L. interrogans* serotyp canicola. Linie 4–5: *L. interrogans* serotyp Mozdok. Linie 6–7: *L. interrogans* serotyp Copenhageni. Linie 8–9: *L. interrogans* serotyp Ballum. Linia 10: *L. interrogans* serotyp canicola (ATCC). Linie 11–12: *L. biflexa* serotyp Patoc (ATCC). Linia 13: *Treponema pallidum* spp. (ATCC). Linia 14: oznaczenie masy 720-20 pb

Antibody Measurements

The IgG response generated by the immunization (two doses) with the different monovalent formulations against the Canicola, Mozdok, Copenhageni and Ballum serovars are shown in Figure 2. In all the groups the antibodies' response against the heterologous serovars were similar to the negative controls and pre-immune values. The IgG rsponse in the group immunized with the Canicola monovalent was revealed in 70% of the animals. After applying the second dose, the values of IgG generated were very significant relative to the values obtained after the first dose. Also, after the first dose 100% of the animals in the immunized groups were positive. A similar response was observed with other monovalents; the main differences were in the percents of responder animals after the first dose (60–80%) and intensity of response. The more immunogenic strains were Canicola and Ballum, and in all groups the IgG response were detected until death or euthanized.

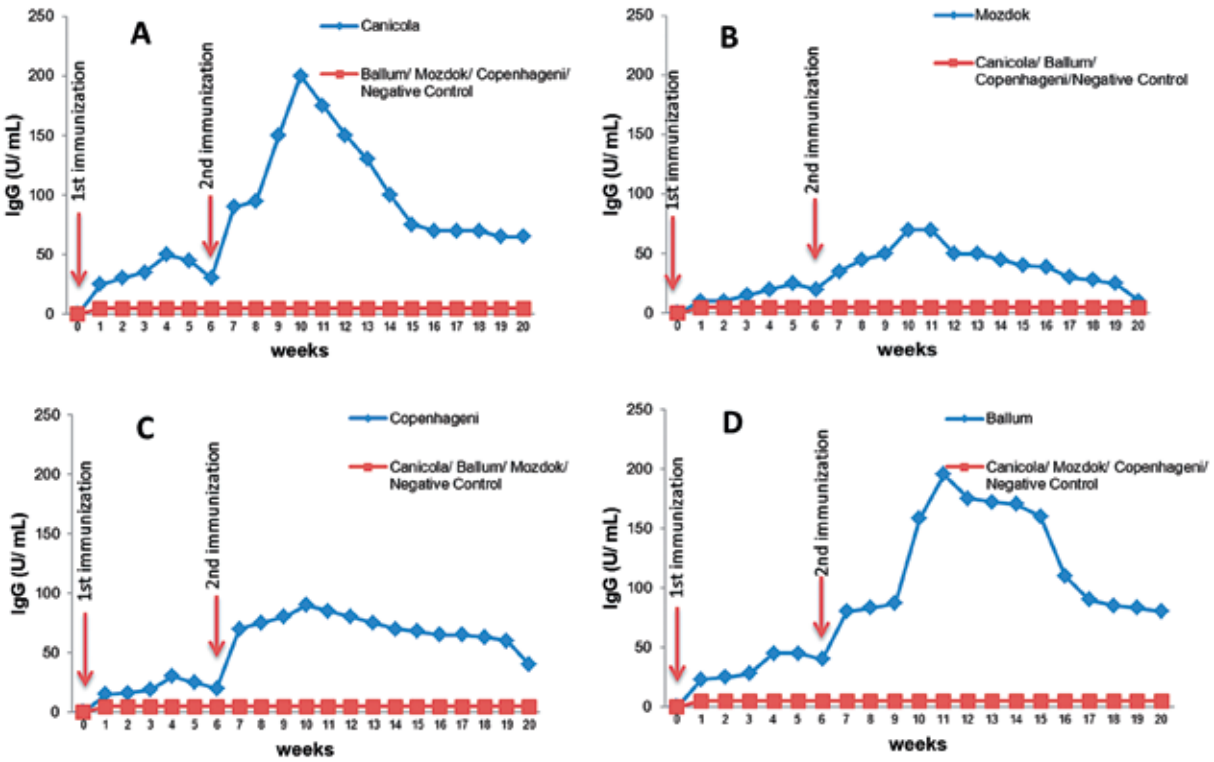

Fig. 2. IgG response induced in hamsters against *Leptospira* serovars after the immunization with two doses of monovalent formulations [A] Canicola, [B] Mozdok, [C] Copenhageni, [D] Ballum

Ryc. 2. Odpowiedź IgG wywołana u chomików przeciwko serotypom Leptospira po szczepieniu dwoma dawkami formulacją jednowartościową a) *canicola*, b) *Mozdok*, c) *Copenhageni*, d) *Ballum*

Experimental Challenge and Post-Mortem Examinations

Figure 3 summarizes the survival rate of vaccinated and unvaccinated animals challenged with the different strains. Hamsters vaccinated with the experimental monovalent formulations and later challenged with a homologous strain showed no clinical signs of leptospirosis and there was no post-challenge mortality. These animals were also culture-negative, with no gross or histological pathological lesion. The same results were obtained in animals vaccinated and not challenged, used as safety controls for each of the monovalent formulations. The unvaccinated challenged hamsters displayed the greatest morbidity (an average histopathological score of 2) with 100% of the hamsters showing clinical signs and 100% mortality.

Mortalities in vaccinated animals challenged against heterologous strains occurred after the vaccination period. For the groups of hamsters inoculated with a Ballum monovalent formulation and challenged with Mozdok and Copenhageni, severe clinical signs were observed in 60–100% of the animals between 8 and 20 days post-challenge, giving mortality rates of 100% and 70% respectively; in contrast, the animals challenged with Canicola did not show mortality or clinical signs. The animal groups inoculated with Canicola monovalent formulation and challenged with Copenhageni and Ballum showed a 100% survival rate without clinical signs, but the animals in this group challenged with Mozdok did not survive: They showed 100% mortality, occurring between 6 and 18 days post-challenge, with very mild clinical signs (average histopathological score = 1). The Copenhageni formulation partially protects against a Canicola challenge (80%), while it protects against Ballum and Mozdok only 30% and 0% respectively (average histopathological score = 2). Finally, the animals immunized with a Mozdok monovalent formulation and challenged with heterologous strains showed 100% mortality; death occurred between 10 and 23 days (average histopathological score = 2).

The organ culture results and gross histopathological lesions from the animals that died or were euthanized are summarized in Table 2. The most severe lesions included hemorrhages of the kidneys and liver. The major histopathological lesions at the kidney level displayed renal tubular necrosis with occasional polymorphonuclear cell infiltration and loss of normal liver architecture.

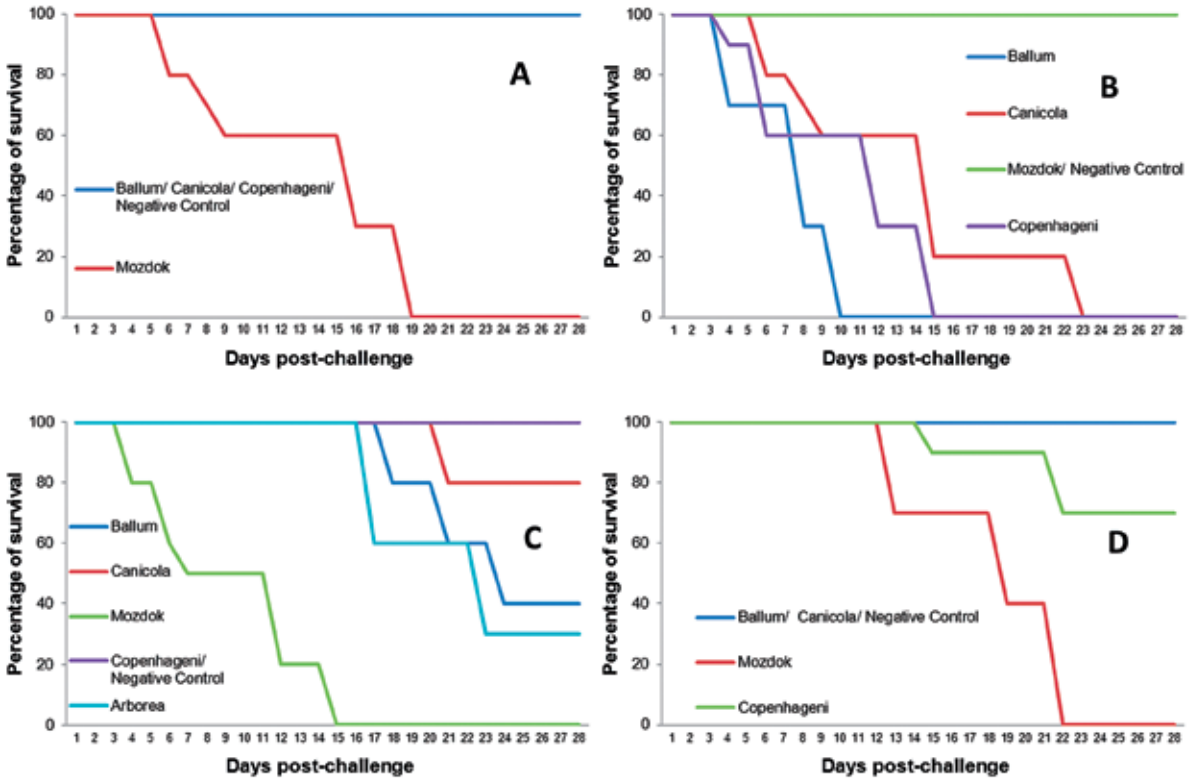

Fig. 3. Percentage of surviving hamsters immunized with monovalent formulations [A] Canicola, [B] Mozdok, [C] Copenhageni, [D] Ballum 21 days post-challenge with 100 DL_{50} homologous and heterologous strains

Ryc. 3. Odsetek przeżywających chomików immunizowanych jednowartościowym preparatem a) *canicola*, b) *Mozdok*, c) *Copenhageni*, d) *Ballum* 21 dni po prowokacji 100 szczepami DL50 homologicznie i heterologicznie

Discussion

To test the hypothesis that cross-protection can be established among unrelated serovars of pathogenic *Leptospira* strains, the authors evaluated the capacity of whole cellular monovalent formulations to confer protection against a lethal challenge in hamsters. The selection of the strains for this assay was based on their epidemic importance for Cuba. All the isolates tested were positive for the *lipL*32 gene, which is found in pathogenic *Leptospira,* and also showed high levels of virulence in Golden Syrian hamsters, which are the animal model most frequently [23] because acute infections exhibit a similar symptomatology to the human infectious process [24, 25]. The virulence of the strains used is an important characteristic to assure the accuracy of the results, because several differences have been observed in leptospirosis pathogenesis using virulent and nonvirulent isogenic strains [26–29]. Extracellular proteins, external membrane lipoproteins and adhesins are expressed more by virulent strains than nonvirulent ones [26, 27]. This unequal antigen architecture translates into a different protector capacity [27]. The LD_{50} experiment results showed that hamster death occurred between 7 and 16 days post-challenge. The timing of the deaths and clinical signs observed were consistent with those of other LD_{50} experiments in hamsters using these serovars [14].

The homologous and heterologous challenge of immunized hamsters with monovalent formulations of inactivated whole cells with these virulent strains evidenced the existence of a complex immune response able to establish cross-protection only among some of the studied serovars, establishing in most of the cases only a one-way protection – that is, the protection conferred by one serovar against another is not necessarily a reciprocal relation. Immunization with the Canicola serovar establishes protection against the rest of the serovars, with exception of Mozdok; on the other hand, immunization with Copenhageni does not guarantee complete protection against a challenge with Canicola. Similarly, immunization with Ballum protects the animals against a challenge with Copenhageni in 80% of the cases, while immunization with Copenhageni only protects the animals challenged with Ballum in 40% of the cases.

Another element of interest in this study is the absence, in all cases, of IgG response against the heterologous serovars, including those in which a partial or total cross-protection was observed.

Some of the causes that can explain high protection levels in the absence of specific IgG antibodies may be the presence of protective IgM antibodies and/or the influence of the cellular response [5]. In Golden Syrian hamsters the presence of IgM antibodies is more relevant, because in this species the immune response against leptospirosis is predominantly humoral [5] and it is characterized as directed fundamentally against LPs [28]. The protection conferred by LPs is serovar specific [29]; in contrast, the response against external membrane protein is related to cross-protection among unrelated serovars [30]. During a natural infection the humoral response against membrane proteins is of much lower relevance than LPs, possibly due to the fact that membrane proteins are present in smaller quantities than LPs and are less accessible to the immune system. The major consequence of this is a lack of long-term protection (IgG antibody response) and a marked decrease in the cross-protection [5, 29, 30].

The obtained results confirm that the cross-protection in *Leptospira* is a complex phenomenon that depends, among other factors, on 1) the serological characteristic of the evaluated strain, and 2) the host immunology system. The authors suggest further evaluation of the Canicola and Mozdok strains as candidates for developing a new whole vaccine formulation capable of generating a broad heterologous protective spectrum.

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