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OmpL1 DNA Vaccine Cross-Protects against Heterologous *Leptospira* spp. Challenge

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SUMMARY Available leptospirosis vaccines made up of inactivated bacteria or their membrane components elicit immunity which is serovar specific and unsatisfactory immunological memory. A vaccine that protects across *Leptospira* serogroups/serovars, *i.e.* broad spectrum, and induces long-lasting memory is needed for both human and veterinary uses. In this study, a plasmid DNA vaccine was constructed from cloning gene encoding a transmembrane porin protein, OmpL1, of pathogenic *Leptospira interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni into a mammalian expression vector pcDNA3.1(+). The protective efficacy of the *ompL1*-pcDNA3.1(+) plasmid DNA vaccine was studied by immunizing hamsters intramuscularly with three doses of the vaccine (100 μg per dose) at two week intervals. The empty pcDNA3.1(+) and PBS were used as mock as negative vaccine controls, respectively. All animals were challenged with the heterologous *Leptospira interrogans,* serogroup Pomona, serovar Pomona (10 LD50), at one week after the last vaccine booster. The *ompL1*-pcDNA3.1(+) plasmid DNA vaccine rescued some vaccinated animals from the lethal challenge and delayed death time, reduced morbidity, *e.g.* fever, and/or the numbers of *Leptospira* in the tissues of the vaccinated animals. While the results are encouraging, further studies are needed to optimize the immunization schedule, vaccine dosage and formulation in order to maximize the efficacy of the vaccine.

Leptospirosis, a zoonosis caused by bacteria of the genus *Leptospira,* is an important emerging infectious disease worldwide. A number of severe cases with high fatality were recognized during the past two decades, not only in the rural tropics, but also in the temperate urbans.[1-3] Leptospirosis eradication is difficult because there are abundance of the animal reservoirs, both wild and domestic, of *Leptospira* spp. and the long-term survival of the bacteria in the environment.[4] Avoiding contact with the animals chronically infected with *Leptospira* spp. (reservoirs) or their environments, such as soil and water contaminated with the animal urine or carcasses, is the most effective means of the disease intervention. However, the measure is difficult to practice especially in the countries where agriculture is the foremost activity and the environmental sanitation is compromised. Vaccines to prevent against the leptospirosis prepared from inactivated whole bacterial cells or outer membrane components of pathogenic *Leptospira* spp. elicit immunity which is limited to the homologous infection. Also the vaccines failed

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to induce long-lasting immunity. Thus, there is a need of more effective vaccine that not only elicits immunity across the heterologous *Leptospira* spp. serovars but also induces long-lasting immunological memory and easy to manufacture and handle, for both human and veterinary uses. Recently, the genomes of three *Leptospira* spp. serovars, *i.e.* Copenhageni, Lai, and Borgpetersenii have been completely sequenced[5,6] and the whole-genome (*in silico*) analysis has been used for identifying the broad spectrum *Leptospira* vaccine candidate genes.[7] However, while such approach, *i.e.* reverse vaccinology, may be relatively convenient, it is required that several genes should be studied concurrently as many of the selected candidates or their expressed proteins counterparts may turned out to be either poorly immunogenic or confer only limited immunity.[8]

In the companion article,[9] proteomes of two *Leptospira* species, *i.e. L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni and *L. borgpetersenii*, serogroup Tarassovi, serovar Tarassovi were studied by using two dimensional-gel electrophoresis (2DE), LC/MS-MS and database search. The antigenic components (immunomes) of the *Leptospira* spp. were determined by probing the 2DE-blots with immune serum of a mouse immunized with pathogenic *Leptospira* spp. homogenate. It was found that there are many antigenic components shared by the two pathogenic *Leptospira* serovars and they are likely to be broad spectrum vaccine candidates. Nevertheless, we have chosen to study the immunogenicity and protective efficacy of a DNA vaccine made up of the gene encoding the outer membrane protein, *i.e.* OmpL1, for the reasons that *ompL1* DNA vaccine has not been studied previously. Besides, this protein, which is a transmembrane molecule, might be exposed on the bacterial surface and is accessible by the host immune apparatus. Immunological blocking the function of the protein may interfere with the *Leptospira* metabolic activity rendering, more or less, benefit to the host.

MATERIALS AND METHODS

Leptospira spp.

Leptospira interrogans, serogroup Icterohaemorrhagiae, serovar Copenhageni was grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, Detroit, Michigan, USA) at 30°C under aerobic conditions. They were used for preparing *ompL1* DNA vaccine. *L. interrogans*, serogroup Pomona, serovar Pomona which was isolated from blood of a Thai patient admitted for treatment of leptospirosis at Khon-Kaen Provincial Hospital in 1999 and maintained in 50% glycerol at -70°C was used for challenging the vaccinated hamsters and controls in the vaccine protection tests.

Preparation of *Leptospira* whole cell homogenate

A whole cell homogenate was prepared from a log phase *Leptospira* culture. The culture was centrifuged at 12,000 x *g* at 25°C for 30 minutes. The bacterial pellet was washed with phosphate buffered saline, pH 7.4 (PBS) three times by centrifugation and finally was resuspended in a small volume of ultrapure distilled water (UDW). The preparation was sonicated at 20 kHz (Model VC750, Vibra Cell™ SONICS & MATERIALS Inc., Connecticut, USA) in an ice bath for 5 minutes. The protein content of the homogenate was determined.[11] The preparation was used for determining the serum antibody titers of the immunized hamsters by an indirect enzyme-linked immunosorbent assay (ELISA).

Preparation of *Leptospira* genomic DNA

Genomic DNA was extracted from whole cells of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni grown to log phase growth by using phenol-chloroform extraction method.[12] The bacterial cells were washed as above and the cells in the pellet from the last centrifugation were resuspended in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The preparation was added with a lysis buffer containing 10% SDS, 3 μl of 20 μg/ml proteinase-K, 5 μl of 20 mg/ml RNase A and incubated at 37°C for one hour. Then 5 M sodium chloride and cetyltrimethylammonium bromide (CTAP) were added appropriately. An equal volume of phenol-chloroform-isoamyl was added to the mixture. The preparation was centrifuged at 12,000 x *g* at 25°C for 5 minutes. The top phase was transferred to a new tube and isopropanol was added to precipitate the DNA. The DNA pellet was collected after centrifugation, washed with 70% ethanol and air-dried. The dried DNA was dissolved in TE buffer

and the quality of the preparation was checked by polymerase chain reaction (PCR) amplification of the 16S RNA gene using two oligonucleotide primers, *i.e.* forward: 5'-GGCGGCGCGTCTAAACAT-G-3' and reverse: 5'-TTCCCCCCATTGAGCAAG-3' generated from 16S rDNA sequence of *L. interrogans*, serovar Canicola strain Moulton.[13,14] The PCR amplicon was subjected to 1% agarose electrophoresis, ethidium bromide staining and visualized under a UV Transilluminator (Biodoc-IT™ Imaging System, UVP Trans-illuminator, Cambridge, UK). Good quality DNA was obtained and the preparation was subsequently used as a template for *ompL1* amplification.

Primer design and *ompL1* amplification by PCR

The oligonucleotide primers for amplification of the *ompL1* by PCR were designed from the DNA sequence (ID LIC 10973) encoding OmpL1 of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni of the GenBank database. The forward primer sequence was 5'-GGCTCGAGCCA-TGGCATTATCTTCGGCTGCA-3' with Kozak sequence and *Xho*I restriction site at the 5' and the reverse primer sequence was 5'-GCTCTAGACTGT-AGATTTGCCCACCGA-3' with *Xba*I restriction site at the 3'. A gradient PCR was performed for determining the optimum primer annealing temperature. The PCR mixture consisted of: 16.25 µl of UDW, 2.5 µl of buffer (10x), 2 µl of 200 nM dNTPs mix, 1 µl each of the primers, 2 µl of DNA template, 0.25 µl of *Taq* polymerase (2 U/µl). The temperature gradients were 52-61°C. An aliquot of each DNA amplicon was subjected to agarose gel electrophoresis and ethidium bromide staining in order to determine the annealing temperature that yielded the highest amount of the target DNA with the least non-specific DNA.

Cloning of *ompL1* amplicon into vectors and selection of *E. coli* transformants

The *ompL1* amplicons were purified by using the ethanol precipitation method. The purified DNA was ligated into pGEM®-Easy vector (Promega, Wisconsin, USA) *via* the overhang T of the plasmid and A of the DNA. The recombinant vector was cloned into JM109 *E. coli* competent cells by using chemical transformation protocol.[12] Selected transformed *E. coli* colonies (white colonies on Luria-Bertani ([LB]-ampicillin [100 µg/ml], 100 mM IPTG and 5% X-gal) agar plate were individually inoculated into LB-ampicillin (50 µg/ml) broth. The cultures were incubated in a shaking incubator at 37°C for 18 hours. Bacterial cells were collected from each culture by centrifugation at 4,000 x *g* at 25°C for 5 minutes. Plasmids were extracted from the cell preparations by using standard alkaline lysis method[12] and cut by using *Xho*I and *Xba*I endonucleases. The cut plasmid preparation was analyzed by 1% agarose gel electrophoresis, ethidium bromide staining and visualization under a UV transilluminator. A transformed *E. coli* clone harboring the recombinant *ompL1*-plasmid was selected for further use.

Preparation of *ompL1*-plasmid vaccine

The *ompL1* was subcloned into pcDNA3.1-(+) vector (Invitrogen, Carlsbad, California, USA). Briefly, the *ompL1*-pGEM-T plasmids were extracted from the transformed JM109 *E. coli* cells, cut with the endonucleases and subjected to 1% agarose gel electrophoresis. The *ompL1* DNA was purified from the agarose gel slice using GENECLEAN-II kit (Bio101, LA, USA) and was ligated into similarly cut pcDNA3.1(+) using T4 DNA ligase. The recombinant plasmids were introduced into Top10 *E. coli* by using the chemical transformation protocol. The transformants harboring *ompL1*- pcDNA3.1(+) were selected on LB-ampicillin agar plate.

For preparation of the *ompL1*-plasmid DNA, single colony of the transformed Top10 *E. coli* grown overnight on the LB-ampicillin agar plate was inoculated into 500 ml of LB-ampicillin broth in a 2-liter sized-flask and incubated at 37°C with shaking at 200 rpm for 18 hours. The cells were collected by spinning the culture at 4,000 x *g* at 25°C for 30 minutes. The cells in the pellet were washed with normal saline solution (NSS) and the plasmid was extracted from the cells by using the alkaline lysis method and purified by using the phenol-chloroform extraction.

Transcription of the *ompL1*-plasmid DNA in COS-7 mammalian cells

In order to ascertain that the *ompL1*-pcDNA3.1(+) plasmid could be expressed in the

mammalian cells, the recombinant plasmid was used to transfect COS-7 cells grown in a tissue culture and the transcribed *ompL1* mRNA in the transfected COS-7 cells was detected by RT-PCR. Briefly, COS-7 cells were grown in Dulbecco minimum essential medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum in a 25 cm^2 tissue culture flask at 37°C in a 5% CO_2 incubator until ~75% confluent growth was obtained. The cells in the flask were washed with sterile PBS, pH 7.4 and then 5 ml of fresh serum supplemented DMEM containing 1% penicillin-streptomycin were added to the flask. The recombinant *ompL1*-pcDNA3.1(+) plasmid (10 μg in 10 μl DMEM) was mixed thoroughly with 15 μl Polyfect (Qiagen, Germany) and the mixture was maintained at 25°C for 15 minutes before adding to the cells in the flask. The pcDNA3.1(+) without *ompL1* mixed with the Polyfect was added to cells in another flask and served as control. The two flasks were incubated at 37°C in the 5% CO_2 incubator for 48 hours. The cells in both flasks were separately harvested and washed extensively with sterile PBS, then total RNA were extracted from the cells using Trizol reagent. DNA in each preparation was eliminated by adding DNase emzyme. Complementary DNA was then synthesized from the total RNA preparations using cDNA synthesis kit (Invitrogen). The cDNA preparations were used as templates in PCR for *ompL1* amplification using the above forward and reverse primers.

Immunization of hamsters and *Leptospira* challenge

Animal experiments were performed according to the guideline of the National Research Council of Thailand.

Twenty-four female Golden Syrian hamsters, four-weeks old, were purchased from The National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhon-pathom province, Thailand. They were housed at the Faculty of Allied Health Sciences, Thammasat University, for one week before commencing the experiments. Blood samples were taken from individual hamsters before the first immunization and serum samples were collected (day 0). The animals were divided into four groups of six hamsters. Each hamster of group 1 was intramuscularly immunized with 100 μg of *ompL1*-pcDNA3.1(+) in 100 μl PBS (vaccinated group) while the animals in group 2 were each injected *via* the same route with pcDNA3.1(+), *i.e.* mock immunization. Hamsters of groups 3 and 4 were similarly injected with 100 μl PBS. The injections were repeated two more times at two weeks intervals using the same dose and route. Blood samples were taken from individual hamsters to collect serum samples on days 7, 14, 28 and 42 after the first injection. The hamsters of groups 1-3 were then intraperitoneally challenged with 10 LD50 (1,000 cells) of *L. interrogans*, serogroup Pomona, serovar Pomona on day 49. The animals of group 3, thus, served as the leptospirosis positive controls while the animals of group 4 were negative leptospirosis controls. All hamsters were returned to the cages with food pellets and water *ad libitum*. They were observed daily for morbidity (rectal temperature, body weight and food and water consumption), and mortality until the experiments were terminated at day 21 after the *Leptospira* challenge (day 70 from commencing the experiments).

Recovery of live *Leptospira* from the tissues of the experimental hamsters

Heart, lung, liver, spleen and kidney were collected from the animals that died within 21 days after the *Leptospira* challenge and also from the animals that survived until the end of the experiments. All tissues were cultured in liquid EMJH medium supplemented with 5-fluorouracil and incubated at 30°C for recovery of live *Leptospira*.

RESULTS

Preparation of *ompL1* vaccine

Genomic DNA encoding OmpL1 was successfully extracted from *L. interrogans* serogroup Icterohaemorrhagiae, serovar Copenhageni, as the 16S rDNA could be amplified from the genomic DNA preparation (lane 1, Fig. 1A). The DNA was used as a template for amplification of *ompL1* by gradient PCR and the annealing temperature at 54.6°C was chosen. The *ompL1* amplicon at 917 bp is shown in lane 2 of Fig. 1A. The *ompL1* was successfully subcloned into pcDNA3.1(+) vector and the recombinant vector, *i.e. ompL1*-pcDNA3.1(+), was introduced into Top10 *E. coli.* Lane 3 of Fig. 1A illus-

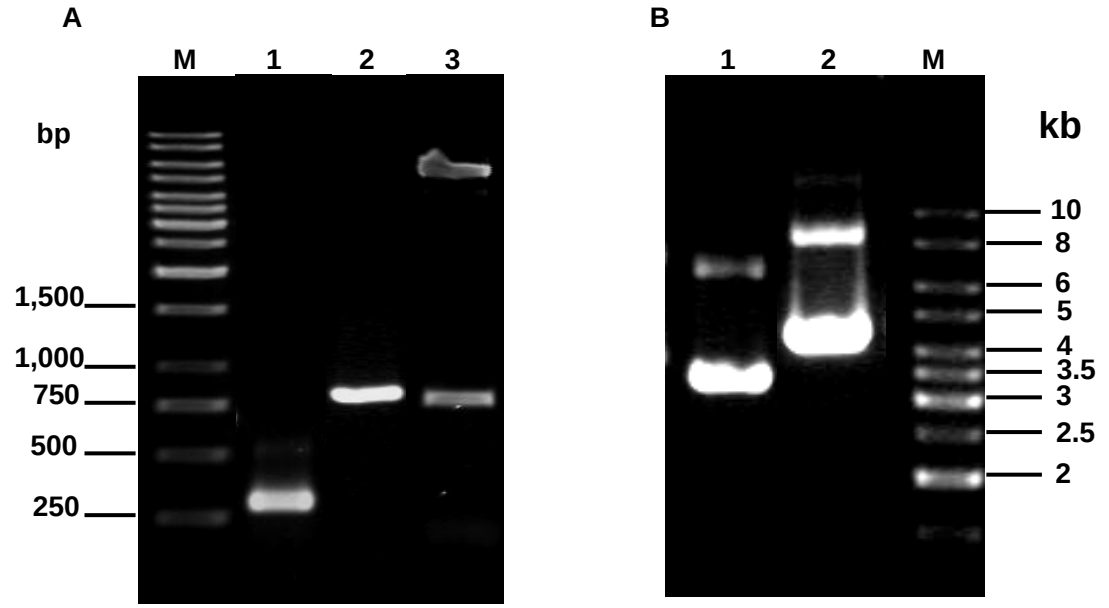

Fig. 1 PCR amplicons of various DNA preparations. Lanes M of both A and B are 1 kb DNA ladder. **(A)**, Lane 1, 16S rDNA amplicon PCR-amplified from genomic DNA of *L. interrogans*, serovar Copenhageni; lane 2, *ompL1* amplicon; lane 3, *ompL1* DNA fragment digested from recombinant pcDNA3.1(+) plasmid extracted from a transformed Top10 *E. coli.* **(B)**, Lane 1, pcDNA3.1(+) plasmid; lane 2, recombinant pcDNA3.1(+) plasmid harboring *ompL1* gene. Numbers at the left of A and the right of B are DNA molecular sizes in kb.

trates DNA banding pattern of the *Xho*I and *Xba*I cut *ompL1*-pcDNA3.1(+) plasmid extracted from the transformed Top10 *E. coli* after 1% agarose gel electrophoresis and ethidium bromide staining. The *ompL1* DNA band is seen at ~917 bp.

The selected transformed Top10 *E. coli* clone harboring the *ompL1*-pcDNA3.1(+) and the *E. coli* clone carrying only pcDNA3.1(+) were separately grown in LB-ampicillin broth and the plasmids were extracted and purified by using phenol-chloroform method (Fig. 1B).

Transcription of the *ompL1*-plasmid DNA in COS-7 cells

RT-PCR amplicon using cDNA prepared from total RNA of the COS-7 cells transfected with *ompL1*-pcDNA3.1(+) as a template is shown in lane 2 of Fig. 2 implying successful transcription of the recombinant plasmid in the mammalian cells. No RT-PCR product was detected when cDNA synthesized from total RNA of the COS-7 transfected with pcDNA3.1(+) alone was used as a template (lane 1, Fig. 2).

Protective efficacy of the *ompL1* vaccine in hamsters

Hamsters in all groups did not show any sign of morbidity after the immunization. The vaccine [100 μg of the *ompL1*-pcDNA3.1(+) plasmids] and the mock [pcDNA3.1(+) alone] were well tolerated by the animals of groups 1 and 2. After the *Leptospira* challenge of hamsters in groups 1-3, all animals showed signs of morbidity, *i.e.* less food and water consumption and body weight loss. Nevertheless, hamsters of group 1 did not have fever while all of the animals of groups 2 and 3 had raised rectal temperature, 37.74 ± 0.35°C and 37.81 ± 0.41°C, respectively (normal range of the hamster's rectal temperature is 36.2 to 37.5°C). Hamsters of group 4 were all healthy with normal appetite throughout the experiment.

All hamsters of group 4 (negative leptospirosis controls) survived until the end of the experiment (day 70). Numbers of hamsters that died after receiving the *Leptospira* challenge in all groups are shown in Fig. 3. Hamsters of group 2 (mock) and group 3 (positive leptospirosis) were all dead between days 5

Table 1 Results of *Leptospira* cultures from various tissues of the hamsters at death or sacrificed hamsters at the experiment termination (day 21 post-infection)

Group	No. of hamsters with *Leptospira* growth in tissue /total (%)				
	lung	heart	spleen	liver	kidney
1: *ompL1*-pcDNA (vaccinated)	*4/6 (66)	1/6 (16)	1/6 (16)	1/6 (16)	3/6 (50)
2: pcDNA (mock)	5/6 (83)	2/6 (33)	3/6 (50)	3/6 (50)	5/6 (83)
3: Leptospirosis control	4/6 (66)	3/6 (50)	3/6 (50)	4/6 (66)	5/6 (83)
4: Negative control	nd	nd	nd	nd	nd

The tissue cultures were examined under dark field microscopy at the 6th week of the incubation.
*lung culture of 1 of 2 survivors was positive for *Leptospira* growth
nd, not done

and 6 post-challenge. For the vaccinated group, two hamsters of group 1 were dead on day 9, one each died on days 11 and 18, respectively. Two hamsters survived the lethal *Leptospira* challenge until day 21 when they were sacrificed.

Results of *Leptospira* cultures from various tissues of the hamsters at death of groups 1-3 and the two survivors of group 1 are shown in Table 1. All cultures were examined under the dark field microscopy at 6 weeks after the tissue incubation. Percentages of culture positive in the respective tissue cultures of the vaccinated hamsters (group 1) were obviously lower than those of the animals in groups 2 and 3.

No significant antibody response was detected in serum samples of hamsters of all groups by the indirect ELISA (data not shown).

DISCUSSION

Even though most OMPs are present in small amount but they play several important roles in the bacterial survival and pathogenesis. OMPs were found to function as bacterial adhesin[15,16] porins[17] and receptors for soluble molecules, such as siderophores.[18] Because they are more or less exposed to the extracellular milieu they should be accessible by the host immunological factors such as antibody. In fact, OMP of bacteria has been shown to be a target of bactericidal antibody.[15,19,20] They could be bound *in situ* by the host complement proteins[21] rendering the bacteria more approachable by the host phagocyte; thus enhances phagocytosis.

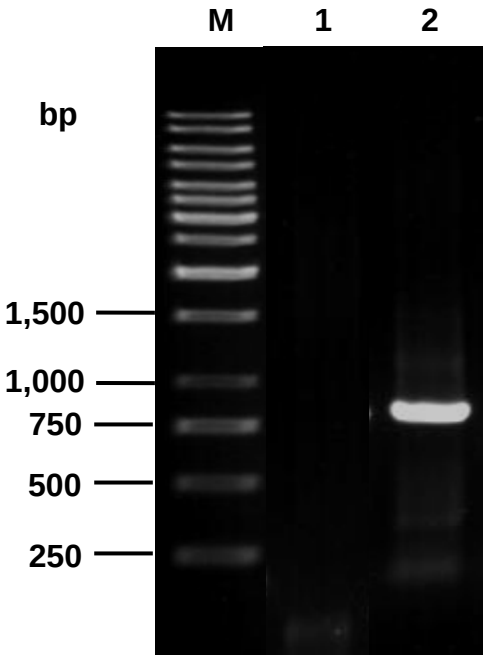

Fig. 2 RT-PCR amplicon of the cDNA synthesized from total RNA extracted from COS-7 cells transfected with *ompL1*-pcDNA3.1(+) plasmids (lane 2), implying expression of the *ompL1* in the mammalian cells. M, 1 kb DNA ladder; lane 1, negative RT-PCR amplicon using cDNA synthesized from total RNA extracted from COS-7 cells transfected with pcDNA3.1(+) plasmids (negative control).

OmpL1 is an immunogenic porin protein found in pathogenic *Leptospira* spp.[9,10] OmpL1 and LipL41 proteins exhibited synergistic immunoprotection against leptospirosis.[22] In this study the *ompL1*-plasmid DNA, constructed by using genomic

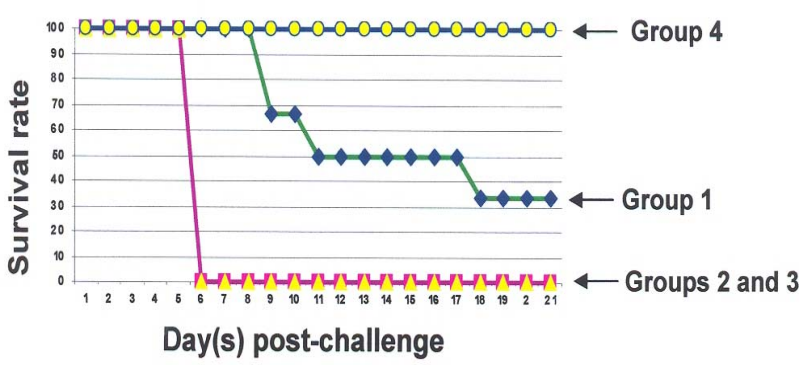

Fig. 3 Survival rate (% survival) of vaccinated and control hamsters. Group 1, pcDNA-*ompL1*; Group 2, Original pcDNA; Group 3, PBS positive leptospirosis control and Group 4, PBS leptospirosis negative control.

DNA of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni, and a mammalian expression plasmid vector, was tested for the protective efficacy in the leptospirosis susceptible animal model, *i.e.* hamsters, after three consecutive intramuscular doses at two weeks intervals. The vaccine was found to be well tolerated by the immunized animals and confer immunity that protected some immunized hamsters against the heterologous lethal challenge by *L. borgpetersenii*, serogroup Tarassovi, serovar Tarassovi isolated from patient with leptospirosis. The vaccine was shown to confer the delay in the death time and reduced morbidity in the vaccinated animals when compared to animals immunized with the plasmid alone or PBS. Further experiments are needed, however, to optimize the immunization schedule, dosages as well as the vaccine formulation in order to maximize the protective efficacy.

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