

Inactivated Trivalent Vaccine for *Leptospira* in Andhra Pradesh

Dr. D. Rani Prameela¹, Dr. D. Sreenivasulu², Dr. P. Eswara Prasad³, Dr. P. Vijayachari⁴,
Dr. S. Vijaya Lakshmi⁵

^{1,2,5}Department of Veterinary Microbiology, C.V.SC., Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

³Department of Veterinary Biochemistry, C.V.SC., Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

⁴Director, ICMR, Port Blair, Center for Diagnosis and typing of leptospira, Andaman and Nicobar Islands

Abstract: *Leptospirosis, the world wide zoonosis is considered as re-emerging disease. Besides economic losses caused by Leptospira to animal production, its zoonotic character makes it an important public health problem. Seroepidemiological studies conducted using MAT indicated the dominance of L.hardjo, L.grippotyposa and L. autumnalis in the state. Hence, serovars hardjo, grippotyposa and autumnalis were selected as vaccine candidates for the preparation of trivalent vaccine. The homology and purity of the vaccine candidates were checked using reference antisera procured from ICMR, Port Blair, Center for Diagnosis and typing of leptospira, Andaman and Nicobar Islands and also using hyper immune sera raised against the respective serovars in the present study. The candidate vaccine strains were adapted to low protein medium containing 0.2 percent Bovine serum albumin (BSA) and adjusted the concentration to 2×10^9 cells/ml. The leptospiral organisms were inactivated with 0.5 percent formalin and adjuvanted with aluminum hydroxide (vaccine-I and Montanide (vaccine-II). The vaccines prepared were evaluated for sterility, safety and potency tests. The immune responses were studied in rabbits using both the types of adjuvanted vaccines and could not find any significant differences between the vaccines. The immune response was found to be satisfactory with both the types of vaccines up to 150 days of post vaccinated period tested. The present work has been carried out during 2006 to 2010.*

Key words: Re-emerging, vaccine candidates, low protein medium and Immune response

1. Introduction

Leptospirosis is a wide spread disease [23] caused by a spirochete *Leptospira*, which affects almost all mammals [4]. Vaccination is the only reliable means of controlling leptospirosis in all species of animals. Inactivated and attenuated vaccines are especially suitable as veterinary vaccines [24]. The majority of vaccines are formalin inactivated bacterins which contain one or more serotypes [22], [13] mixed with aluminum hydroxide. Immunity to leptospire is serovar specific [7]. The study was conducted to identify the prevailing serovars in Andhra Pradesh.

A total of 2,320 sera samples representing different districts of the state were subjected to MAT and observed higher prevalence against *L. grippotyphosa*, *L. hardjo* and *L. autumnalis*, the predominant serovars circulating in Andhra Pradesh. *L. hardjo* was also isolated and are being maintained in the laboratory. The local serovars could not be used in the study for development of vaccine for want of detailed antigenic and genotypic characterization. Hence, the reference strains i.e. grippotyposa, hardjo and autumnalis obtained from reference center ICMR, Port Blair, Andaman and Nicobar Islands were used for preparation of vaccine.

2. Material and Methods

2.1 Selection of Serovars of *Leptospira*

L. grippotyphosa, *L. hardjo* and *L. autumnalis*, the commonly circulating serovars were used for the preparation of vaccine. *Leptospira* bacterins were prepared as per the production outline of British Pharmacopoeia [5].

2.2 Cultivation of leptospire

Preparation of low protein medium

Low protein medium was prepared accordingly to the protocol followed at Regional medical research center, Port Blair, Andaman and Nicobar islands.

2.3 Preparation of charcoal treated tween 80

Charcoal treated (CT) Tween 80 was prepared according to the method described by Bey and Jhonson [3]. Stock solutions of charcoal treated tween 80 were prepared by dissolving 20g of Tween 80 in 200ml of distilled water. While the solution of tween is stirring, 40g of activated charcoal was added gently to ensure homogeneous suspension of charcoal particles. The Tween was then carefully decanted from the charcoal sediment and centrifuged at 10,000 g for 1h and sterilized using 0.22µm filter membrane. The stock solution of charcoal treated tween was stored at 4°C.

2.4 Adaptation of leptospire to low protein medium

EMJH media containing 10 percentages, 7.5 percentage, 5percentage, 2.5 percentage, 1 percentage and 0.2 percentage of BSA were prepared. They were distributed into 4ml of aliquots and checked for sterility by keeping at room temperature for a week. 5 to 7 day old cultures containing 2×10^9 organisms per ml were added to media containing 10percentage BSA and subcultured once in a week for three times. Later leptospiral serovars were further subcultured serially in media containing 7.5 percentages, 5

percentage, 2.5 percentage, 1 percentage and 0.2 percentage of BSA for adaptation.

3.5 Preparation of leptospira suspension

The three serovars namely *L.autumnalis*, *L.grippotyphosa*, and *L.hardjo* selected for preparation of vaccine were grown separately in cell culture bottles containing 18ml of the low protein medium. Two ml of leptospiral seed stock cultures were inoculated and incubated at 29°C ± 1°C for 10 to 12 days. Cultures containing more than 2X10⁹ organisms per ml were used as inoculum for further cultivation of organisms in bulk for preparation of vaccine.

Twenty ml of inoculum thus prepared containing *L.autumnalis*, *L.grippotyphosa* and *L.hardjo* was inoculated in each of the three bottles containing 180ml of low protein medium. The cultures were incubated at 29°C ± 1°C for 10 to 12 days. The concentration of leptospires was adjusted to 2X10⁹ leptospires per ml of each serovar for preparation of vaccine.

Before inactivation of the organisms the purity of the organisms were checked using dark field microscopy. If suspected for minor contamination the leptospiral organisms were passed through 0.22µm filters for clarity. Further, the cultures were also checked for sterility using blood agar medium, Saubourads dextrose agar medium and Thioglycollate medium in duplicates.

3.6 Preparation of Vaccines

The cultures thus obtained were inactivated with 0.5% formalin and adjuvanted with two types of adjuvants namely aluminium hydroxide and Montanide ISA 206 separately.

3.6.1 Formalin inactivation

The cultures thus obtained were inactivated by adding 1ml of formalin (0.5percentage) to each bottle containing 200ml of leptospiral suspension. Later cultures were tested for success of inactivation. Inactivated cultures along with fresh leptospira cultures of *L.autumnalis*, *L.grippotyphosa*, and *L.hardjo* were inoculated into 4.5ml of fresh EMJH liquid media, incubated for 14 days at 29°C ± 1°C and examined the growth of leptospira under dark field microscope.

After confirming the successful inactivation the cultures were incubated at 37 °C under stirring. The cultures were harvested by centrifugation at 14,000 g at 4°C. The sediment was resuspended in PBS and washed thrice with PBS. Then the formalized leptospires were resuspended in PBS to a concentration of 2X10⁹ organisms per ml.

All the bacterins thus prepared were mixed in equal quantities together and bacterin suspension was aliquoted equally in two separate bottles.

3.6.2 Aluminium hydroxide

The bottle containing the bacterins were subjected to centrifugation and separated the pellet. The pellet was mixed with 20ml of aluminum hydroxide gel thoroughly and made upto 100ml using PBS. The vaccine produced was

distributed in 2ml glass bottles labeled as Vaccine I and stored in refrigeration.

3.6.3 Montanide ISA 206

Similarly another bottle containing bacterins was also subjected to centrifugation and pellet was mixed with 46.2ml of Montanide ISA 206 and homogenized thoroughly. Later the volume was made up to 100ml with PBS. The vaccine was distributed into 2ml glass bottles, labelled and stored in refrigerator for future use as Vaccine II.

3. Standardization of the Vaccine

Standardization of the vaccines was done according to the British pharmacopoeia [5].

4. Results

Serovars namely *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* were selected as candidate vaccine strains for the development of inactivated vaccine based on the studies conducted on seroprevalence of leptospirosis in Andhra Pradesh. *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* were found to be predominant serovars circulating in the state.

Antigenic property of the selected leptospiral serovars i.e. *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* were examined using hyper immune sera inoculated into rabbits against the respective serovars. The selected *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* reacted with respective reference sera and found to have higher titers in 1:10240, 1:2560 and 1:20480 respectively. Rabbits inoculated with *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* were also found to have the titers of 1: 81920, 1:40960 and 1:40,960 respectively (Table.I).

Table 1: Results of antigenic properties of Leptospiral vaccine strains

Serovar	Titer with Reference Serum	Titer with Hyperimmune serum
<i>L.grippotyphosa</i>	10,240	81,920
<i>L.hardjo</i>	2,560	40,960
<i>L.autumnalis</i>	20,480	40,960

The organisms were adopted to grow from high protein to low protein (up to 0.2 percent) concentration of BSA. In the process of adaptation, growth of leptospires in low protein medium containing 0.2 percent BSA, the organisms were found to grow at a concentration of 2 x 10⁹ organisms per ml by 10th to 12th day of inoculation. The details of growth of leptospires are shown in Table.2 & Fig.1.

Table 2: Growth pattern of Leptospiral vaccine strains in Low Protein medium (0.2%BSA)

S. No	Indicating percent	No. of leptospires in millions per ml		
		Autumnalis	Grippotyphosa	Hardjo
1	0	8.5	8.0	9.0
2	1	17.0	16.5	18.5
3	2	26.5	27.5	32.0
4	3	37.0	40.0	43.5
5	4	98.5	121.5	119.0
6	5	123.0	160.0	145.5
7	6	195.5	290.0	230.0
8	7	340.0	474.0	435.0
9	8	876.5	1025.0	980.5
10	9	1825.0	235.0	225.0
11	10	2850	3100	3200
12	11	3200	3450	3675
13	12	2725	2325	2550
14	13	1900	1885	1975
15	14	850	974	875
16	15	230	310	280

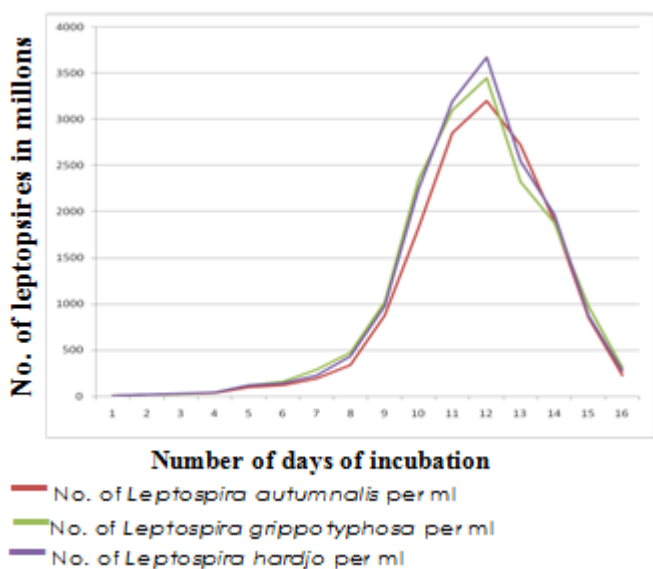


Figure 1: Growth pattern of Leptospiral vaccine strains in low protein medium (0.2%BSA)

The leptospiral serovars namely *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* adjusted to the concentration of 2×10^9 organisms/ml were inactivated using 0.5 ml of formalin for 24 hrs.

Table 3: Potency test in Guinea pigs for evaluation of experimental leptospira vaccines

S. No	Test	Sample examined	Details of serovars								
			Autumnalis			Grippotyphosa			Hardjo		
			V-I	V II	Control	V I	V II	Control	V I	V II	Control
1	MAT	Serum on 14 th day	4.78	4.87	-	4.85	4.89	-	4.77	4.83	-
2	GMT in /log ²	Serum on 35 th day	4.86	4.86	-	4.78	4.87	-	4.82	4.87	4.81

The MAT titers against *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* in guinea pigs after 14 days of vaccination with vaccine-I was 4.78, 4.77 and 4.85 and with vaccine-II was 4.87, 4.83 and 4.89 respectively. The MAT titers on 20 days after post challenge with vaccine-I was 4.86, 4.82 and 4.78 and with vaccine-II was 4.90, 4.87 and 4.87 respectively.

The un-vaccinated control animals died within 7-10 days in case of autumnalis and grippotyphosa but death was not

5.1 Standardization of Vaccine

Vaccine was prepared with inactivated leptospira using aluminium hydroxide (vaccine-I) and mountanide (vaccine-II) as adjuvants. The adjuvanted vaccines, Vaccine-I (aluminium hydroxide) and Vaccine II (Mountanide) were tested for sterility, safety and potency.

5.2 Sterility Test

Blood agar, nutrient agar, sabroaud's agar and thioglycolate medium inoculated with vaccine-I and vaccine-II did not showed any growth of microorganisms and fungi during incubation of 14 days at 30°C and 37°C regularly.

5.3 Safety Test

All the four guinea pigs inoculated with the vaccine preparations did not show any local and systematic reaction and no elevated temperature up to 20 days, the period of observation. No gross lesions were observed in any of the organs in the guinea pigs sacrificed on 20th day of inoculation. Examination of the liver and kidney tissue impression smears using ADMAS modified silver staining method did not reveal any leptospire.

5.4 Potency Test

The vaccinated guinea pigs with vaccine-I and vaccine-II challenged with virulent leptospire of autumnalis, hardjo and grippotyphosa did not show any clinical signs of leptospirosis where as un vaccinated animals die between 7-10 days after challenging with serovars grippotyphosa and autumnalis. However, the guinea pigs challenged with hardjo did not die. The weekly mean body temperature s of the guinea pigs is presented in a table.3. An increase in body temperature in un-vaccinated animals was observed when compared with vaccinated animals after challenge.

The MAT titers against *L. grippotyphosa*, *L. hardjo* and *L. autumnalis* of vaccinated and un-vaccinated guinea pigs are shown in table.3.

observed in animals challenged with hardjo and further it induced antibody titer of 4.87.

5.5 Immune Response in Rabbits

The antibody response in vaccinated rabbits was studied by using MAT and results are shown in table.4.

Table 4: Immune response in rabbits against leptospira experimental vaccines

S. No	DPT	Autumnalis			Grippotyphosa			Hardjo		
		Vac-I	Vac-II	Control	Vac-I	Vac II	Control	Vac-I	Vac -II	Control
1	0	-	-	-	-	-	-	-	-	-
2	7	4.77	4.83	-	4.73	4.78	-	4.74	4.81	-
3	30	4.83	4.87	-	4.77	4.82	-	4.79	4.84	-
4	60	4.87	4.90	-	4.85	4.89	-	4.86	4.88	-
5	90	4.84	4.87	-	4.82	4.84	-	4.83	4.82	-
6	120	4.79	4.84	-	4.76	4.84	-	4.78	4.81	-
7	150	4.77	4.84	-	4.76	4.82	-	4.77	4.79	-

The rabbits in the group-I received vaccine-I containing aluminium hydroxide responded to antigen showed the GMT log² titer of 4.77 to autumnalis, 4.74 to hardjo and 4.73 to grippotyphosa on 7th day. GMT log² titers of serum samples collected on 7th day of post vaccinated in response to vaccine-II against *L. autumnalis*, *L. hardjo* and *L. grippotyphosa*, were found to be 4.83, 4.81 and 4.78 respectively. The GMT log₂ titer values against autumnalis, hardjo and grippotyphosa were found to be 4.77, 4.77 and 4.76 respectively by day 150th of the observation period for the vaccine -I.

The rabbits in the group II receiving vaccine-II containing mountaine showed seroconversion to autumnalis 4.83, hardjo 4.79 and grippotyphosa 4.82 in response to vaccine-II by day 7th (table..). In the group the peak GMT log 2 titers against autumnalis (4.90), hardjo 4.88) and grippotyphosa (4.89) were noticed on 60th day. The GMT log₂ titer values of 4.84, 4.77 and 4.82 were noticed against autumnalis, hardjo & grippotyphosa on 150th day post vaccination period Fig2.

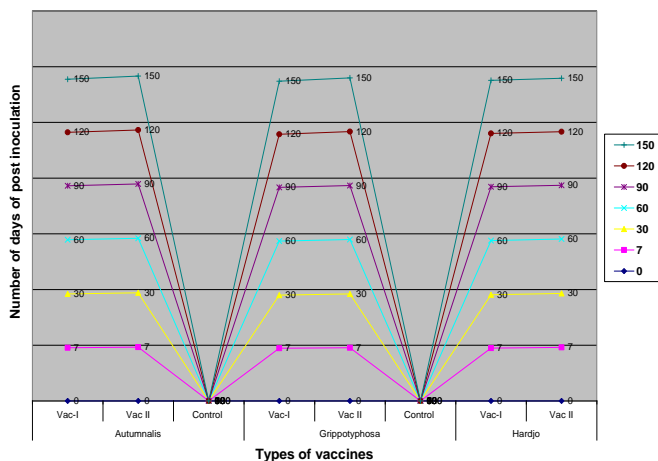


Figure 2: Immune response in rabbits against leptospiral Experimental Vaccines

5. Discussion

Leptospirosis can be prevented and controlled through active immunization. Seroepidemiological studies indicated the dominance of *L. hardjo*, *L. grippotyphosa* and *L. autumnalis* in the state. Hence, serovars *L. hardjo*, *L. grippotyphosa* and *L. autumnalis* were selected as vaccine candidates for the preparation of trivalent inactivated vaccine.

In the present study vaccine strains were adopted to low protein medium containing 0.2% BSA to minimize the

adverse reactions such as pyrogenicity and dermal toxicity caused by serum albumin [3]. Adaptation of leptospires to low protein media did not alter the immunogenicity [1]. Activated charcoal powder was used to reduce the toxicity of tween [3] Formalin at the concentration of 0.5 percent was used for inactivation of leptospiral organisms for the preparation of vaccine in the study. Earlier several workers were also used formalin as an inactivating agent in the preparation of leptospiral vaccine [15], [22], [13], [12] and [16]. Further, it was also reported that formalin treated antigen induces strong antibody response [18] and [14].

The vaccines thus prepared were evaluated for sterility, safety and potency tests. Under safety test, no raising temperature, local & systemic reactions are seen in inoculated Guinea pigs and it is due to the usage of low protein medium for cultivation of vaccine strains. [8] was also used low protein medium for the growth of leptospiral while preparing the vaccine and obtained similar results.

Sterility of vaccine I (Alum), Vaccine II (Montenied) were checked using nutrient agar, blood agar, thioglycolate media and sabourauds dextrose agar media. Both the vaccines were found to be free from viable bacteria and fungi during incubation of 14 days at 30° C and 37 ° C respectively maximum period tested, meeting the requirements[5] and [9].

Potency of the vaccine is tested in guinea pigs by measure in antibody titers [16]. In the present study, the guinea pigs vaccinated with vaccine-I and vaccine-II were challenged after 14 days of post vaccination with virulent leptospires of *L. autumnalis*, *L. hardjo* and *L. grippotyphosa* did not shown any clinical signs of leptospirosis indicating 100 percent protection. But, un vaccinated animals were died between 7 to 10 days after challenge with serovars *L. grippotyphosa* and *L. autumnalis*, where as the guinea pigs challenged with *L. hardjo* did not die and instead induces antibody titer of 4.81 (GMT log 2 titers). This is in agreement with earlier report of [6]. Some strains may be virulent in some hosts but not in the experimental models. *L. noguchii* isolated from clinically suffering sheep fails to cause the disease in hamsters [21].

In vaccinated guinea pigs 14 days after vaccination antibody titer was measured using MAT. GMT log₂ values of MAT titers against vaccine I were 4.78, 4.77 and 4.85 and with vaccine II 4.84, 4.83 and 4.89 respectively against *autumnalis*, *hardjo* and *grippotyphosa*. Similarly MAT titers (GMT log₂ titers) on day 20th of post vaccination with vaccine I were 4.86, 4.82 and 4.78 and with vaccine II were 4.90, 4.82 and 4.87 respectively. All the unvaccinated control guinea pigs died within 7 to 10 days in case of *autumnalis* and *grippotyphosa* and no death was observed in

guinea pig challenged with *hardjo*. Thus, vaccine I (aluminium hydroxide) and Vaccine II (Montanide adjuvanted) full filled the criteria of potency suggested by [16].

Immune responses against two types of adjuvanted vaccines namely vaccine-I (aluminum hydroxide) and vaccine-II (mountanide adjuvanted) were studied in rabbits for a period of 150 days. Both the vaccines induced detectable level of antibodies against all the three serovars incorporated in the vaccine on day 7th of post vaccination. The GMT log₂ MAT titer values followed by vaccine-II was slightly higher than vaccine-I which is significant. The antibody response on the day 30th to 90th day was high against all the three serovars in both the vaccines and slight decrease in the antibody titer on the 20th to 150th day of immunization (table.5 & fig.4). No statistical significant difference was observed in immune responses elicited by both the types of adjuvanted vaccines in rabbits. The antibody response was observed up to 150 days of post vaccination, the maximum period of study.

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Author Profile

Dr. D. Rani Prameela received Bachelor degree of Veterinary and Animal Sciences in 1990, Masters in Veterinary Microbiology in 1992 and Doctoral degree programme in 2010 at College of Veterinary Sciences, Sri Venkateswara Veterinary University, Tirupati and worked as Assistant Professor from 2001 to 2012 and Associate Professor from 2012 to till date. Presently working as Head at State Level Animal Disease Diagnostic Laboratory, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India.