Inactivated Trivalent Vaccine for Leptospira in Andhra Pradesh

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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. Seroprevalence studies conducted using MAT indicated the dominance of L.hardjo, L.grippotyphosa and L. autumnalis in the state. Hence, serovars hardjo, grippotyphosa and autumalis 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 adopted to low protein medium containing 0.2 percent Bovine serum albumin (BSA) and adjusted the concentration to 2x10⁹ cells/ml. The leptospiral organisms were inactivated with 0.5 percent formalin and adjuvanted with aluminum hydroxide. 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 leptospires 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 vaccine for want of detailed antigenic and genotypic characterization. Hence, the reference strains i.e. grippotyphosa, 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. Leptospires bacterins were prepared as per the production outline of British Pharmacopoeia [5].

2.2 Cultivation of Leptospira

 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.

3.4 Adaptation of leptospires 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 2X10⁹ 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...
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 2X10^9 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 2X10^7 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 (0.5%percentage) 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 leptospora 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 2X10^9 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). 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 10^9 organisms per ml by 10th to 12th day of inoculation. The details of growth of leptospires are shown in Table.2 & Fig.1.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Titer with Reference Serum</th>
<th>Titer with Hyperimmune serum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L.grippotyphosa</em></td>
<td>10,240</td>
<td>81,920</td>
</tr>
<tr>
<td><em>L.hardjo</em></td>
<td>2,560</td>
<td>40,960</td>
</tr>
<tr>
<td><em>L.autumnalis</em></td>
<td>20,480</td>
<td>40,960</td>
</tr>
</tbody>
</table>

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 10^9 organisms per ml by 10th to 12th day of inoculation. The details of growth of leptospires are shown in Table.2 & Fig.1.
The un-vaccinated control animals died within 7-10 days of vaccination with L. autumnalis and grippotyphosa but death was not 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 2: Growth pattern of Leptospiral vaccine strains in low protein medium (0.2%BSA)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Indicating percent</th>
<th>No. of leptospires in millions per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autumnalis</td>
<td>Grippotyphosa</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>17.0</td>
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<td>3</td>
<td>2</td>
<td>26.5</td>
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<td>4</td>
<td>3</td>
<td>37.0</td>
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<td>5</td>
<td>4</td>
<td>98.5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>123.0</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>195.5</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>340.0</td>
</tr>
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<td>9</td>
<td>8</td>
<td>876.5</td>
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<td>10</td>
<td>9</td>
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<td>11</td>
<td>10</td>
<td>2850</td>
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<tr>
<td>12</td>
<td>11</td>
<td>3200</td>
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<td>13</td>
<td>12</td>
<td>2725</td>
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<td>14</td>
<td>13</td>
<td>1900</td>
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<tr>
<td>15</td>
<td>14</td>
<td>850</td>
</tr>
<tr>
<td>16</td>
<td>15</td>
<td>230</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>S. No</th>
<th>Test</th>
<th>Sample examined</th>
<th>Details of serovars</th>
<th>Autumnalis</th>
<th>Grippotyphosa</th>
<th>Hardjo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>V-I</td>
<td>V-II</td>
<td>Control</td>
<td>VI</td>
</tr>
<tr>
<td>1</td>
<td>MAT</td>
<td>Serum on 14th day</td>
<td>4.78</td>
<td>4.87</td>
<td>-</td>
<td>4.85</td>
</tr>
<tr>
<td>2</td>
<td>GMT in /log²</td>
<td>Serum on 35th day</td>
<td>4.86</td>
<td>4.86</td>
<td>-</td>
<td>4.78</td>
</tr>
</tbody>
</table>

5.1 Standardization of Vaccine

Vaccine was prepared with inactivated leptospires 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, sabraud’s agar and thioglycolate medium inoculated with vaccine-I and vaccine-II did not show 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 leptospires.

5.4 Potency Test

The vaccinated guinea pigs with vaccine-I and vaccine-II challenged with virulent leptospires 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 grippotyposa 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.
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 grippotyposa on 7th day. GMT log2 titer of serum samples collected on 7th day of post vaccinated in response to vaccine-II against L. autumnalis L. hardjo and L. grippotyposa, were found to be 4.83, 4.81 and 4.78 respectively. The GMT log2 titer values against autumnalis, hardjo and grippotyposa 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 montane showed seroconversion to autumnalis 4.83, hardjo 4.79 and grippotyposa 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 grippotyposa (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 & grippotyposa on 150th day post vaccination period Fig2.

### Table 4: Immune response in rabbits against leptospira experimental vaccines

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

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.

### Figure 2: Immune response in rabbits against leptospiral experimental vaccines

#### 5. Discussion

Leptospirosis can be prevented and controlled through active immunization. Seropidemiological studies indicated the dominance of L. hardjo, L. grippotyposa and L. autumnalis in the state. Hence, serovars L.hardjo, L. grippotyposa 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].

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 grippotyposa. 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 (aluminium 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.

References


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.