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COMPARISON OF AGGLUTINATING AND GROWTH INHIBITION ANTIBODIES LEVELS IN SOWS IMMUNIZED WITH TWO EXPERIMENTAL INACTIVATED MONOVALENT VACCINES OF CANICOLA SEROVAR: WHOLE CULTURE WITH ALUMINUM HYDROXIDE AND LIPOPOLYSACCHARIDE SUBUNIT WITH LIPID A

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ABSTRACT

It was compared the antibody response of sows immunized with two experimental vaccines produced with *L.interrogans*, serovar Canicola, strain LO-4, isolated in Brazil.One of the vaccines was the usual bacterin (whole culture inactivated with phenol and adjuvanted with aluminum hydroxide -WC-$AlOH_3$) and the other one was a subunit vaccine produced with a lipopolysaccharide (LPS) fraction extracted from the bacteria outer envelop and with the lipid A, also extracted from the leptospira wall as adjuvant (LPS-MPLA). Experiment was as follows: group 1 (n = 11), not immunized control, group 2, (n = 11): two immunization with 30 days interval of LPS-MPLA vaccine and group 3 (n = 11): two immunization with 30 days interval of WC-AlOH3 vaccine All three groups were simultaneously immunized, independently of pregnancy stage. Both agglutinin and neutralizing post vaccination antibodies levels were measured respectively by the microscopic sera agglutination with live antigens test (MAT) and the in vitro leptospira growth inhibition test (GIT). Sera collections were performed each 30 days during four months after the first vaccination. Non vaccinated control group animals presented no agglutinating antibodies against Canicola serovar during the whole experiment. At 32 and 68 post vaccination days the agglutinating antibodies levels of group 2 (LPS-MPLA) were significantly higher than the observed in group 3 (WC $AlOH_3$), respectively, $p = 0.013$ and $p = 0.031$. The differences observed in the growth inhibition antibodies titers of the two vaccines tested were not significant ($p > 0.05$). Despite the peak of post-vaccination agglutinins have been registered at 68 days after first immunization, higher levels growth inhibition antibodies were detected at 30 days of first vaccination. Subunit vaccine presented the same immunogenic capacity for the production of neutralizing antibodies as the whole culture one.

KEY WORDS: Swine, leptospirosis, vaccine, active immune response, lipopolysaccharide LPS vaccine, whole culture vaccine.

RESUMO

COMPARAÇÃO DOS NÍVEIS DE ANTICORPOS PÓS-VACINAIS, AGLUTINANTES E INIBIDORES DO CRESCIMENTO DE LEPTOSPIRAS EM FÊMEAS SUÍNAS IMUNIZADAS COM DUAS VACINAS INATIVADAS EXPERIMENTAIS MONOVALENTES DO SOROVAR CANICOLA: CULTIVO TOTAL INATIVADO COM HIDRÓXIDO DE ALUMÍNIO E SUBUNIDADE DE LIPOPOLISSACARÍDIOS COM MONOFOSFORIL LIPÍDIO A. Foi comparada a resposta imune de fêmeas suínas adultas imunizadas contra a leptospirose, com vacinas monovalentes produzidas com *L.interrogans*, sorovar Canicola estirpe LO4, isolada no Brasil. A vacina foi empregada em duas formas: cultura de bactérias totais inativada e acrescida do adjuvante de hidróxido de alumínio (WC-$AlOH_3$) e a do tipo de subunidade constituída apenas por uma fração de lipopolisacarídios (LPS) extraídos do envelope externo da bactéria tendo como adjuvante o monofosforil lipídio A, também extraído da parede da leptospira (LPS-MPLA). O delineamento experimental incluiu: grupo 1 (n = 11): controle não imunizado; grupo 2 (n = 11): imunizado com duas aplicações em intervalo de 30 dias da vacina LPS MFLA; Grupo 3 (n = 11): imunizado com duas aplicações em

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intervalo de 30 dias da vacina WC-$AlOH_3$. Todos os grupos foram imunizados simultaneamente sem ser considerado o estágio de gestação dos animais. Os níveis de anticorpos pós-vacinais, aglutinantes e neutralizantes foram avaliados, respectivamente, pelos testes de soroaglutinação microscópica com antígenos vivos (SAM) e o de inibição do crescimento de leptospiras *in vitro* (ICLIV). O monitoramento sorológico foi efetuado a cada 30 dias durante quatro meses após aplicação da primeira dose da vacina. Os animais do grupo controle, não vacinados, não apresentaram anticorpos aglutinantes para o sorovar Canicola durante todo o período experimental. Aos 32 e 68 dias da primo-vacinação, os níveis de anticorpos aglutinantes do grupo 2 (LPS-MPLA) foram significativamente superiores aos observados no grupo 3 (WC $AlOH_3$), respectivamente $p = 0,013$ e $p = 0,031$. As diferenças observadas nos níveis de anticorpos inibidores do crescimento de leptospiras *in vitro*, induzidos pelas duas vacinas, não foram significativas ($p > 0,05$). A despeito do pico de anticorpos aglutinantes pós-vacinais ter sido registrado aos 68 dias da primeira imunização, os níveis mais elevados de anticorpos inibidores do crescimento de leptospiras já foram observados aos 30 dias da primo-vacinação. A vacina de subunidade apresentou a mesma capacidade de indução de anticorpos neutralizantes que a vacina de bactérias totais.

PALAVRAS-CHAVE: Suíno, leptospirose, vacinas, resposta imune ativa, lipopolissacarídio, vacina LPS e vacina bactéria total.

INTRODUCTION

Vaccines used in swine leptospirosis control are inactivated cultures of Pomona, Icterohaemorrhagiae, Hardjo, Canicola, Grippotyphosa and Bratislava serovars. However, these bacterins do not induce cross protection against different pathogenic leptospira serogroups. Usually these vaccines protect only against clinical illness, but do not impair the kidney infection (Brange r et al., 2005).

Leptospira subunit vaccines could be an option for the prevention of leptospirosis, since they are usually constituted by antigens that can stimulate immune response, such as lipopolysaccharide (LPS) and its glycolipids, lipoproteins, membrane proteins, phospholipids and peptidoglycan (Cinco et al., 1996).

Midwintwer et al. (1990) detected immune response in hamsters using Pomona and Hardjo LPS-derived immunoconjugates anti-leptospira vaccines, with the maximal production of agglutinin titers between the sixth and tenth post vaccination weeks. Sonrier et al. (2000) observed complete protection against homologous serovars and partial protection against heterologous ones in hamsters treated with leptospira LPS.

Koizumi; Watanabe (2004) developed an anti-leptospira vaccine by the identification of two homologous immunogenic lipoproteins, the Lig A and Lig B, obtained from *Leptospira interrogans* serovar Manilae, strain UP- MMC- NIID.

In this paper it was compared the antibodies profile induced in sows by two experimental anti-leptospira vaccines, a LPS subunit with lipid A (LPS-MPLA) and a whole culture with aluminum hydroxide (WC-$AlOH_3$). The vaccines were produced with a *L. interrogans*, serovar Canicola, strain LO-4 isolated in Brazil and It was performed the comparison of agglutinating and growth inhibition antibodies titers during a period of 123 days after the first vaccination.

MATERIAL AND METHODS

The experiment approved by the Bioethic Commission of the Faculdade de Medicina Veterinária Zootecnia of Universidade de São Paulo, Brazil was performed in a swine commercial breeding herd with 180 sows at Ibiúna Municipality, São Paulo State, Brazil, during four months from December 2005 to April 2006.

Animals and management conditions

Thirty three cross bred sows (*Sus scrofa*), Landrace (LD) x Large White, (LW), aging from 210 to 783 days old, at first to fourth parturition, which have never been vaccinated against leptospirosis and characterized as not reagent in the microscopic agglutination test (MAT) with 24 strains, were selected from the herd. Animals were submitted to clinical examination for the control of body condition and absence of injuries and their clinical history was obtained from the individual files of the pig farm. The animals were maintained in a pen with three meters high and 1 swine/2.5 m^2. Each sow was daily fed with 2.2 kg of commercial swine food divided in two meals. Water was ad libitum in an automatic drinking system. The risk of a leptospira infection was prevented with adequate liquid waste destination, rodent control, daily hygiene practices, disinfection with quaternary ammonium compounds and serological surveillance of the not vaccinated control group of with the MAT with 24 leptospira strains every month during the whole experiment.

Leptospira strain used for vaccine preparation

The vaccines were produced with a *L. interrogans* serovar Canicola, strain LO-4 isolated from the liver of a swine killed in a slaughterhouse of Paraná State, Brazil (Freitas et al., 2004,) and typed with

monoclonal antibodies in the Royal Tropical Institute, Amsterdam, Netherlands. This strain is pathogenic for hamsters.

LPS and MPLA extraction

The LPS extraction was performed as follows: a seven to ten days old culture of *L. interrogans* serovar Canicola strain LO4 , 7 to 9 x 10^8 leptospira/mL was inactivated with thimerosal, centrifuged at 12,000 g, during 20 minutes, lyophilized into a pellet and submitted to LPS extraction with chloroform and methanol (2:1) three times (F1a); chloroform and methanol (1:1) four times (F1b); chloroform and methanol (1:2) four times (F1c) and chloroform/methanol/water (10:20:8) four times (F1d), butanolic partition (F3); butanol/water (1:1) water phase (F3a); butanolic phase (F3b) followed by constant shaking during four hours and centrifuged at 1,500xg. The LPS was collected in six fractions: F1a, F1b, F1c, F1d, F3a and F3b according to the chloroform and methanol proportions and using a chromatographic purification and hidrofobic interation in a Octyl Sepharose column (Almeida et al., 1994; Almeida et al., 2000; Almeida et al., 2003). The immunological reactivity of the fractions was evaluated with a in house capture ELISA test using a polyclonal rabbit antisera against serovar Canicola produced by the Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinäermedizin, Berlin, Germany. The fraction F1d obtained with the proportions of $CHCl_3$:MetOH:H_2O (10:20:8) presented the highest titers in the ELISA test and was chosen for immunization. The lipid A, monophosforil A lipid (MPLA) was obtained by acid hydrolysis of the same F1d fraction (Almeida et al., 2003; Campos et al., 2004).

LPS subunit vaccine (LPS- MPLA)

The LPS subunit vaccine was produced with LPS (F1d) fraction at a 2.4 nmol concentration of inorganic phosphate/µL and MPLA 1.0 nmol (0.28 nmol of inorganic phosphate/µL).

The whole culture inactivated vaccine (WC-$AlOH_3$)

The whole bacteria vaccine was produced by the cultivation of leptospiras in EMJH medium (DIFCO) with incubation during seven days at 28º Celsius. Subcultures were performed each seven days until it was obtained a proper volume with the concentration of 7 to 9 x 10^8 leptospira/ mL. Culture was inactivated with thimerosal, 0.5% (v/v) and phenol 37%, and incubated at 37º Celsius during two days. The concentration of leptospiras was obtained by counting in a Petroff-Hausse chamber. The cultures were centrifuged at 3.500 g during two hours at 4º Celsius. The pellet was resuspended in PBS in the same volume of the initial culture volume and centrifuged at the same conditions. The second pellet was resuspended in PBS at the proportion of 1/5 of the initial volume and the amount of leptospiras was evaluated achieving the level of 10^8 leptospiras/mL. The final composition of the vaccine in one milliliter was 10^8 leptospiras, Aluminum hydroxide 0,15% final concentration, thimerosal 0.01%, final concentration and PBS (Masuzawa et al., 1991).

Swine vaccination and blood collections

The experiment was done with three groups of 11 animals receiving the first vaccination or saline solution without considering the pregnancy phase.

Group 1: control, received two doses of saline solution with 30 days interval.

Group 2: received two doses of the LPS-MPLA *L. interrogans* serovar Canicola strain L0-4 vaccine with 30 days interval.

Group 3: received two doses of WC- $AlOH_3$ *L. interrogans* serovar Canicola, strain LO-4, whole culture bacterin with 30 days interval.

Vaccines and saline solution were injected subcutaneously at the ear of the animals in a volume of 1.0 mL per animal, at the first vaccination and 2.0 mL at the booster dose.

Blood collection was performed aseptically with disposable needles (size 40 X 12) by cranial cava vein punction each 30 days during four months after first vaccination.

Serological tests

MAT test for the measurement of agglutinin antibodies titers was performed with the microtechnique of microscopic agglutination test, Cole et al. (1973) and Galton et al. (1965). Live cultures in modified EMJH medium (Alves et al., 1996) with four to eight days of culture, ½ of Mac Farland scale turbidity. Twenty two pathogenic leptospira strains and two avirulent ones were used: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Sentot, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Panama, Pomona, Pyrogenes, Wolffi, Hardjo, Shermani, Tarassovi, Javanica, Andamana and Patoc. Sera were diluted at 1:50 in PBS and tested against the whole collection of live antigens. The final dilution of antigen-sera mixture was 1:100 and considered as the cut off point of the reaction. Positive sera in the trial phase were titrated in two fold dilution series with the respective reactive serovars. The titer was the reciprocal of the highest sera dilution presenting 50% of leptospiras agglutinated under dark field microscopy.

The neutralizing antibodies titers were evaluated with the in vitro leptospira growth inhibition test

(GIT) (Triphaty et al., 1973; Tabata et al., 2002). The procedure was adjusted to the laboratory conditions with leptospiras cultures of four to eight days of age in liquid modified EMJH media (Alves et al., 1996; Turner et al., 1970). Serum samples were inactivated in water bath for 30 minutes at 56º Celsius. Each no contaminated sample was examined in four dilutions, (1:2, 1:4, 1:8 and 1:16) five tubes per dilution. In each tube it was included 2.5 mL of EMJH modified media (Alves et al., 1996; Turner et al., 1970), 0.2 mL of serum dilution and 0.1 mL of leptospiras culture. The tubes were incubated at 28 to 30º Celsius during two weeks. After this period it was done the observation of the leptospiras growth. The tubes that presented no turbidity by naked eye observation and less than ten leptospiras by dark field microscopy examination, 120 magnification, were considered positive for inhibition of leptospiras growth. When a serum sample presented contamination it was filtered in swinny Millipore filters 0,22 μm.

Statistical analysis

As the variables in study were quantitative and expressed in arithmetic mean, the group size of eleven animals were adopted. The results of swine vaccination were analyzed with the comparison of reagent animal rates and agglutinin and neutralizing antibodies mean titers. The agglutinating antibodies titers were expressed in base 10 logarithm. Value 1 was attributed to non reagent animals, and it was added value 1 to calculate base 10 logarithm for titers of 100, 200, 400, 800, 1600 and 3200, resulting in log 2.004, 2.303, 2.603, 2.904, 3.204 and 3.505, respectively. The growth inhibition antibodies titers were calculated by the method of Reed; Müench (1938). The comparison of arithmetic means of agglutinating and growth inhibition antibodies titers was performed by Kruskall Wallis non-parametric tests or U of Mann Whitney, when indicated, using SPSS, 12.0 version program. Significance level was 5%. The GIT titers detected in the control group were considered unspecific and arithmetic mean of this group was subtracted from results of the vaccinated ones. Confidence intervals (95%) of growth inhibition antibodies were calculated according to Pizzi (1950).

RESULTS

Agglutinating antibodies

On the 32nd post vaccination day the proportion of reactant animals immunized with the WC-$AlOH_3$ vaccine was 0/11 and to the LPS-MPLA vaccine it was 6/11, with an arithmetic mean titer of 0.91 and titer standard error of 1.047. On the 68th post vaccination day with the WC-$AlOH_3$ vaccine the proportion of reactant animals was 8/11, with the arithmetic mean titer of 1.144 and standard error of 1.438 but to the LPS-MPLA vaccine at the same day the results were respectively 11/11, 2.303 and 0.134. On the 94th post vaccination day with the WC-$AlOH_3$ vaccine the proportion of MAT reactant animals was 2/10, with an arithmetic mean titer of 0.230 and standard error of 0.728, with the LPS-MPLA vaccine, at the same day, the results were respectively 3/11, 0.446 and 0.995. On the 123rd post vaccination day with WC-$AlOH_3$ vaccine the proportion of MAT reactant animals was 3/9, with an arithmetic mean titer of 0.479 and standard error of 0.953, with LPS-MPLA vaccine, at the same day, the results were respectively 2/7, 0.286, 0.757.

Figure 1 shows the results of MAT tests for groups 2 and 3 of vaccinated sows and time of blood collection. There were a higher production of agglutinating antibodies titers after 68 days of first vaccination ($p = 0.035$) in group 2, vaccinated with LPS- MPLA vaccine. The animals of group one remained as not reagents in the five performed evaluations.

Arithmetic means for LO-4 strain Canicola serovar agglutinin titers from sows immunized with LPS-MPLA subunit vaccine were higher than those observed for WC-$AlOH_3$ vaccine: $p = 0.013$ and $p = 0.031$, respectively, for days 32 and 68 after first vaccination. On the other blood collections the observed differences were not significant ($p > 0.05$).

Neutralizing antibodies

The arithmetic median titer and standard error observed in the no vaccinated group was 0.113, so the value was always subtracted from the results obtained in the vaccinated groups.

On the 32nd post vaccination day with the WC-$AlOH_3$ vaccine the proportion of reactor animals was 7/11, with arithmetic mean titer and 95% confidence interval of 0.329 ± 0.017, but with the LPS-MPLA vaccine the obtained results were respectively 5/11 and 0.175 ± 0.021. On the 68th post vaccination day with WC-$AlOH_3$ vaccine the proportion of reactor animals was 6/10, with arithmetic mean titer and 95% confidence interval of 0.118 ± 0.093 as with the LPS-MPLA vaccine the results were respectively 9/11 and 0.194 ± 0.063. On the 94th post vaccination day with WC-$AlOH_3$ vaccine the proportion of reactor animals was 6/10, with arithmetic mean titer and 95% confidence interval of 0.042 ± 0.043 as with the LPS-MPLA vaccine the results were respectively 4/11 and 0.035 ± 0.036. On the 123rd post vaccination day with WC-AlOH3 vaccine the proportion of reactor animals was 1/10, with arithmetic mean titer and 95% confidence interval of 0.061 ± 0,000 but with

LPS-MPLA vaccine the results were, respectively, 4/10 and 0.200 ± 0.061.

Results of the GIT in groups 2 and 3 at the moment of each blood collection are presented in Figure 2. In the blood collections performed at 68 and 123 days, the differences observed between the LPS-MPLA subunit vaccine and the WC-$AlOH_3$ were not significant ($p > 0.05$).

DISCUSSION

The agglutinating antibodies levels of group 2, at 32nd and 68th post vaccination days were significantly higher than the respectively observed in group 3. Midwintwer et al. (1990) also found immunological response in hamsters immunized with anti-leptospira LPS bivalent vaccine serovars Pomona and Hardjo with agglutinin titers peaks at 42 and 70 days after vaccine application. Sonrier et al. (2000) observed that hamsters immunized with leptospira LPS presented peaks of agglutinating antibodies after the booster dose and neutralizing antibodies during a period of 120 days post vaccination.

In the present work, at 94th days from the first vaccination, there was a strong agglutinin titer reduction either for the subunit vaccine as well as for the whole culture one. This short vaccine response period, mediated by the brevity of the presence of agglutinating antibodies were also reported in cattle by Cacchione et al. (1969) and in water buffaloes by Nardi et al. (2007).

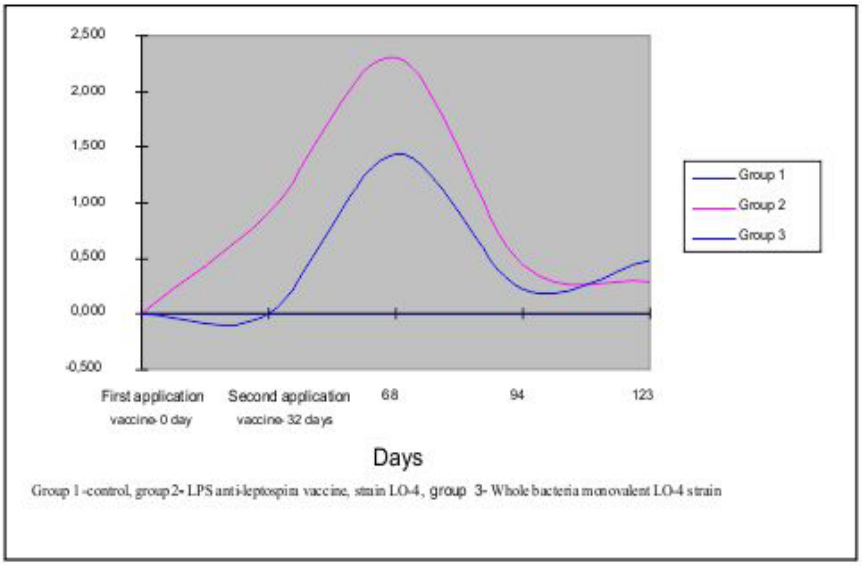

Fig. 1 - Arithmetic means of agglutinating antibody titers expressed in logarithm base 10 to sample LO-4 serovar Canicola in sows with experimental vaccines produced with the same sample, in relation to the group and the time expressed in days after first vaccination.

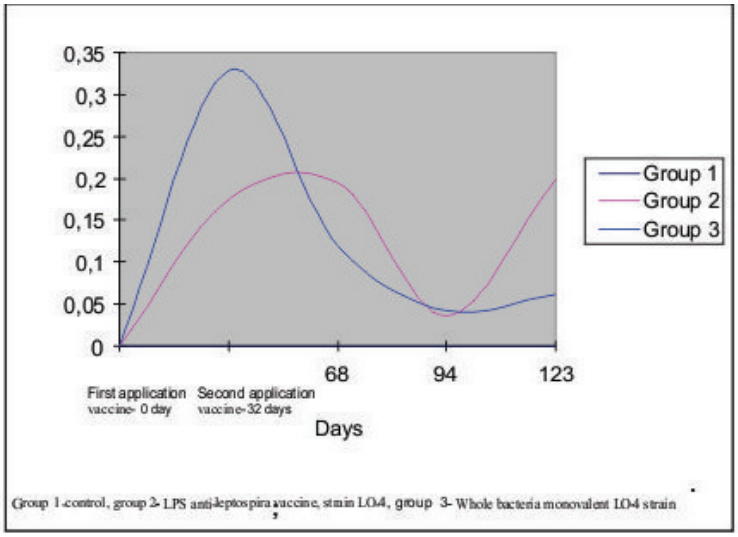

Fig. 2 - Arithmetic means of neutralizing antibody titers expressed in logarithm base 10 to sample LO-4 serovar Canicola in sows with experimental vaccines produced with the same sample, in relation to the group and the time expressed in days after first vaccination.

In the present investigation, in swine immunized against leptospirosis the post vaccination in vitro GIT antibodies presented no correlation with the agglutinating ones. The same was observed by NARDI et al. (2007) in water buffaloes.

In spite of the observation of a strong GIT antibodies decrease at the 94th post first vaccination day for both tested vaccines, it is probable that a longer persistence of these antibodies could be achieved if it was used higher vaccine doses. In bovines vaccinated with anti-leptospirosis whole culture vaccine it was observed that the level of post-vaccine agglutinins is dependent of the antigen concentration in the vaccine (FÁVERO et al., 1997).

The results presented in Figure 2, showed that the booster vaccination with both vaccines tested, at thirty days from the first vaccination did not promote an increase in the GIT antibodies levels. It seems even that there was a decrease of these antibodies just after the second vaccination. It is probable that this condition could be caused by the antibody consumption due to the vaccine antigen. This possibility was analyzed and discussed in rabies prophylaxis (ITO et al., 1991; MORSE et al., 1958). On the 123rd day after the first vaccination it was observed an increase in the GIT antibodies induced mainly by the LPS-MPLA vaccine, but this difference was not significant ($p > 0.05$).

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