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Antibody Development in Swine Against a Hog Cholera Lethal Strain

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Abstract: Pathogenic Hog Cholera (HC) Virus (V) generally kills inoculated pigs before the development of detectable antibodies, making difficult to produce an immune sera against the ALD exposition pathogenic live-virus. With this last purpose a procedure was evaluated by inoculating 2 HCV seronegative pigs, initially with highly diluted dosages of challenge HCV (ALD strain/with an initial titer of $10^{4.99}CCID_{50}$/mL) and then progressively with more concentrated dosages (from 10^{-11} to 10^{-1}). This allowed to produce the disease with a long incubation period. The 2 pigs presented fever and HC clinical signs and survived 30 and 38 days after inoculation, respectively. In a second phase, in an attempt to produce a HC immune serum, 5 groups of 4 susceptible pigs were immunized as follows Group (G) I, Negative Control; G II vaccinated with the PAV-250 HC vaccine; G III, inoculated with 1 mL ALD-HCV ($10^{4.0}$/mL); G IV, vaccinated and challenged with ALD-HCV; G V, vaccinated twice andALD-HCV challenged. G I was negative. HC-antibody titres of ≥1:10 were detected at 14 Post Challenge (PC) days in Control Groups II, IV and V and at 15 PC. days in Group III. HC antibodies developed in G III because pigs had enough time for antibody production after being inoculated. The utilized challenge dilution ($10^{4.0}$ /mL) was satisfactory for obtaining immune sera against the pathogenic live HCV-ALD, in G III; Control groups II, IV and V also produced satisfactory immune sera.

Key words: Hog Cholera, classical swine fever, pav-250 vaccinial strain, ald pathogenic strain, immunization

INTRODUCTION

Classic swine fever or Hog Cholera (HC) has been one of the economically most important viral diseases for swine production worldwide (Van Oirschot, 1999). It is caused by a virus from the *Flaviviridae* family, *Pestivirus* genus (*Heinz et al*., 2000). Several clinical presentations of this disease can be observed in susceptible swine, caused by HC Virus (HCV) strains with diverse virulence degrees (Meyers and Heinz-Jurgen, 1996): Peracute form, produced by very virulent strains that practically kill all pigs 2-5 days postinfection (Sierra *et al*., 1994), affected swine do not have time to produce detectable antibody levels in serum against HCV before death, Acute form, caused by virulent strains with 2-6 days of incubation period, death occurs 10-20 days postinfection, after a relatively slow course (Sierra *et al*., 1994; Van Oirschot, 1992). The subacute form has a longer incubation period, with a mortality rate lower than 30% and mortality happens between 20-30 days postinfection and Chronic form, with a very slow course which causes that pigs survive more than 30 days postinfection (Terpstra, 1991; Sierra *et al*. , 1994; González, 2000). In the last two cases serum antibody production is more feasible. Other authors classify strains in similar ways (Van Oirschot, 1988; Van Oirschot and Terpstra, 1989; Meyers and Heinz-Jurgen, 1996).

The aforementioned clinical presentations of HC can also be seen when a group of pigs is inoculated with progressively reduced doses of a same HCV. As it was observed by Dahle and Liess (1992) tittering a virulent Alfort strain in pigs, concluding that the minimum infecting dose capable of producing the lethal disease was less than 10 infection doses for cell cultures ($CCID_{50\%}$) per pig. The disease was successfully produced using the highest viral dilution, nonetheless, only one out of four animals died. Meanwhile, the surviving three pigs developed neutralizing antibodies.

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On the other hand, when Pasteur developed his successful rabies vaccine method, he inoculated rabbits with the fix rabies virus, collected their spinal cords and dried them for 1-14 day-periods. On the fist day of vaccination schedule he applied 14 day-spinal cord suspensions and consecutively daily doses of spinal cords less dried each time; until 2 or 3 day-spinal cord suspensions were used. Meaning that, as spinal cords lost humidity, the virus was apparently more attenuated or, maybe the viral quantity was reduced (Brunner and Gillespie, 1973).

Antibodies against HCV can be detected only 14-21 days postinfection (Ehrensperger, 1988; Van Oirschot, 1988, 1992). Therefore, it is difficult to achieve the experimental production of antibodies using the HCV virulent strains, due to the fact that pigs die before developing antibodies.

Based on the aforesaid, it is suggested the hypothesis that in order to produce immune serum against a very pathogenic HCV, on one hand the Dahle and Liess (1992) procedure must be followed and, on the other hand according to Pasteur, a small group of pigs should be immunized as it was done for rabies vaccine, based on the initial application of a minimal dose which is progressively increased. The aim is that the animal inoculated with HCV does not die before producing the needed antibodies.

The objective of this study was to evaluate a procedure in which pigs are inoculated with the exposition ALD virulent virus of the HC at doses that allow them to survive the time required for serum antibody developing before dieing.

MATERIALS AND METHODS

HC virus: A live attenuated vaccine against the HC PAV-250 strain (lot 93-01)[d] reproduced in PK-15 cells was used and the ALD pathogenic strain that was grown in primary cultures of swine spinal cord (Mendoza, 1995). Both strains were tittered by the Immunoperoxidase (IP) technique (Ramirez *et al*., 1998).

Experiment 1: Inoculation with increasing infecting doses of the ALD virulent strain: Through out a first test on pigs, the dilution of the ALD pathogenic virus was assessed, being able to produce the disease but with a long incubation period in order to achieve antibody production before dieing. The procedure was as follows: 2 pigs, 45-day-old, serologically negative to HC were obtained from a farm free from this disease; dilutions progressively more concentrated of the ALD strain were applied to these animals. Previously, logarithmic dilutions of the strain were prepared from 10^{-1} to 10^{-11} ; 1 mL of dilution was inoculated to each pig by Intramuscular way (IM), starting with the highest dose (10^{-11}) down to 10^{-1}; doses were given every 2 or 3 days. Rectal temperature and clinical signs were daily supervised on the pigs since day 0 of the experiment up to day 38 after inoculation.

Experiment 2: In a second phase, a group of pigs was immunized using the same lot and dilution of the ALD strain in order to determine if the chosen dose (in Experiment 1) was capable of inducing antibody production; a group of non-vaccinated pigs was the negative control and, several groups of pigs vaccinated were used as vaccinated controls with several treatments, such as PAV-250 vaccine application while others were vaccinated and challenged. Twenty, 45-day-old pigs were used, all susceptible to HC. Five groups of 4 pigs each were formed, which received the following treatments:

Group I: Non-treated controls.

Group II: Vaccinated with a dose of the PAV-250 strain; but non-challenged.

Group III: Inoculated with the pathogenic ALD strain, with the dilutions chosen by Experiment 1.

Group IV: Vaccinated with one dose (2 mL) of the PAV-250 vaccine and after 10 days were challenged with the ALD strain.

Group V: Vaccinated with two applications of the PAV-250 strains and, after that the pigs were challenged with the ALD pathogenic strain.

The groups of pigs were housed inside Isolation Units. Groups I and II were managed by different personal than groups III, IV and V. The pigs were observed during the first 4 days. Then they were identified and blood samples were collected to obtain the sera, in order to verify that they were serologically negative to HC. Vaccination was done on day one of the experiment. One dose of the vaccine (2 mL) was applied IM to the vaccinated groups (II, IV and V). The second vaccine application (Group V) was carried out on day 10 of the experiment, the same day that Groups III, IV and V were challenged with 1 mL IM of the ALD strain at a $10^{-4.0}$ /mL dilution.

Clinical signs: Pigs were clinically observed every day during the experiment, along with rectal temperature

measurement. All the animals were bleed on the day one of the assay (before vaccination) to obtain blood serum and then every 5 days until day 30. On the last day, the pigs were euthanized by total bleeding. Necropsy was done to observe lesions and to collect tonsil samples for viral confirmative studies. The obtained sera were identified and kept at -20°C until use. The qualitative Immunoperoxidase (IP) (Ramirez, *et al.* , 1998) test was done to verify if they were initially negative to HCV and later to detect the presence of antibodies against HCV.

Direct immunofluorescence test: On day 30 after vaccination after necropsy, tonsils from Groups I, II, IV and V were collected to perform confirmative viral studies; along with those from Group III, in which animals got sick and/or died after challenge. The Direct Immunofluorescence (DIF) test was applied to these samples, cutting the frozen tissues using a specific CSF conjugated diluted at 1:60 (Coba and Correa, 1993).

RESULTS

HC virus: The PAV vaccinial strain used in this experiment showed an IP titer of $10^{3.8}$ $CCID_{50}$ / 0. 2 mL. The IP initial titer of the pathogenic ALD strain was $10^{4.99}$ $CCID_{50}$/0. 2 mL.

Experiment 1: Clinical signs and serological results observed when increasing infecting doses of the virulent ALD strain were applied. The two pigs from the preliminary assay, inoculated with increasing concentrated ALD strain dilutions, showed elevated temperature from day 24 and 25, respectively, after the application of the 10^{-11} dilution; meaning 7 and 8 days after the $10^{4.0}$ dilution application and 4 and 5 days after the application of the $10^{3.0}$ dilution. Both animals presented HC signs, increased rectal temperature, anorexia, prostration, heaping together, rough hair, lordosis, neurological signs, tonic and clonic movements, continuous blinking, congestion of the ocular conjunctive, skin cyanosis on the ears, depressions in the lateral abdomen. One of them died on day 30 and the other on day 38 showing hypothermia and diarrhea. Serological tests only yielded 1:20 titers for IP antibodies in the last pig on day 38 of the experiment.

Experiment 2: Observed clinical signs: The following was observed along the developing of the study.

Group I: Non-treated controls: Pigs remained normally without increased rectal temperature or clinical signs; no lesions were found at necropsy.

Table 1: Neutralizing antibodies detected in the different groups by the immunoperoxidase test

	Sampling days						
Groups	1	5	10	15	20	25	30
Group I	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Group II Vac[a]/1d[b] Pav-250	Neg	Neg	Neg	≥1:10[c] (1/4)[d]	≥ 1:10 (3/4)	≥ 1:10 (4/4)	≥1:10 (4/4)
Group III Inoc[e] ald	Neg	Neg	Neg	Neg	Neg	≥ 1:10 (1/4)	≥1:10 (1/4)
Group IV Vac 1d Pav-250 y Chal[f] ald	Neg	Neg	Neg	≥ 1:10 (4/4)	≥ 1:10 (4/4)	≥ 1:10 (4/4)	≥1:10 (4/4)
Group V Vac 2d[g] Pav-250 y Chal ald	Neg	Neg	Neg	≥ 1:10 (3/4)	≥ 1:10 (3/4)	≥ 1:10 (4/4)	≥1:10 (4/4)

VAC[a] = Vaccinated; 1D[b] = One dose; [c] = Antibody titer; [d] = Number of pigs with antibody titers/No. of treated; INOC[e] = Inoculated; CHAL[f] = Challenged; 2D[g] = Two doses

Group II: Vaccinated with PAV-250 vaccine: There were no elevated rectal temperature except in one pig that showed a 0. 4° C increase (40. 4°C) during day 3 of the experiment. Only 2 vaccinated pigs had lightly hemorrhagic lymph nodes at necropsy.

Group III: Inoculated with the virulent ALD strain: All the pigs showed hyperthermia, 1 from day 5, 2 from day 10 and the rest from day 11 after inoculation. Besides, the pigs presented the rest of the HC clinical signs between two and 3 days after the onset of the hyperthermia. One pig died on day 10 (having the HC signs for 5 days) and 2 on day 16 (after 6 days with HC clinical signs). All of the pigs had HC lesions at necropsy.

Group IV: Vaccinated with one dose of the PAV-250 strain and exposed to the ALD pathogenic strain: These pigs remained normal except two that showed slight hyperthermia (40. 0 and 40. 3°C, respectively) on day 16 after challenge; no other signs were seen. Scarce lesions were found at necropsy; one had spleen infarcts. There were no pathological findings in the rest of the pigs, except for scarce petechiae in the bladder mucosa.

Group V: Vaccinated with 2 doses of the PAV-250 strain and challenged with the ALD pathogenic strain: No hyperthermia was observed, except for one pig with light temperature elevation (40° C) on day 26 after inoculation; none of the animals died, there were scarce lesions suggesting HC at necropsy in the lymph nodes, bladder and spleen.

Direct immunofluorescence test: The following results were found: Group I, the tonsils studied were negative; Group II, the tonsils from the vaccinated non-challenged pigs, were negative; tonsils from pigs in groups III, IV and V were positive.

Serology: Sera from the 5 groups of pigs were initially negative to antibodies against HCV by the Immunoperoxidase (IP) test, according to the first sampling, done before vaccination. Later it was determined that the Controls (Group I) did not develop antibodies against HCV; meanwhile antibodies were present in Group II from days 15 and 20 after vaccination. Only one out of the four pigs inoculated with the ALD pathogenic strain in Group II had antibodies from day 15 after inoculation. Antibodies were detected from day 15 after vaccination in Groups IV and V (Table 1).

DISCUSSION

Different forms with several incubation periods of the disease caused by the HCV infection can be observed; even the length of the disease is variable. The type of HCV strain determines the incubation period and length of the disease; therefore, antibody development depends on these 2 factors. The virulent HCV stains (field and reference virus) that cause the acute form of the disease are characterized by producing short incubation periods and disease courses. Because of that, animals have no time to create detectable serum antibodies. The low virulence strains produce long incubation periods and disease courses, so the affected pigs have time to develop their own specific serum antibodies (Van Oirschot, 1992). On the other hand, since HCV vaccinial strains are harmless, they also stimulate the production of specific serum antibodies, practically without clinical signs (Correa, 1998).

Due to the aforementioned, it is hard to achieve the experimental production of antibodies against the HCV virulent strains because the inoculated pigs usually die before developing antibodies. Because of this, it was necessary to use a procedure that uses a minimal infecting quantity of the ALD virulent strain, which could be able to produce the disease when applied, but not being lethal, at least for some of the inoculated pigs. It was expected that the minimal doses used would cause that the incubation period and course of the disease were long enough to allow antibody production and then produce the immune serum necessary to perform another study (Coba, 2000).

Therefore, though out the Experiment 1, it was possible to infect susceptible pigs with very low virus quantities that allowed using such doses successfully in the following experiment. It was achieved for the 2 pigs to survive during 30 and 38 days after inoculation, respectively, showing the typical signs of the disease, but with a long course, according to the results yielded by the first experiment. This was similar to producing a chronic disease, as it has been reported (Van Oirschot, 1988; Terpstra, 1991; Sierra *et al*., 1994). Through the application of the same doses to the pigs in the second experiment, it was possible to gain the time needed for antibody production. The $10^{4.0}$/mL dilution used, was adequate to obtain immune serum against the ALD-HCF pathogenic strain.

Regarding the inoculation of different doses in Experiment 1, data concur with Van Oirschot (1988) and Dahle and Liess (1992) reports, who mention that the length of the disease depends on the virus dose inoculated to the pigs. On the other hand, Van Oirschot (1988) also mentions that viral replication progress was slower when a low virulence HCV was used and then would be enough time for developing an immune response; by this, the broad viral diffusion in the body of the affected pig would be prevented. In the same sense, Dahle and Liess (1992) report that by doing a viral titration and inoculating weaned pigs with the Alfort virulent strain, they observed that the minimum dose capable of producing a lethal disease corresponded to less than ten 50% Cell Culture Infecting Doses (CCID) per pig. Furthermore, when the highest viral dilution was applied producing the disease, only one out of four animals died, while the surviving pigs developed neutralizing antibodies. This agrees with the observed in the present study since it was possible to produce chronic infections in the inoculated pigs applying a minimal quantity of the virulent virus.

Based on the results yielded by the first experiment, in the second one the ALD pathogenic virus was used at an initial 104. 99 CCDI/mL titer, which was determined by the IP test. Ten-fold dilutions were done from this original titer and 1 mL of the last dilution was inoculated to each pig in Groups III, IV and V. This procedure allowed to infect the pigs. Carbrey (1988) mentions that pigs are 10-times more sensible than cell cultures to detect the HCV. The aforesaid concurs with the observed in this study since pigs from Group III, inoculated with the mentioned dilution, showed HCF signs and lesions, antigen in the tonsils and one pig could develop antibodies; while all pigs from Groups IV and V developed antibodies against HCF since they were vaccinated andexposed (Table 1). On the other hand, negative controls from Group I remained normal, without clinical signs or lesions at necropsy: their tonsils were negative to the DIF test and there were no

VN-IP antibodies (Table 1). Animals in Group II (vaccinated with one PAV-250 dose) behaved clinically normal,in the expected way. Tissues were apparently normal at necropsy and tonsils were negative to DIF. The absence of the HCF antigen in the tonsils of these groups verifies that the antigen of the PAV-250 vaccinial strain is not detected 30 days after vaccination. This agrees with previous studies that demonstrated that in pigs vaccinated with the PAV-250 vaccine and euthanized after different periods, the vaccinial antigen was detected in several tissues on days 5 and 7after vaccination, while it was not present on days 10 and 40 (Coba and Correa, 1995 non-published). In the present research, it was seen that VN-IP antibodies against PAV-250 were detected since day 15 after vaccination. Other researchers have also observed that VN-HCF antibodies are detectable from day 15 or between the second and third weeks after the animals were in contactwith the HCV (Ehrensperger, 1988; Van Oirschot, 1988; Van Oirschot, 2000). All pigs in Group III (inoculated with ALD) presented HCF signs and lesions and only one out of the four inoculated pigs survived during the 30 days of the experiment. This concurs with the described by Sierra *et al.* (1994) and by Dahle and Liess (1992) who reported that there are remarkable differences regarding pig individual susceptibility to different HCF strains; their tonsils were positive to the DIF test. One of the four inoculated pigs in this group developed VN antibodies against HCV, which were detected from day 15 after inoculation with titers>1:10 by the IP technique. These antibodies were still detected until day 30 in which the experiment ended and animals were euthanized (Table 1). These data were similar to those found by Ehrensperger (1988) and Van Oirschot (1988, 1992, 1999). VN-IP antibodies were not detected in the other three inoculated pigs from Group III at the used dilution (1:10), since one of them died on day 10. Even though the other two pigs died on day 16 after inoculation, it could had happen that they might had developed VN antibodies ≤1:10 to the IP test and then titers would have been so low that could not be detected by the assay.

In general, pigs in Group IV, vaccinated with one PAV-250 dose and inoculated with the ALD virulent strain, did not show signs or lesions. VN-IP antibodies in all the vaccinated animals were detected since day 15 and remained until day 30. Tonsils were positive in the vaccinated and inoculated pigs, which proved that the ALD virus antigen was present on day 30 after exposition, in contrast to the PAV-250 vaccine. In Group V, pigs vaccinated with two doses of the PAV-250 vaccine and inoculated with the ALD pathogenic strain, only one pig had mild hyperthermia for one day and none of the pigs died; while tonsils were positive again. VN-IP antibodies were detected on days 15 and 20 after the first vaccination in this group (Table 1).

The development of hyperimmune serum against the ALD pathogenic strain was achieved through out the described procedure, corresponding to Group III, by inoculating pigs and demonstrating the presence of antibodies against HCF. Immune sera from Groups II, IV and V worked correctly as controls, just as serum from the negative control Group.

CONCLUSION

- The right dilution ($10^{4.0}$ /mL) of the ALD strain (with an initial titer of $10^{4.99}$ $CCID_{50}$/mL) needed to produce immune serum against the exposition strain was assessed through out the described procedure inoculating increasing doses of the virus.
- Furthermore, the immune serum against the PAV-250 vaccinial strain was obtained and against PAV-250 and the ALD pathogenic strain; which worked correctly as positive controls, along with the serum from the negative controls.
- The viral-neutralizing antibodies (IP) produced by the pigs vaccinated with PAV-250 were detected from days 15 and 20 after vaccination.
- Seroconversion was detected by IP from day 25 in the pigs inoculated with the ALD strain.
- (IP) were detected from day 15 and 20 after vaccination when one and two doses of the vaccine were applied and later the ALD strain was inoculated.

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