Assessment of the Ecuadorian ECJB 2000 isolate of classical swine fever virus as challenge strain

Evaluación del aislamiento ecuatoriano ECJB 2000 del virus de la peste porcina clásica como cepa de desafío

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ABSTRACT: Classical swine fever (CSF) is an infectious disease responsible for high economic losses in pigs of all ages. Several programs for the control and eradication of this disease have been implemented at a global level, being vaccination the principal mechanism. This research had the purpose of identifying the pathogenicity of an isolated circulating strain in Ecuador, and determining the efficacy of the vaccine applied for prevention. Animals were challenged with decreasing doses of the viral strain ECJB 2000 to obtain 10 median infective doses of this strain in pigs (DI 50). Afterward, the efficacy of a vaccine of the lapinized Chinese strain was evaluated against the virus of porcine cholera adapted to a cell culture line derived from a miniature pig kidney (MPK-LC-HCV), averaging a trial potency following the standard protocol of the World Organization for Animal Health (OIE). The vaccine was applied at different dilutions, and 14 days later, a viral challenge was done with DI 50. The unimmunized pigs presented clinical signs from day three post-infection, and the statistical results did not show significant differences in the body temperature registers and leukocyte count. Spearman-Kärber’s trial determined that the vaccine contained 239.88 protective doses (DI 50): taking into account that an effective vaccine against classical swine fever virus (CSFV) must have a value of > 100. This study demonstrated that the circulating strain in Ecuador is of elevated pathogenicity and that the vaccine is effective for the control of the disease.

Key words: Classical Swine Fever, efficacy, challenge, immunization, strain ECJB 2000.

RESUMEN: La peste porcina clásica (PPC) es una enfermedad infecciosa responsable de altas pérdidas económicas en cerdos de todas las edades. Varios programas para el control y la erradicación de esta enfermedad se han implementado a nivel mundial, pero el mecanismo principal es la vacunación. Esta investigación tuvo como objetivo determinar la patogenicidad de una cepa circulante aislada en Ecuador y determinar la eficacia de la vacuna aplicada para la prevención. Los animales fueron desafiados con dosis decrecientes de la cepa viral ECJB 2000 para obtener 10 dosis infectivas medianas de esta cepa (DI 50) en cerdos. Posteriormente, se evaluó la eficacia de una vacuna de la cepa china lapinizada contra el virus del cólera porcino, adaptado a una línea de cultivo celular derivada de riñón de cerdo en miniatura (MPK-LC-HCV), promediando una potencia de prueba y siguiendo el protocolo estándar de la Organización Mundial de Sanidad Animal (OIE). La vacuna se aplicó a diferentes diluciones y, a los catorce días de aplicada, se realizó un desafío viral con DI 50. Los cerdos no inmunizados presentaron signos clínicos desde el día 3, posterior a la infección. Los resultados no mostraron diferencias significativas en los registros de temperatura corporal y en el recuento de leucocitos. El ensayo de Spearman-Kärber determinó que la vacuna contenía 239,88 dosis protectoras (DI 50); tomándolo en cuenta que una vacuna efectiva contra el virus de la peste porcina clásica (CSFV) debe tener un valor > 100. Este estudio demostró que la cepa circulante en Ecuador tiene una patogenicidad elevada y la vacuna es efectiva para el control de la enfermedad.

Key words: cepa ECJB 2000, desafío, eficacia, inmunización, Peste Porcina Clásica.

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INTRODUCTION

Classical swine fever (CSF) is an infectious disease of wild and domestic pigs, causing a high morbidity and mortality in the infected animals. Outbreaks of CSF usually cause high mortality rate in pigs of all ages and decrease domestic and international trade of pigs and pork products. Nowadays CSF is considered a transboundary disease and it is included in the OIE list of notifiable animal diseases (1,2,3). CSF has been eradicated in countries of the European Union and North America, but it is still present in countries of Asia, Central and South America like Ecuador (4), where it is an endemic disease, currently causing great economic losses to pig farmers in several Ecuadorian provinces.

In the year 2000, FAO (Food and Agriculture Organization of the United Nations) established a continental plan for the control and eradication of CSF for the Americas and the Caribbean, which will last until the year 2020 (5). In Ecuador, the National Program for the Control and Eradication of Classical Swine Fever has worked since 2011 (6). This program approved the classical swine fever virus (CSFV) attenuated Chinese strain based vaccines and it demands the mandatory vaccination of weaned pigs (45 days old), sows and boars.

The vaccine efficacy test is regulated by OIE as quality control of vaccine batches. In the test, the efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge and it is expressed by the number of 50 % protective doses (PD50) (7).

There is a need to unify the efficacy test protocols for all vaccines produced in Ecuador by using a unique Ecuadorian isolated virus as challenge strain. The aim of the present study was to obtain an Ecuadorian CSF isolate and assess its pathogenicity for using it as challenge strain in the efficacy test of CSF attenuated vaccine batches. Two trials were conducted, the first one for assessing the virulence of the Ecuadorian CSF ECJB 2000 isolate. In the second trial, the suitability of CSF ECJB 2000 isolate as challenge strain was evaluated through the efficacy test of a CSF attenuated vaccine batch.

MATERIALS AND METHODS

The isolate ECJB 2000 had been obtained from collected organs from a pig with clinical signs of CSF at the year 2000. The 10% organ homogenate in phosphate buffered saline (PBS) was prepared and inoculated by intramuscular way into two healthy and CSF seronegative pigs, tested by ELISA ID Screen ® Classical Swine Fever E2 competition. The blood of these infected animals was taken at the pike of fever and inoculated again in other two pigs for second passage. The blood collected from these animals was fractionated into 10 mL aliquots and stored at -80 °C, until be used.

The presence of CSFV RNA in ECJB 2000 isolate was identified by real time RT-PCR. For the molecular characterization of E2 gen, the virus was isolated in PK-15 cell cultures growing in 24 well plates, after two passages. The presence of CSFV in the supernatant of infected PK15 cells was detected by two methods: two steps real time RT-PCR and IPMA using the CSF monoclonal antibody 21.2 (Prionic). The protocol for real time CSF detection included RNA extraction with High Pure RNA Purification Kit (Roche Ltd) from blood samples and TRIZOL method for RNA extraction from organ samples. The reverse transcription was performed using Transcriptor First Strand cDNA Synthesis kit® (Roche Ltd). The probes and primers used in the real time PCR were described by Hoffman et al (8). The RNA of strain Alfort 187 was used as positive control and the samples that presented a value of Ct<40 were considered positive.

The E2 gen of ECJB 2000 second passage was amplified and the nucleotide sequence was obtained by Sanger method and named EC-PE2-JB2p with Genbank accession number KX586773. The isolate used in this study was phylogenetically classified into 1.1 genotype of CSFV (9). Twelve cross breeding Landrace/Yorkshire swine of 6-8 weeks old, weighting about 20 kg, serologically negative to CSFV, and belonging to a non-vaccinated and CSF free herd, were used in both experiments. The animals were housed in separate experimental rooms with Biosecurity Containment Level 3 and they were handled according to international guidelines for experimentation with animals. The experiments
were carried out under the supervision of the veterinary authorities in Ecuador (AGROCALIDAD). ELISA test (ID Screen® kit Classical Swine Fever E2 competition) was used in order to test the presence of CSF specific antibodies in the serum samples of each animal.

The first trial consisted in the titration of ECJB 2000 virus in pigs and the evaluation of time course of viremia and clinical signs to determine the virulence of the viral isolate. Pigs were divided into four groups of three pigs each in order to determine only the median lethal dose 50 (LD$_{50}$). They were intramuscularly inoculated with 2 mL of the virus at decreasing dilutions from group 1 (10$^{-4}$), group 2 (10$^{-5}$), group 3 (10$^{-6}$), and group 4(10$^{-7}$).

The efficacy test (second trial) was conducted as described by OIE (7). The vaccine batch had been prepared from the Chinese lapinized strain adapted to grow in the miniature pig kidney cell culture (MPK-LC-HCV). Briefly, two groups (A and B) of five pigs were intramuscularly vaccinated with 2 mL each of 1:40 and 1:160 vaccine dilutions respectively. Group C consisted of two non-vaccinated animals. Fourteen days after vaccination, all pigs were challenged with 10$^5$ LD$_{50}$ of ECJB 2000 isolate by intramuscular injection. All animals were euthanized approximately 20 days post-challenge (d.p.c.), with the exception of animals from group C that were sacrificed in order to avoid suffering.

Rectal temperature and clinical signs were recorded three days before the viral challenge and daily during the trial, 10 days for the first trial and 20 d.p.c. Blood samples with anticoagulant were taken on day 0, each five d.p.i. and before euthanasia. These samples were used for determining leukocyte count and the presence of CSFV RNA by real time RT-PCR. The assessment of the clinical signs was carried out through the semi-quantitative scoring system developed by Mitteholzer et al. (10). All pigs showing severe clinical signs were euthanized to avoid suffering, and their organs (tonsils, spleen, lymph nodes, kidney, and ileum) were collected for CSFV RNA detection by real time RT-PCR. The post mortem macroscopic pathological lesions were recorded in the euthanized pigs.

RESULTS AND DISCUSSION

In the virulence assessing trial, the inoculated pigs with decreasing dilutions of ECJB 2000 virus presented severe clinical signs of the disease, characterized by fever, progressive deterioration and death before the 15th day after inoculation. The onset of fever and typical CSF clinical signs began after 3-5 d.p.i., the rectal temperature mean increased up to 40°C-41°C. The groups inoculated with 10$^{-4}$, 10$^{-5}$ and 10$^{-6}$ dilutions were euthanized 6 -8 d.p.i. The fever and other typical clinical signs of CSF were evident on the third d.p.i (Figure 1). Two pigs from group 4 (inoculated with 10$^{-7}$ dilution) showed rectal temperatures above 40 °C after 4 d.p.c. The remaining pig (number 12) was slaughtered at 10th d.p.i., without clinical signs.

The pigs inoculated with 10$^{-3}$ dilution showed leukopenia since the fifth d.p.i, while at time of the euthanasia, leukopenia was identified in groups inoculated with the dilutions 10$^{-5}$, 10$^{-6}$, and the two symptomatic pigs of the 10$^{-7}$ group. At necropsy, the presence of typical hemorrhagic lesions was observed in the target organs of all diseased pigs. Those pathological lesions were not observed in the surviving pig, at the time of necropsy.

The average score of the clinical signs of the challenged pigs was calculated (10), obtaining these results: 20,6 points for group 1; 17,6 points for group 2; 15,5 points for group 3; and 15 points for group 4, respectively (Table 1).

The titre of ECJB 2000 isolate was 10$^{7.25}$ LD$_{50}$/2mL, for calculating the dilution of the viral preparation used for challenging in the assessment of the efficacy of the vaccine batch. The results of the real time RT-PCR demonstrated the presence of viral CSF RNA in all the animals challenged with ECJB 2000 of CSF, with the exception of pig number 12 in which, CSFV was not detected at 5 d.p.i. (table 2A).

In accordance with Mittelholzer et al. (10) and other studies conducted with CSFV isolated in other European countries (11,12), the score of the Ecuadorian isolate is in the range of high virulent CSFV strain (peak CS>15). Also ECJB 2000 isolate showed a great capacity for replication and invasion, producing viremia at just a few
d.p.i., and persisting until death (13). This could be why it was possible to detect CSF RNA in the organs from all pigs, including the surviving pig number 12, that was subclinical infected. The viral load and the virulence of the strain determined the course of CSFV infection to acute or subclinical form as it has been previously reported (13).

In the second trial after challenge, five pigs from group A (n=5/5) and four pigs from group B (n=4/5), all of them vaccinated, did not present clinical signs and survived. Group C (n=2), corresponding with the control pigs and pig No 22 (Group B), showed CSF clinical signs and died 6-8 d.p.c. On day 5 d.p.i., a decrease in the average leukocyte count was observed in all groups, but increased in the vaccinated groups. In group C, leukopenia persisted. Macroscopic lesions were not observed in the organs of the pigs that survived the trial. In the control pigs and No 22 pig, there was a characteristic CSFV infection in the spleen, mesenteric lymph nodes, tonsils, and intestines.

After these results, the calculated protective doses for CSF vaccine batch was 239.88 DP50, fulfilling the requirements established by OIE.
In the same way, the research carried out by Campos et al. (14) and Stojanovic et al. (15) have shown the efficacy of the vaccine strains against CSFV using the OIE standard protocol. In those studies, they obtained more than 100 DP in each vaccine as a result. It should be noted that although this research resulted in the death of several immunized pigs, the vaccine exceeded the OIE requirements (7).

Regarding blood samples from vaccinated animals, viral RNA was not identified on day 14 post vaccination, only after 5 d.p.c. CSF RNA was detected in the blood samples from all challenged pigs, including the two control pigs. One animal from each vaccinated group showed the presence of CSFV RNA in all blood samples (Table 2B). In the remaining vaccinated pigs, the presence of the virus was not detected in blood or organs.

The Chinese vaccine strain tends to produce a substantial protection, starting three days after vaccination, induced by the production of IFN-γ.

Table 2A. Detection of Classical Swine Fever virus RNA by real time RT-PCR in blood and organ samples. / Detección del ARN del virus de la peste porcina clásica mediante RT-PCR en tiempo real en muestras de sangre y órganos

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<th>Day 15</th>
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<th>S</th>
<th>I</th>
<th>M.L.N</th>
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A. Pigs inoculated with 10-4 -10^{-7} serial dilutions of ECJB 2000 isolate / Cerdos inoculados con diluciones seriadas 10-4 -10^{-7} del aislado ECJB 2000.

Table 2B. Detection of Classical Swine Fever virus RNA by real time RT-PCR in blood and organ samples. / Detección del ARN del virus de la peste porcina clásica mediante RT-PCR en tiempo real en muestras de sangre y órganos

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<th>M.L.N</th>
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+ (Positive); - (Negative); E (Euthanasia); K (Kidney); S (Spleen); I (Ileum); M.L.N.(Mesenteric Lymph Nodes); T (Tonsils); NA (Not applicable).

B. Efficacy Test of vaccine batch. Pigs vaccinated with vaccine 1:40 and 1:160 diluted and challenged with 105LD50 of E ECJB 2000 CSF virus / Prueba de eficacia del lote de vacunas. Cerdos vacunados con la vacuna 1:40 y 1: 160 diluidos y desafiados con 105LD50 del virus E ECJB 2000 CSF.
and neutralizing antibodies directed against the viral proteins E2 and NS3 (16). On the other hand, the presence of the CSFV in two immunized pigs could be because qRT-PCR had increased sensitivity and it is a specific method for the detection and quantification of nucleic acids (17,18). The study demonstrated that the viral strain ECJB 2000 contains $10^{7.25}$ PID$_{50}$/2mL. Its inoculation in unimmunized pigs produced clinical signs of CSF compatible with high pathogenicity viral strains. The vaccine MPK-LC-HCV was effective against the viral strain circulating in Ecuador, containing more than 100 DI50, and its application produced protection of CSF clinical signs and a transitory viremia.

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The authors of this work declare no conflict of interest.
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