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Article

# Survival of classic swine fever virus in hams made from the meat of pigs vaccinated with the PAV-250 strain and unvaccinated pigs

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**Abstract:**

The study was to determine the presence of Classical Swine Fever virus (CSFv), in the meat of vaccinated pigs with the PAV-250 strain and then challenged using the same strain. Five treatment groups were established (each with four pigs). Group A: Pigs that

were fed with processed hams from negative animals; Group B: Pigs that were fed with processed hams from commercial pigs inoculated with the ALD (reference strain) (titre of $10^{4.0}$/ml); Group C: Pigs fed with processed hams from pigs infected with the virulent ALD strain (titre of $10^{2.5}$/ml); Group D: Pigs fed with processed hams from pigs vaccinated with the PAV-250 strain and challenged with the ALD strain (titre of $10^{1.1}$/ml); and Group E: Pigs fed with processed hams from pigs vaccinated with two doses of the PAV-250 strain and challenged with the ALD strain (negative). Blood samples were taken at d 1, 5, 10, 15 and 20 for biometric analysis. Groups B, C and D manifested clinical signs of CSFv: 40 °C temperature, anorexia, paralysis, vomiting, diarrhea, tremor, hirsute hair and cyanosis. Pigs were slaughtered and necropsies performed to identify lesions in tissues. Results of direct immunofluorescence testing of tissues were positive and the virus was recovered. Under these study conditions, it was found that CSFv resisted the cooking method at 68 °C for 40 min in hams from unvaccinated pigs, and that the virus was able to transmit the disease to healthy unvaccinated pigs, whereas the hams from the vaccinated animals did not transmit the virus.

**Key words:** Classic swine fever, PAV-250 vaccine, Hams, Mexico.

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# Introduction

The Classic swine fever virus (CSFv) belongs to the Flaviviridae family, genus *Pestivirus* and is closely associated to the viruses that cause bovine viral diarrhea/mucosal disease and border disease, which can also infect pigs[1,2]. The CSF is a highly contagious disease, whose acute form affects the nervous system, the vascular endothelium, and the reticuloendothelial cells[2]. Contagion occurs primarily through contact with different types of secretions and nasal, lacrimal, urinary, and faecal excretions, but it can also be mechanically transmitted by mosquitoes, birds, utensils, contaminated food waste, infected or contaminated pork meat[3-7]. The virus can remain infective in pigs and pork by-products for months, constituting an epizootic factor of considerable importance. Additionally, the virus can survive in infected pigs or swine by-products for months or even years, when the meat is stored in frozen or refrigerated conditions[8-10].

In Mexico, backyard and subsistence pig-raising represent a production and commercialization system characterized by a continuous purchase and sale cycle of animals after short fattening periods; although in some cases, the producers raise their own pigs by keeping one male and as many as five breeding females. However, the facilities are rustic, located near homes, and the workers are usually family members[11]. Traditionally, the feed for pigs raised under these conditions consists of scraps and waste (*escamocha*) from household food preparation and consumption or from restaurants, hotels, hospitals, markets, food distribution centres and agricultural industries, among others. These materials often include pork products or by-products. For these reasons, feeding backyard pigs with scraps and waste constitute an important source for CSFv, susceptible pigs can acquire the disease when fed with inadequately heat-treated kitchen waste or food scraps, as it has happened in Mexico for many years**.** It has been proven that feeding pigs with food scraps and waste is one of the main causes of CSF outbreaks, because the virus can resist in bone marrow for long periods, especially if cooking temperatures are not sufficiently high to inactivate the virus[12]. McKerker *et al*[13] found that the persistence of African Swine Fever virus in the *serrano* and Iberian hams (112 d post-processing) was up to 5-to-6 mo, which was supported by the findings from Botija[14].

Effective vaccines against CSF are available and their use can reduce costs and limit the spread of this disease. However, the efficacy of vaccines depends on their ability to induce a strong immune response, which can be obtained using the modified live vaccine, which was very successful to control CSF in countries where it was endemic. Serial transmission studies in pigs of the live-attenuated CSF vaccine have demonstrated that reversion to virulence does not occur[15]. Around 2005, Mexico was engaged in the swine fever control and eradication program, where the country was divided into three regions: Region 1 had an intensive vaccination program; Region 2, where the eradication of the virus was obtained through vaccination; and Region 3, which corresponded to a disease-free phase. Because phases 1 and 2 require intensive vaccination, the restricted use of the vaccine prompted the research team -along with the Lelystad Institute- to perform a study for determining the antigenic composition of the currently use of vaccine and compare it to others that were applied in the past. The Mexican field strains included in this study revealed heterologous reactions in their secondary epitopes, which were distantly related to the conserved neutralization sites. This variation was restricted to the secondary neutralization sites of CSFv. Only the PAV-250 vaccine has been used in phases 1 and 2 of Mexico's CSFv eradication campaign. Pigs vaccinated with PAV-250 and challenged on the 14$^{th}$ post-vaccination day with the virulent ALD-CSFv did not transmit the challenge virus to susceptible pigs. However, a low releasing level of the virulent virus from vaccinated pigs was detected following this challenge; therefore, the virus remained at levels below the infectious dose. The PAV-250 vaccine proved to be superior to other swine vaccines available in Mexico[16,17].

The aim of this study was to determine the presence of CSFv in the meat of pigs vaccinated with the PAV-250 strain and then challenged. Additionally, to ascertain the virus status in hams prepared from meats belonging to those animals.

# Material and methods

## Meat from experimentally vaccinated and challenged pigs

Meat used in this experiment came from a previous one that had five treatments: Group I, meat from negative control pigs; Group II, meat from commercial pig legs inoculated with the ALD strain with a titre of $10^{6.0}$/ml; Group III, meat from pigs infected with the virulent ALD strain with a titre of $10^{4.7}$/ml; Group IV, meat from pigs vaccinated with the PAV-250 strain and challenged with the ALD strain with a titre of $10^{3.1}$/ml; and Group V, meat from pigs vaccinated with two doses of the PAV-250 strain and challenged with the ALD strain with negative titre[18-21].

## Preparation of the hams

The legs from each experimental group were selected and processed independently. The average weight of the legs before processing was 2.2 kg. Bone was removed from the legs, and then the meat was injected with 20 % of brine and kept for 18 h at 4 °C before cooking. The brine contained the following ingredients: 20 g of NaCl, 0.24 g of sodium nitrite, 0.24 g of sodium nitrate, 6.0 g of food grade phosphate, 0.66 g of sodium ascorbate, 3.6 g of refined sugar, 0.18 g of monosodium glutamate and 0.11 g of hydrolyzed vegetable proteins. Hams were wrapped with a cotton mesh and placed in molds[22] and then cooked to an internal temperature of 68 °C for 40 min. A thermometer was inserted at the piece core to measure the temperature, as specified by the NMX-F-123-S-1982[23]. The molds containing the hams were refrigerated for 24 h and then hams were washed with water at 28 °C. Finally, the hams were stuffed into plastic casings and stored at 4 °C until its use to feed the animals.

## Animals

A total of 20 commercial Yorkshire × Landrace cross pigs obtained from a commercial farm and with a weight of 16 to 18 kg were used. The pigs were serologically negative for CSF, PRRS and Aujeszky virus according to the ELISA technique[24-26].

## Ham from experimentally vaccinated and challenged pigs

Five treatment groups were sorted with four pigs each. Group A corresponded to pigs that were fed with processed hams from negative animals; Group B belonged to pigs that were fed with processed hams from commercial pig legs inoculated with the ALD strain (with a titre of $10^{4.0}$/ml); Group C corresponded to pigs fed with processed hams from pig legs infected with the virulent ALD strain (with a titre of $10^{2.5}$/ml); Group D was formed with pigs fed small pieces of the ham processed hams from pig legs vaccinated with the PAV-250 strain and challenged with the ALD strain (with a titre of $10^{1.1}$/ml); and Group E belonged to pigs fed with processed hams from pig legs vaccinated with two doses of the PAV-250 strain and challenged with the negative ALD strain. All the pigs of the four groups were fed with shredded ham and were given approximately 200 g for each pig in a single occasion.

## Clinical signs

All pigs in each group had their rectal temperature monitored and clinical signs evaluated on a daily basis, during a period of 7 to 21 d.

## Blood biometrics

Blood samples were taken from all animals. A baseline sample was drawn at the beginning of the experiment; the following samples were then taken every 5$^{th}$ day for a total of 5 (d 1, 5, 10, 15 and 20). At the end of the experiment (i.e. d 21), the pigs were slaughtered[27].

## Slaughter and pathology evaluation

Necropsies were performed on all animals that died during the experiment and also on the slaughtered pigs. Euthanizing was carried out by sedation with 3 mg/kg of azaperone and deep anaesthesia with 0.3 ml/kg of a mixture of xylazin and tiletamin with zolazepam, followed by exsanguination[28]. The study design was approved by the Ethics Committee for Animal Experiments of the Veterinary Medicine Faculty at UNAM (Universidad Nacional Autónoma de México); the experiment was conducted in compliance with the Mexican Regulations for Animal Care and Maintenance[29].

## Immunofluorescence test

The tonsils, ganglia and spleens of the experimental pigs were collected. The conjugate for CSF diagnosing was added to previously fixed tissue sections, which were incubated immediately for 30 min at 37 °C in a humid chamber. The lamellae were washed and mounted with glycerol/PBS 1/1 and observed under immunofluorescence microscopy[24-26].

### Viral isolation and titration

The virus was isolated in the PK-15 cell line from a suspension that included tissues from the lymph nodes, spleens and tonsils, as well from the bone marrow of the femur. Viral titration was performed using direct immunofluorescence testing[20-21].

### Statistical analysis

A factorial design with random data from the CSFv-infected groups was analysed using the SAS software by means of a +/- standard deviation (SD) or standard error above the mean (SEM) with an ANOVA.

## Results

### Preparation of the hams

The legs from the above-mentioned pig groups were selected and processed by the cooking method, in order to obtain the hams. Under these study conditions, CSFv was found after cooking (68 °C for 40 min) in hams from unvaccinated pigs, as the viral titer decreased.

## Viral isolation and titration

Virus isolation and titration revealed the following: CSFv was not isolated from Group A hams. For Group B commercial meat that had an inoculum of the ALD strain of the CSF virus at $10^{4.0}$/ml was used. The ham of Group C had a titre of $10^{2.5}$/ml; whereas the ham of Group D had a titre of $10^{1.1}$/ml. Finally, the meat belonging to Group E had a negative titre.

## Temperature and clinical signs

After feeding the pigs with ham pieces made from the legs of (1) PAV-250 vaccinated animals, (2) non-vaccinated but challenged pigs with the ALD reference strain and (3) pigs inoculated directly with the ALD strain, the findings in the infected pigs revealed a variety of clinical signs characteristic of CSFv (Table 1).

**Table 1**: Clinical signs: Expressed as percentages

| Groups (n=4 ) | Group A | Group B | Group C | Group D | Group E |
|---|---|---|---|---|---|
| Temperature 40 °C | 0 | 100 | 100 | 100 | 0 |
| Anorexia | 0 | 100 | 100 | 100 | 0 |
| Paralysis | 0 | 100 | 100 | 50 | 0 |
| Vomiting | 0 | 25 | 100 | 75 | 0 |
| Diarrhea | 0 | 25 | 100 | 75 | 0 |
| Tremor | 0 | 0 | 100 | 50 | 0 |
| Hirsute hair | 0 | 75 | 100 | 25 | 0 |
| Cyanosis | 0 | 100 | 100 | 50 | 0 |

## Hematic biometry

The blood values for Group C were markedly different from the ones of those pigs that were fed with free CSFv hams, as their red blood cell count was $10x^6$ μl. However, there were no statistical differences in terms of the percentages of cellular packet, haemoglobin (g/dl), monocytes and eosinophils ($10^3$ μl) with $P<0.05$. Table 2 shows the cell values that were affected after the ingestion of hams infected with CSFv in Groups B and C. Those animals showed leukopenia, which is characteristic of this disease and other highly significant values, such as decreased leucocytes, indicating that Group A behaved quite similarly to Group E. These findings indicate that the hams fed to the vaccinated and challenged animals had no viral particles capable of causing CSF infections.

## Necropsies

The animals were slaughtered for the evaluation of the macroscopic tissue lesions in the different experimental groups (Tables 2 and 3).

**Table 2**: Blood values observed in the experimental groups

| Groups (n=4) | Group A | Group B | Group C | Group D | Group E |
|---|---|---|---|---|---|
| Leucocytes x $10^3$ μl | Average: 17.31[a]<br>DE: 4.02<br>SEM: 1.42 | Average: 11.97[b]<br>DE: 2.67<br>SEM: 0.94 | Average: 10.77[b]<br>DE: 4.02<br>SEM: 1.42 | Average: 11.56[b]<br>DE: 3.02<br>SEM: 1.42 | Average: 16.88[a]<br>DE: 4.02<br>SEM: 1.42 |
| Lymphocytes x $10^3$ μl | Average: 3.68[a]<br>DE: 1.14<br>SEM: 0.43 | Average: 1.18[b]<br>DE: 0.22<br>SEM: 0.07 | Average: 2.68[b]<br>DE: 1.24<br>SEM: 0.43 | Average: 1.38[b]<br>DE: 1.24<br>SEM: 0.43 | Average: 3.38[a]<br>DE: 1.24<br>SEM: 0.43 |
| Segmented neutrophils x $10^3$ μl | Average: 7.50[a]<br>DE: 2.18<br>SEM: 0.77 | Average: 3.50[b]<br>DE: 2.18<br>SEM: 0.77 | Average: 4.60[b]<br>DE: 2.18<br>SEM: 0.77 | Average: 3.30[b]<br>DE: 2.18<br>SEM: 0.77 | Average: 7.80[a]<br>DE: 2.18<br>SEM: 0.77 |

[abc]Means with different superscript in the same row are different significantly ($P<0.05$).

**Table 3:** Lesions observed in the experimental groups

| Groups (n=4) | Group A | Group B | Group C | Group D | Group E |
|---|---|---|---|---|---|
| Haemorrhagic and oedematous lymphonodus | 0 | 100 | 100 | 25 | 0 |
| Kidneys and/or bladder with petechial haemorrhaging | 0 | 50 | 75 | 25 | 0 |
| Cyanotic skin | 0 | 0 | 75 | 50 | 0 |
| Infarcted spleen | 0 | 100 | 75 | 50 | 0 |
| Ileocecal valve and intestine with ulceration | 0 | 100 | 75 | 50 | 0 |
| Pulmonary lesions | 0 | 100 | 75 | 25 | 0 |

## CSFv identification by IFD in organs and tissues and viral isolation

The fluorescence assay performed in all groups was positive, except for Group A (i.e. negative control). Viral isolation was performed using a mix of lymph nodes, spleens and tonsils (named as the suspension), and bone marrow from each animal's femora. The found titres varied widely, ranging from $10^2$ to $10^4$ (Table 4).

**Table 4:** CSFv identification by direct immunofluorescence testing and viral isolation

| Groups (n= 4) | Group A | Group B | Group C | Group D | Group E |
|---|---|---|---|---|---|
| Organs and tissues positives by IFD, expressed in percentages | | | | | |
| Tonsils | 0 | 100 | 100 | 75 | 0 |
| Lymphonodus | 0 | 100 | 100 | 75 | 0 |
| Spleen | 0 | 75 | 50 | 50 | 0 |
| Viral isolation | | | | | |
| Mix of lymphonodus; spleens and tonsils suspension | Negative | Titre: $10^{4.3}$ | Titre: $10^{3.9}$ | Titre: $10^{2.2}$ | Negative |

# Discussion

The main result is that the PAV-250 vaccine reduced the infection titres in the vaccinated and infected animals, which means that cooking does not eliminates the virus.

The contribution of this study is that it was shown that the PAV-250 vaccine, in addition to protecting the animals, contributed to maintain low infection titers after the challenge. For several years, Mexico maintained a control and eradication campaign of the Classic Swine Fever, until the country was declared free of this disease in 2012[30], whereas the CSF was eradicated in the USA during the year of 1978[31]. However, the outbreak danger of this disease in high swine-density areas in Central America and south-eastern Mexico persists. It is important to keep in mind that pork meat plays a key role in food safety. Although Classic Swine Fever does not affect humans, previous studies show that it can be transmitted in poorly processed food products that may be used to feed pigs, which increases the risk of disease re-emergence[32]. Although many swine-raising operations are large-scale in modern production systems, there are also small-scale domestic or 'backyard' pig raising practices, where the animals are fed with scraps and waste. Therefore, these practices represent a high risk for large-scale production units, as well.

A study by Mebus *et al*[33] that utilized meat from animals inoculated with CSFv demonstrated the presence of the virus in different types of meats and sausages after preparation, revealing a high-risk scenario. The meat from those animals contained large amounts of the virus in certain products and by-products. The Italian and USA meat industries determined a period of 189 d for deactivating the CSFv virus in hams produced by the *Prosciutto di Parma* technique. In other dried or pickled products, the CSFv survived for 70 d in bone marrow and 90 d in fat and muscle; these results agreed with those from other studies[34-36]. However, the virus persisted for long periods in the lymph nodes, bone marrow and fat from the products used in the study. The test used to determine the presence of CSFv showed that inoculated animals were sensitive and substantial amounts of viral particles were detected. These results agree with the present research, since it was isolated the virus in the PK15 cell line, as well as in viral titres. In the study by Mebus *et al*[33], the inoculated pigs developed CSF during *in vivo* tests using Iberian ham. Of the three-studied viruses, the CSFv persisted for a longer period in the tested products.

This was proven by observation of classic clinical signs of CSF in the experimental pigs fed with those hams[35]. The analysis of haemograms showed the presence of leukopenia and normal counts, while those levels fell below 9,000 and reached a low of 3,000 (between d 4-7) in the CSF pigs. It is important to consider that healthy pigs under 5 wk of age normally have lower leucocyte counts. Upon disease onset, the animals showed a low white blood cell count, weakening of the immune system and multiple internal haemorrhages that occurred when the bacteria invade the animals[27]. The pigs' organs presented macroscopic lesions caused by the CSFv found in the experimental hams. Moreover, this was confirmed by direct immunofluorescence testing of the tissue sections obtained from the experimental pigs. These results showed that the cooking treatment reduced the viral titer by 2 logarithms in the legs inoculated directly with the ALD reference strain (Group B) and those from pigs infected with this same strain (Group C). Another way to prove the presence of this virus, involved viral isolation using a suspension prepared with a mixture of lymphatic ganglia, spleens and tonsils, and bone

marrow from the femora. The titres obtained using this method ranged from $10^2$ to $10^4$. Based on these tests and animal observation throughout the experiment, it was possible to prove that the virus was indeed present in the hams made from the legs of infected pigs.

As has been shown, the CSFv persists for different periods in the tissues of exposed pigs: 14 d in blood, 21 d in ganglia, and 94 d in intestinal 'button' ulcers. In addition, it has been detected in the spleen of approximately 1.2 % healthy animals from exposed groups sent to slaughter and in waste materials from restaurants, which contain meat and bone remains from sick pigs that were killed at the slaughterhouse. In ham, hotdogs and similar products, the virus can survive up to 85 d, whereas in the bone marrow of salted meat, it can persist for 73 d and 60 d in salami. Furthermore, it can also resist refrigeration and freezing for 5-10 yr. Feeding pigs with inadequately cooked scraps was the main factor responsible for the 18 and 22 % of outbreaks that occurred in the USA between 1972 and 1973, respectively[8]. On the other hand, the virus can survive for 25 d in bacon. It has been proven that feeding pigs with scraps is one of the main outbreak causes of CSF, because the virus survives for long periods in bone marrow, especially when cooking temperatures are too low for effectively inactivate any virus particles that may be present. Often, pigs infected with cholera are sent to the slaughterhouse and the meat enters the market, where it is consumed in restaurants. The food scraps that contain incompletely cooked bones are generally sold as food for swine, constituting another way in which the infection cycle can be perpetuated and cause an infection. CSFv also survives for 2 d in open corrals and from 2-to-4 d in manure. In fact, this virus survived for several weeks in experimentally infected manure; while in another experiment, the CSFv was detected along 1,600 m of an open sewage canal that ran from laboratories that produce vaccinations against CSF[12].

The vaccination strain PAV-250 produces good immunity in pigs and does not allow the spreading of the virus in the field among herds on the same farm. These data were confirmed by the experiment, since the pigs from Group E did not show any clinical signs or pathological lesions. Additionally, they had negative results on the DIF tests and a zero recovery of the virus from lymphoid tissues and bone marrow. In Mexico, the use of the vaccination strain PAV-250 during the Control and Eradication CSF Program, succeeded through the implementation of a vaccination strategy based on zones, which not only controlled the CSF but also achieved a total eradication of the disease[9,14,27,29].

The eradication of FPC in Mexico was carried out only with the use of vaccination, it was a very exceptional process and it is not mentioned in any other publication. Yes, Mexico is already free of CSFv was due in large part to the benefits of the PAV-250 vaccine, while in other Latin American countries continue to have this big problem, basically due to the use of several vaccine strains of FPC and continue to feed them with food waste[15,30,37].

The use of this vaccine for the process of obtaining ham, is not referred to in any other publication. Although Mexico is already free of CSFv, which is largely due to the benefits

of the PAV-250 vaccine, however, in other Latin American countries they still have this great problem, due to the use of several vaccine strains and to feed with squid.

## Conclusions and implications

The PAV-250 vaccine reduced the infection titers in the vaccinated and infected animals, which means that cooking does not eliminate the virus. Under these study conditions, it was found that CSFv resisted the cooking method at 68 °C for 40 min in hams from unvaccinated pigs, and that the virus was able to transmit the disease to healthy unvaccinated pigs, whereas the hams from the vaccinated animals did not transmit the virus. The eradication of FPC in Mexico was carried out only with the use of vaccination. The contribution of this study is that it was shown that the PAV-250 vaccine, in addition to protecting the animals, contributed to maintain low infection titres after the challenge.

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