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Survival of several porcine viruses in different Spanish dry-cured meat products

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The purpose of this study was to determine the effect of the Spanish dry curing process on the inactivation of viruses of main economic importance: African swine fever virus (ASFV), classical swine fever virus (CSFV), foot and mouth disease virus (FMDV) and swine vesicular disease virus (SVDV) in Serrano cured hams and Iberian cured hams, shoulders and loins. 31 to 35 Iberian, or white pigs were infected with the respective viruses, and slaughtered at the estimated peak of viremia. The meat products were processed using the commercial procedures currently used in Spain. Samples collected at slaughter and at predetermined intervals during processing were analysed for virus survival by *in-vitro* and *in-vivo* assays. The results demonstrated that ASFV, FMDV, CSFV and SVDV are inactivated during Spanish commercial curing process in the dry-cured products studied except in the case of the Serrano ham infected with SVDV, in which the curing time required for inactivation of the virus in lymph nodes exceeded the maximum commercial curing time. © 1997 Elsevier Science Ltd

INTRODUCTION

Regulatory agencies in countries need information to develop criteria for import and commercialization of dry-cured meat products. To set up these rules, it is necessary to obtain accurate information about the inactivation of the different viruses in the cured product, to assure that they will not pose a risk to their livestock.

At present, different studies have been carried out in order to evaluate the survival of different porcine viruses in uncured meat and mainly dried pepperoni or salami sausages, bacon and ham cured products (Cottral *et al.*, (1960); Savi *et al.*, (1962); McKercher *et al.*, (1974, 1980), Dhennin *et al.*, 1980). There has been an Italian–US study, in which the persistence of several porcine viruses was evaluated in hams produced by the 'Prosciutto di Parma' process (McKercher *et al.*, 1985, 1987).

In this work, we present the effect of the dry curing process on the inactivation of the most important swine viruses: African swine fever virus (ASFV), classical swine fever virus (CSFV), foot and mouth disease virus (FMDV) and swine vesicular disease virus (SVDV), in Spanish serrano cured hams and Iberian cured hams, shoulders and loins. The study was performed by applying commercial methods and industrial procedures currently in use in Spain for producing these types of dry cured meat products. Since a part of the study was conducted in the USA, it was necessary to freeze the meat during shipment while, in Spanish industrial processing, meat is not frozen before salting, drying and curing procedures. For that reason, this work also includes a study performed in Spain to evaluate the effect of freezing on the persistence of the virus in tissues.

MATERIALS AND METHODS

Viral inocula

The foot and mouth disease virus was serotype C, Dutch origin—lot10/89 passed five times in MVPK cell line. Its infectivity titer was $10^{8,9}$ $TCID_{50}$ ml^{-1}. The inoculum used in this study consisted of 1 ml of 1:10 dilution of the stock virus injected intravenously.

The African swine fever virus was the fifth buffy coat passage of INIA E-70, lot D-89. The inoculum consisted

of 1 ml of undiluted stock virus ($10^{6,5}$ $TCID_{50}$ ml^{-1}) injected intramuscularly.

The classical swine fever virus was the Ames strain in defibrinated blood, lot 86–07. The inoculum titer was $10^{5,3}$ $TCID_{50}$ ml^{-1}. One ml of a 1:100 dilution of this stock virus was injected intravenously.

The swine vesicular disease virus inoculum was the UKG 72 isolate that had a history of four swine passages and two IB-RS-2 cell passages. The viral titer was 4.5×107 pfu m^{-1}. Three ml of 1:3 dilution of this stock virus was injected intravenously.

Animal studies

Black and domestic white pigs weighing between 133–162 kg and 92–108 kg, respectively, and negative for antibodies against FMDV, ASFV, CSFV and SVDV, were used for the experiment. Four non-infected pigs were processed as controls. Separate groups were inoculated with each of the viruses and the number of swine inoculated in each group was for FMDV, 31 black pigs and 31 white pigs; for ASFV, 35 black pigs and 32 white pigs; for CSFV and SVDV, 32 black pigs and 32 white pigs. In order to obtain meat with high viral titers, the time of slaughter was selected based on knowledge of the disease process and body temperature. In this study, the FMD-infected pigs were slaughtered at 1 day post-inoculation (DPI), the ASF-infected pigs at 5 DPI, the CSF-infected white pigs at 5 DPI, and the CSF-infected black pigs at 4 DPI.

The study was conducted in three phases: collection, production and confirmation. The collection phase of the study was conducted in Spain. This phase included animal inoculations, slaughter, sample collection and freezing of products involved in this technical study (Iberian ham, shoulder and loin produced from the Spanish black pig and the Serrano ham produced from the domestic white pig). At that time, an evaluation was made of the effect of freezing treatment in tissue samples from three ASF-infected black pigs. The high security animal facility used was decontaminated between each of the virus inoculations.

The production and confirmation phases were performed in the United States, at Plum Island Animal Disease Center (PIADC). These technical phases included processing of hams, shoulders and loins, consisting of salting, post-salting long term drying and curing and all applicable remaining sample collection as well as all infectivity *in-vitro* and *in-vivo* assays on samples collected at various periods during the study.

All the procedures such as animal handling, slaughter, preparation and processing of the meat products, were done trying to simulate commercial conditions in Spain. At the time of slaughter, a heparinized blood sample was collected for infectivity assay. The carcasses were dehaired by placing in a 60°C scalding vat for 5 min followed by scraping. The carcasses were eviscerated and halved. Muscle, fat, bone marrow and lymph node were collected from each animal. At this time, tissue samples from three ASF-infected black pigs, AB02, AB04 and AB14, were obtained for evaluation of viral titers before freezing. The carcasses were hung at 4°C overnight. Cuts were stored in a −40°C freezer and held at this temperature until they arrived

Table 1. ASF virus titers in non-frozen and frozen samples collected from three black pigs after slaughter

	Non-frozen samples of pig No:			Frozen samples of pig No:		
Tissue	Ab–02	Ab–04	Ab–14	AB–02	Ab004	Ab–14
Bone marrow						
Right Ham	3.9	4.2	4.2	2.9	3.9	2.9
Left Ham	3.4	4.2	3.2	3.4	3.4	2.9
Right Shoulder	2.9	3.2	2.9	2.9	4.4	2.9
Left Shoulder	3.2	4.4	3.2	3.4	3.0	2.3
S.D.: 0.537; Median.: 3.575				S.D.: 0.528; Median: 3.192		
Muscle						
Right Ham	4.4	5.2	4.2	4.2	4.4	3.4
Left Ham	5.1	4.4	4.4	4.2	4.4	4.4
Right Shoulder	3.2	3.9	3.9	4.2	3.2	3.9
Left Shoulder	3.4	3.9	6.1	3.9	4.4	4.4
Right Loin	4.4	4.2	3.9	4.2	4.4	3.2
Left Loin	4.9	4.4	4.2	3.4	4.2	3.2
S.D.: 0.657; Median: 4.339				S.D.: 0.46; Median: 3.978		
Fat						
Right Ham	2.9	3.2	3.9	4.4	4.2	3.9
Left Ham	3.2	3.2	3.2	3.9	2.9	3.4
Right Shoulder	3.4	3.9	4.4	4.2	3.2	3.2
Left Shoulder	3.4	4.2	3.4	3.2	3.4	4.2
S.D.: 0.444; Median: 3.525				S.D.: 0.511; Median: 3.692		

ASFV titers are expressed as the inverse $\log_{10}$ per ml.

at PIADC. The frozen cuts for production of the Iberiam hams, shoulders and loins and the Serrano hams were kept in four separate environmental chambers for controlled thawing. The processing of the products was performed following previously approved protocols.

Collection and *in vitro* assay of tissue samples during processing

Tissue samples were collected, processed and assayed for infectivity as previously described (Mebus *et al* 1993*a*,*b*). Briefly, muscle, fat, popliteal lymph node and bone marrow samples were collected from hams; muscle, fat and bone marrow samples were collected from shoulders; muscle and usually a small amount of fat were collected from loins. Infectivity assay for FMDV samples was performed using lamb kidney cells by a plaque assay. If cytopathic effect (CPE) was not observed in 4–5 days, two additional passages were made. Samples for ASFV were cultured on porcine buffy coat cells. If hemadsorption was not observed in 2–5 days, two blind passages were made. Each sample for CSFV was inoculated into chambered slides and two flasks of PK-15 cells; if no immuno-fluorescence for CSFV was observed in the first passage, a second or a third passage was made and read by immunofluorescence after one day of incubation. Infectivity assays for SVDV were performed using IB-RS-2 cell line. If CPE was not observed in 2–3 days, two blind passages were performed and the cultures read over a period of seven days.

In-vivo assay

When three consecutive samples of a product were negative for a virus by *in-vitro* assay, the frozen supernatants of the last three consecutive negative samples (hams for example, muscle, fat, bone marrow and lymph node) were thawed and pooled for an *in-vivo* assay. Each supernatant was injected intradermally for FMDV and SVDV detection or intramuscularly for ASFV or CSFV detection into two 30 kg pigs. If the pigs became sick, virus isolation was attempted. If the pigs remained clinically normal for four weeks, they were bled and the serum was tested for antibody to the virus of concern.

Evaluation of the freezing treatment at slaughter

The evaluation of the effect of freezing treatment was carried out by means of a comparative study using tissue samples from three ASFV-infected back pigs obtained before and after freezing. Analysis of the non-frozen samples was performed in the following 24–48 hours.

Results

Freezing treatment at slaughter

ASF viral titers after analysis of the tissue samples from three infected blacks pigs (bone marrow from the right and the left hams and right and left shoulders; muscle from the right and the left hams, right and left shoulders and right and left loin; and fat from the right and the left hams and right and left shoulders), prior or post-freezing, are presented in Table 1. Stadistical analysis (Median and standard deviation (S.D.)) revealed non-differences after freezing, indicating this treatment did not affect the infectivity of the virus.

Survival of ASFV, FMDV, CSFV and SVDV in different Spanish dry-cured meat products

A summary of the mean viral infectivity titers in different tissues of the black and white pigs, at the time of slaughter, are presented in Table 2.

The mean ASFV titers in each tissue of the black and white pigs were highest in bone marrow (9.5) and lymph node (8.5); the mean FMDV titers were highest in blood (3.6) and lymph node (3.4); in the case of CSFV, the highest titers were obtained in bone marrow (5.2) and titers were somewhat lower in lymph node and blood (3.8). For SVDV the highest titer was found in lymph node (6,2).

Infected white and black pigs responded similarly to ASFV and CSFV. With FMDV, the mean titer in the blood of the black pigs (4.3) was higher than that in the white pigs (2.8) and the same occurred for lymph nodes of black pigs (4.1) and white pigs (2.6). In general black pigs responded somewhat better than the white pigs. In the case of SVDV, similar and moderate titers in black and white pigs were found in the tissues analysed except for lymph nodes; the mean SVDV infectivity titers in

Table 2. Mean infectivity titers in tissues of the black and white pigs at slaughter

Virus	Tissue				
	Blood	Lymph node	Bone marrow	Fat	Muscle
ASFV	7.8	8.5	9.5	5.4	6.6
FMDV	3.6	3.4	1.9	0.5	0.03
CSFV	3.8	3.8	5.2	0.9	1.0
SVDV	2.1	6.2	1.0	0.3	0.2

FMDV, CSFV, SVDV titers are expressed as the inverse $\log_{10}$ plaque formit units PFU ml^{-1}. ASFV titers are expressed as the inverse $\log_{10}$ per ml.

Table 3. Range of curing and processing times (in days) of the negative samples in the *in vitro* test and corroborated *in vitro*

Product	Curing time (days)	Days of processing after which samples were negative for			
		FMDV	ASFV	CSFV	SVDV
Iberian ham	365–730	168	140	252	560
Iberian shoulder	240–420	112	140	140	196
Iberian loin	90–130	42	112	126	42
Serrano ham	180–365	182	140	140	539

lymph node of the black pigs (6,7) was higher than that in the white ones (5,7).

ASFV was not detected in any of the products between 56 to 112 days, and the *in-vitro* results were confirmed by the *in-vivo* assays.

The persistence of FMDV was very low in muscle since the virus was not detected after 14 days of processing. However, the virus persisted up to 84 days in bone marrow. The *in-vivo* assays confirmed these results.

The *in-vitro* assays for CSFV showed negative results between 98 and 126 days in Iberian shoulders and loins and Serrano hams. However, the virus persisted longer in Iberian ham muscle and lymph nodes (last sample positive *in-vitro* on day 168). The *in-vivo* testing of the Iberian hams was not confirmed by an *in-vivo* assay up to 252 days.

The survival of SVDV was low in Iberian loins and shoulders, up to 14 to 84 days. However, the *in-vitro* testing showed that the virus persisted up to 470 days in Iberian and Serrano hams. The negative *in-vitro* results were confirmed *in-vivo* using a pool of the first three consecutive *in-vitro* negative samples.

A comparison of the range (days) of commercial curing and the days of processing after which the samples were negative in the *in-vitro* tests, corroborated by *in-vivo* assays, are presented in Table 3

Discussion

In this work, we have analysed the effect of the Spanish dry curing process on the inactivation of viruses of main economic importance: ASFV, CSFV, FMDV and SVDV in Serrano cured hams and Iberian cured hams, shoulders and loins.

The commercial methods used normally for production of these kinds of dry-cured meats were used. However, to conduct the experiments with regard to production and elaboration of these products in the USA, it was necessary to freeze the meat for shipment, as traditional procedures in Spain do not include freezing. It was necessary to assess the influence of this treatment in our study. The comparative study carried out between non-frozen and frozen samples of different tissues collected from three black ASF-infected pigs after slaughter demonstrated the freezing step did not affect the results in this study.

Animals inoculated with the viruses of concern showed high viral titers at the time of slaughter, representing the worst case that could be found in case of field infections. Moreover, in a real situation, these animals would not be processed for meat or cured products because they are supposed to be rejected after antemortem inspection.

The results clearly demonstrated that ASFV, FMDV, CSFV and SVDV are inactivated by commercial curing processes. A comparison between the commercial curing time and the processing times required for the inactivation of the different viruses in the different products analyzed are shown in Table 3 . There was only one case in which curing time required for inactivation exceeded the maximum commercial curing time. SVDV persisted in lymphoid tissue in the Serrano ham longer than the commercial time of curing.

The present study shows that the persistence of the viruses differs from one tissue to another and confirms, in general, the survival times of these viruses in other cured products evaluated in previous studies in which a salted and dried process was also used (McKercher *et al.*, 1985, 1987; Cottral *et al.*,1960; Savi *et al.*, 1962, 1964; Dhennin *et al.*, 1980). Thus, in the joint Italian and US studies on the survival of FMDV, ASFV, CSFV and SVDV in hams using the "Prosciutto di Parma" curing process, they reported the following inactivation times: 108 or 170 days for FMDV, 189 or 313 days for CSFV and 310 or 360 days for SVDV, depending on the Italian or US contribution, respectively. For ASFV, the survival time in the "Prosciuto di Parma" hams were longer, 300 or 399 days, than that obtained in this study, (140 days). These differences indicate, and corroborate our findings on, the need to conduct full studies on each curing process and for each product separately prior to importation into countries free of these viruses.

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