Determination of African swine fever virus viability in meat during long-term storage and sous-vide cooking using cell culture and real-time PCR combined with palladium compound pre-treatment methods

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Abstract

African swine fever virus is the causative agent of an acute and highly contagious disease affecting domestic and wild members of the family *Suidae*. The virus can be transmitted by direct contact among infected animals or via a contaminated environment or feed. Since the contaminated meat or products thereof have been characterised as the most probable vehicle in several outbreaks, the aim of the present study was to define viability of the virus in meat under conditions of freezing and chilling (−25 °C and 6 °C) and low temperature cooking (55 °C for 2.5 h and for 1 h). Two independent methods were employed; cell culture as a reference and real-time polymerase chain reaction combined with palladium compound (BB-$PdCl_2$ and $PdCl_2$COD) pre-treatment as an alternative method. Obtained results demonstrated a minimal decrease in the infectious virus titre during storage at −25 °C, and a remaining amount of viruses in meat stored at 6 °C for 14 months that can cause a disease after ingestion. The results obtained by both methods applied on the samples corresponded to each other. In contrast, results related to the virus' persistence in thermal-treated meat indicated much lower stability than previously thought; infectious viruses were not detected by infectivity assay after the treatment at 55 °C for 1 h. The observed difference of one order of magnitude of virus detected using palladium compound pre-treatment suggests presence of intact rather than infectious viruses. A better suitability of $PdCl_2$COD compared to BB-$PdCl_2$ pre-treatment was demonstrated.

Domestic pig, wild boar, pork meat, venison, persistence, infectious virus

African swine fever (ASF) is an acute and highly contagious disease affecting domestic and wild members of the family *Suidae*. The disease is characterised by high mortality (up to 100%) and the symptoms include fever, diarrhoea, bleeding, and generalised reddening of the skin. An enlargement of the spleen and bleeding into the lymph nodes can be observed post mortem. These symptoms are highly nonspecific, with similarities to classical swine fever and porcine dermatitis and nephropathy syndrome (Done et al. 2001). In contrast to classical swine fever which affects mainly young pigs, all age groups are equally susceptible to ASF (Gallardo et al. 2015). Suspected or confirmed ASF occurrence is subjected to mandatory reporting to the Regional Veterinary Administration followed by the introduction of necessary measures. Since the absence of a vaccine against the causative agent strongly limits the possibilities of controlling the spread of the disease, restriction measures involve immediate depopulation of the entire affected farm and limitations in the national and international trade of animals, meat, and products thereof. Thus, ASF poses a significant threat to worldwide pork production and has a major socio-economic impact on the involved countries and regions (Niederwerder 2021).

The causative agent of the disease, African swine fever virus (ASFV), is an enveloped virus whose genome consists of double-stranded DNA. The size of the genome depends on the individual isolates and varies between 170 and 193 kbp. This corresponds with the number of open reading frames (150 to 167) encoding proteins involved in virus replication

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and interaction with host cells. The virus is the only member of the family *Asfarviridae*, genus *Asfivirus* (https://talk.ictvonline.org/taxonomy/). Twenty-four genotypes have been described so far, but only two genotypes are found in Europe. Genotype I is endemic exclusively to Sardinia. Genotype II, which is currently causing an injurious disease situation in Europe, was first identified in Lusaka, Zambia and subsequently in Madagascar, Mozambique, Southern Tanzania and Mauritius. This genotype was located in Georgia in 2007, and began to gradually spread westward to other European countries (EFSA 2014; Gaudreault et al. 2020). In Europe, the epizootiology of ASFV shows differences from that observed in Sub-Saharan Africa where the virus is endemic. The virus is transmitted through direct contact between infected and healthy animals, in particular, by the natural migration of wild boars. Indirect transmission may occur by a contaminated environment. The main source of contamination includes ASFV-positive wild boar carcasses. In addition, irresponsible human behaviour plays a crucial role in virus transmission. The spread of the virus is often caused via contaminated meat and products thereof, fomites, the transport of game meat from the affected zone, and the feeding of domestic pigs with contaminated leftovers or feed (Bosch et al. 2017).

The extreme stability of ASFV in the environment has a major effect on the spread of the disease. The virus is quite resistant to high temperature and is stable at pH ranging between 4 and 10. The presence of a protein-rich environment such as blood, serum, and uncooked meat or products thereof increases its stability (Dee et al. 2018). To assess the risk of ASFV transmission, it is important to determine if viral presence has an infectious potential. Therefore, the main aim of the present study was to define viability of ASFV in meat during long-term storage and popular sous-vide cooking (low-temperature long-time cooking) by two independent methods. Culture-based methods are considered the gold standard for the determination of infectious viruses (Foddai and Grant 2020), however, they are time-consuming and require days or weeks to obtain results. Thus, real-time polymerase chain reaction (qPCR) combined with palladium (Pd) compound pre-treatment was used as a novel and alternative method to reveal ASFV infectivity.

Materials and Methods

Virus strain and sample preparation

African swine fever virus strain Ba71V obtained from the European Union Reference Laboratory for ASF (EURL-ASF, Madrid, Spain) was used in present study. The strain Ba71V was propagated on VERO cells and its concentration was determined by titration assay (see below). The prepared virus suspension was stored at −80 °C until further use.

Pork and venison (loin and leg) acquired locally were cut into pieces weighing 1 g (long-term storage and first sous-vide experiment) and 300 g (second sous-vide experiment). The meat samples were contaminated with 500 µl of Ba71V suspension (1.26×10^7 median tissue culture infective dose/ml; $TCID_{50}$/ml). Surface contamination of meat samples was performed in experiments 1, 2, and 3. Larger pieces of meat (experiment 4) were contaminated in the centre of the sample to imitate natural meat contamination. Subsequently, the samples were incubated at room temperature for 15 min and treated according to the experimental design:

1. long-term storage in the freezer at −25 °C for 30, 60, 90 days, 12 and 14 months
2. long-term storage in the refrigerator at 6 °C for 30, 60, 90 days, 12 and 14 months
3. first sous-vide experiment at 55 °C for 2.5 h
4. second sous-vide experiment at 55 °C for 1 h

Subsequently, 10 ml of Dulbecco's Modified Eagle Medium High Glucose (DMEM; Biosera, Nuaille, France) were added to each meat sample, agitated at room temperature at 587 *g* for 15 min and centrifuged at 5,000 *g* for 5 min. The supernatant was filtered through a 0.45 µm filter (Techno Plastic Products AG, Trasadingen, Switzerland) and concentrated using 30 kDa ultrafiltration columns (PierceTM Protein Concentrator, Thermofisher Scientific, Waltham, MA, USA) to a final volume of 500 µl. The obtained suspension was split into aliquots and used for determination of virus viability by infectivity assay and qPCR combined with Pd compounds pre-treatment methods. To ensure valid results, each thermal/time treatment of meat samples was performed in biological duplicates. Positive (time 0) and negative controls were employed in each experiment. Experiments with potentially infectious ASFV strain Ba71V were carried out under biosafety level-3 laboratory conditions.

ASFV infectivity assay

Infectivity assay was employed as a reference method. The assay was performed using VERO cells and supplemented with 10% (v/v) gamma-irradiated foetal bovine serum (FBS; HyClone Laboratories, Cramlington, UK). A confluent monolayer of cells (cultivated for a maximum of 24 h) was used. Tenfold dilutions up to 10^{-6} were prepared in DMEM and each dilution (100 µl) was used to inoculate five plate wells with target cells. After one hour of virus adsorption at 37 °C under 5% CO_2, 100 µl of DMEM were added to each well, and cells were microscopically examined for signs of cytopathic effect (CPE) after five days of incubation at 37 °C in an atmosphere of CO_2 (5%). The virus infectivity titre ($\log_{10}$ $TCID_{50}$) was calculated using the Spearman-Kärber method (Finney 1964).

Detection of infectious ASFV by qPCR combined with Pd compounds pre-treatment

Detection of viable ASFV by qPCR combined with Pd compounds pre-treatment was performed by a method described by Fraisse et al. (2018). Briefly, 95 µl of 100-fold diluted aliquot was mixed with 5 µl of 20 mM bis(benzonitrile) palladium(II) dichloride (BB-$PdCl_2$) or dichloro(1,5-cyclooctadiene)palladium(II) ($PdCl_2$COD; both Sigma Aldrich, Darmstadt, Germany) and incubated for 15 min at 4 °C. Simultaneously, 95 µl of each 500-fold diluted virus suspension without Pd compound pre-treatment was used to specify the relation between the infectivity assay (reference method), Pd pre-treatment and simple qPCR methods. An isolation of nucleic acid using QIAamp® MinElute® Virus Spin Kit (Qiagen, Hilden, Germany) followed according to the manufacturer's instructions. Isolated DNA was eluted in the total volume of 40 µl.

Detection and quantification of the ASFV genome was done by performing an optimised qPCR according to SOP EURL-ASF (https://asf-referencelab.info/asf/images/ficherosasf/PROTOCOLOS-EN/SOP-ASF-PCR-2_REV2021.pdf), with slight modifications as described previously (Krasna et al. 2022). The qPCR was run in a total volume of 20 l of reaction mix containing: 10 l of LightCycler 480 Probes Master (Roche Molecular Diagnostics, Mannheim, Germany), 10 pmol of King-F, King-R, IAC F and IAC R primers, 4 pmol of King-probe, 2 pmol of IAC probe, 0.2 U of Uracil DNA Glycosylase (Roche), 10^3 copies of internal amplification control DNA and 5 l of template DNA. As a positive control, ten-fold diluted (final concentrations from 1×10^6 to 1×10^1 copies/l) plasmid DNA was employed, serving also for quantitation of ASFV (genomic equivalents; GE). Amplification and fluorescence detection were carried out on a LightCycler 480 Instrument (Roche) under the following conditions: initial denaturation at 95 °C for 4 min and 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 10 s. The results were analysed by the "Fit point analysis" option of the LightCycler 480 Software release 1.5.0 (version 1.5.0.39).

Results

ASFV infectivity assay

The initial rinse of artificially-contaminated meat samples with DMEM led to a decrease in the amount of infectious viruses detected; from 1.26×10^7 $TCID_{50}$/ml of the virus suspension applied for artificial contamination to 5.62×10^6 $TCID_{50}$/ml. This number was used to determine the impact of long-term storage and sous-vide cooking on ASFV infectivity.

During the long-term storage of venison at −25 °C a high infectious dose remained. The virus concentration did not change after 30 days (5.62×10^6 $TCID_{50}$/ml). After 60 days, 90 days, and 6 months, 3.47×10^6 $TCID_{50}$/ml, 3.63×10^6 $TCID_{50}$/ml and 2.82×10^6 $TCID_{50}$/ml were determined, respectively. Prolonged storage of meat samples (12 and 14 moths) did not further affect the concentration of infectious ASFV (Fig. 1).

Long-term storage of venison at 6 °C initiated a reduction in infectious virus concentration by three orders of magnitude (3.98×10^3 $TCID_{50}$/ml) after 30 days, i.e. 99.9 %. Gradual decrease was observed after 60 and 90 days (2.51×10^3 and 6.31×10^2 $TCID_{50}$/ml). Infectious ASFV was detected after storage for 12 and 14 months (3.16×10^3 $TCID_{50}$/ml, both times).

Viable viruses were not detected using infectivity assay (limit of the detection 3.16×10^1 $TCID_{50}$/ml) after the heat treatment of 1 g venison pieces by sous-vide cooking in the first sous-vide experiment; 55 °C for 2.5 h. Heat treatment of 300 g meat samples at 55 °C for 1 h showed an identical result (second sous-vide experiment), i.e. no infectious viruses were detected.

Detection of infectious ASFV by qPCR combined with palladium compound pre-treatment

The concentration of 2.38×10^9 GE/ml of ASFV strain Ba71V was determined by simple qPCR reaction in virus suspension used for artificial contamination of meat samples. Once BB-$PdCl_2$ and $PdCl_2$COD pre-treatments were applied, the concentrations

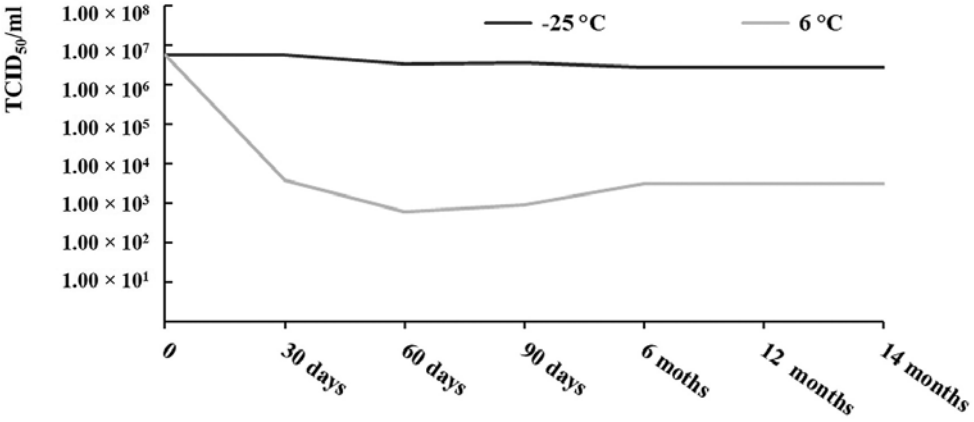

Fig. 1. Overview of African swine fever virus strain Ba71V viability during long-term storage of artificially contaminated meat samples at −25 °C and 6 °C

$TCID_{50}$ - median tissue culture infective dose

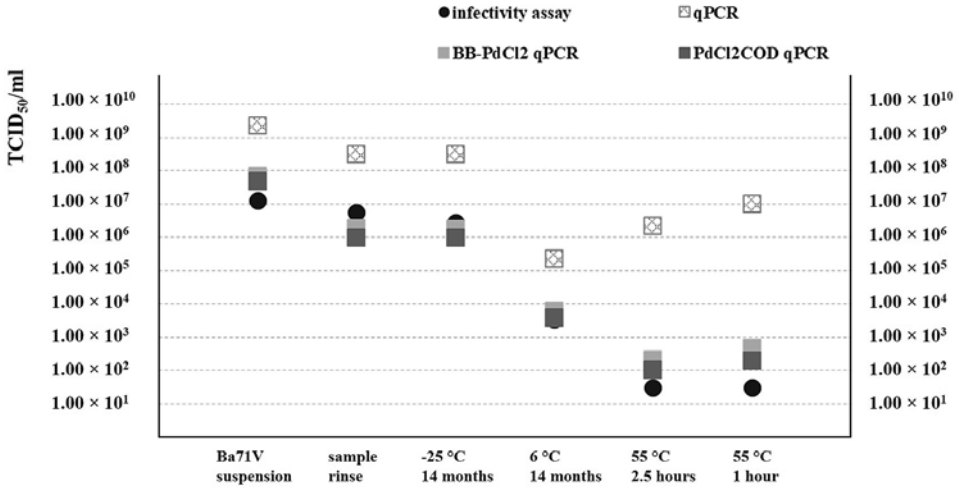

Fig. 2. Comparison of results of culture-based and real-time polymerase chain reaction (qPCR) combined with palladium compound pre-treatment methods applied on artificially contaminated meat samples treated with different conditions

$TCID_{50}$/ml - median tissue culture infective dose per ml virus suspension or rinse of artificially contaminated meat samples; GE/ml - genomic equivalents per ml of virus suspension or rinse of artificially contaminated meat samples; qPCR - real-time polymerase chain reaction without palladium compound pre-treatment used as control; BB-PdC_{l2} qPCR - real-time polymerase chain reaction with bis(benzonitrile)palladium(II) dichloride sample pre-treatment PdC_{l2}COD qPCR - real-time polymerase chain reaction with dichloro(1,5-cyclooctadiene)palladium(II) sample pre-treatment

of 7.21×10^7 GE/ml and 4.96×10^7 GE/ml were observed, respectively. An initial rinse of contaminated meat samples resulted in a reduction to 3.14×10^8 GE/ml, 1.94×10^6 GE/ml, and 1.01×10^6 GE/ml determined by simple qPCR, BB-$PdCl_2$, and $PdCl_2$COD pre-treatments, respectively (Fig. 2).

Long-term storage of venison at −25 °C did not affect the number of GE/ml detected. In agreement with the infectivity assay, long-term storage of venison at 6 °C resulted in gradual reduction in GE concentration, 6.19×10^3 GE/ml determined by BB-$PdCl_2$ pre-treatment and 3.98×10^3 GE/ml determined by $PdCl_2$COD pre-treatment after 14 months. Simple qPCR (control) revealed a decrease to 2.25×10^5 GE/ml (Fig. 2).

The simple qPCR and BB-$PdCl_2$ or $PdCl_2$COD pre-treatments recorded 2.20×10^6 GE/ml, 2.07×10^2 GE/ml, and 1.05×10^2 GE/ml after the heat treatment of 1 g venison pieces by sous-vide cooking (first sous-vide experiment; 55 °C for 2.5 h), respectively. Heat treatment of 300 g meat samples at 55 °C for 1 h (second sous-vide experiment) showed similar results; 1.04×10^7 GE/ml detected by simple qPCR in comparison to 4.77×10^2 GE/ml and 2.01×10^2 GE/ml determined using BB-$PdCl_2$ and $PdCl_2$COD pre-treatments (Fig. 2).

Discussion

The current ASF multiple epizooty (not limited to Europe) was preceded by a period of more than ten years of the virus spreading west from Georgia. Estonia, Lithuania, Latvia and Poland were the first EU countries affected in 2014. In June 2017, the disease was unexpectedly diagnosed in wild boars in the Czech Republic. Now, ASF is prevalent in 14 European countries, with the exception Italy (where the disease has a different origin). The current manifestation of ASF poses a serious threat to the pig industry in other countries. Historically, contaminated meat or products thereof have been characterised as the most probable transmission vehicle of the infectious agent in several ASF outbreaks. The potential of transmission depends in large part on the ability of viruses to persist in the environment, therefore questions arise of how long and under what conditions ASFV is able to survive (Mazur-Panasiuk et al. 2019). The present study was focused on the viability of ASFV in meat during long-term storage under common freezer and refrigerator conditions (−25 °C and 6 °C) and during the currently popular culinary technique of sous-vide cooking; also known as low-temperature long-time cooking. Due to the complicated nature of cell culture assay and determination of infectivity titre of field ASFV strains, the experiments were performed on venison and pork meat artificially contaminated by defined amounts of ASFV strain Ba71V to obtain standardised and valid results. As maximum ASFV titres in blood range from 10^6 to 10^9 $TCID_{50}$/ml during the acute phase of the disease (Mazur-Panasiuk et al. 2019), 500 ml of 1.26×10^7 $TCID_{50}$/ml were used for artificial meat contamination.

A common characteristic of viruses is their prolonged persistence in the environment at lower temperatures (e.g. freezer and refrigerator). The presence of a protein-rich environment such as blood, serum and uncooked meat or products thereof increases their stability. The maturing processes of the meat do not affect the infectivity of ASFV, and frozen, salted, dried or smoked meat may contain infectious particles of the virus (McKercher et al. 1987; Mebus et al. 1997; Bellini et al. 2016). However, several studies presenting data concerning ASFV persistence in raw and processed meat as well as products thereof provided variable results (Mazur-Panasiuk et al. 2019). In frozen raw meat and organs, viable ASFV can be detected over 103 to 118 days, but according to scientific opinion of European Food Safety Authority (EFSA 2014) the virus may remain infectious for up to 1,000 days. The present study demonstrated minimal decrease in infectious virus titre (from 5.62×10^6 $TCID_{50}$/ml to 2.82×10^6 $TCID_{50}$/ml) after 420 days (14 months) of storage at −25 °C (Fig. 1). African swine fever virus can survive

for up to seven months at 4–8 °C in carcass tissues, and meat stored at 4–8 °C provided the infectious viruses for periods lasting from 90 to 183 days. Spleen samples originating from infected animal stored in a refrigerator remained infectious for 240 days (Zani et al. 2020). Although the storage of meat at 6 °C revealed a reduction in virus titre of three orders of magnitude (from 5.62×10^6 $TCID_{50}$/ml to 3.98×10^3 $TCID_{50}$/ml) after 30 days, a relatively high infectious dose (3.16×10^3 $TCID_{50}$/ml) remained even after 420 days (14 moths; Fig. 1). By calculating the detected $TCID_{50}$ per 100 g of meat, the virus concentration would reach the values at which a pig can be infected after ingestion of such meat, as an infectious dose of 10^4 $TCID_{50}$ in solid feed is required (Niederwerder et al. 2021).

Heat-treated meat products are generally considered safe in terms of any viable pathogen presence, which has been experimentally demonstrated. Inactivation of ASFV has been proved at 56 °C after 70 min and at 60 °C after 20 min (Bellini et al. 2016). The sous-vide experiment was focused on venison samples weighing 1 g heat-treated at 55 °C for 2.5 h (the first sous-vide experiment). According to the obtained results, and due to a slower heat transfer into the centre of treated samples, subsequent trials dealt with larger pieces of meat (300 g; second sous-vide experiment). The present study indicates that ASFV stability in thermal-treated meat is much lower than previously thought since the infectious viruses were not detected even after the treatment at 55 °C for 1 h.

Standard protocols for the detection of infectious viruses and thus viral survival under different conditions still involve cell cultures. These methods require the use of susceptible cell lines in which the viruses propagate, and can be time-consuming (Foddai and Grant 2020). Molecular techniques represent an alternative method, which can be used for virus detection (particularly viral genomes). The disadvantage of these methods is that these cannot differentiate between infectious and non-infectious viruses and thus their use in persistence studies is limited. To address the limitations, several new approaches have been developed. Generally, these include sample pre-treatment steps prior to nucleic acid isolation. Compounds used in the pre-treatment step penetrate capsid-compromised non-infectious viruses and form covalent links to genomic DNA/RNA interfering with the binding of DNA polymerase to the DNA or reverse transcriptase to RNA, thereby blocking the amplification of target genome sequences. Therefore, when detecting virus genome, only unmodified DNA/RNA originating from intact virus capsid is amplified during qPCR (Cechova et al. 2022). As initial experiments demonstrated the effect of pre-treatment by Pd compounds on native and heat-treated suspensions of ASFV, qPCR combined with such pre-treatment was employed as a novel and alternative method to standard infectivity assay.

Analysis of the samples by simple qPCR revealed generally higher concentration of GE/ml in comparison to $TCID_{50}$/ml detected by infectivity assay as a reference method. However, the result obtained by pre-treatments by Pd compounds applied to samples originating from both long-term storage experiments (−25 °C and 6 °C) corresponded to those determined by infectivity assay. In this process, the differences in qPCR signal reduction observed after BB-$PdCl_2$ and $PdCl_2$COD pre-treatment indicated better concordance of infectivity assay with employing $PdCl_2$COD (Fig. 2). BB-$PdCl_2$ and $PdCl_2$COD pre-treatments of meat samples originating from sous-vide cooking experiments caused detection of 2.07×10^2 GE/ml and 1.05×10^2 GE/ml (first sous-vide experiment; 55 °C for 2.5 h) and 4.77×10^2 GE/ml and 2.01×10^2 GE/ml (second sous-vide experiment; 55 °C for 1 h), respectively. In contrast, the infectious viruses were not found out by infectivity assay. Due to the limits of the infectivity assay (especially limit of detection; 3.16×10^1 $TCID_{50}$/ml), it is not possible to determine the absence of virus in the sample. When this limit is considered, the difference of one order of magnitude of virus detected was observed between Pd pre-treatments and infectivity assay. Such effect could be caused by the ability of Pd compounds to bind unspecific substances (e.g. proteins, nucleic acids), which may be released during the thermal treatment of meat samples (Omondi et al. 2020). Better

suitability of $PdCl_2$COD pre-treatment was demonstrated in the sous-vide experiment.

Based on the presented results, qPCR combined with $PdCl_2$COD pre-treatment can represent an alternative to culture-based methods and may be used as an effective tool for risk management. However, further studies should be focused on evaluation of the method for the practical use that is, to detect infectious viruses in different kind of samples (e.g. environmental samples such as water or soil, animal feed).

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