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Virus inactivation by salt (NaCl) and phosphate supplemented salt in a 3D collagen matrix model for natural sausage casings

Tinka Wieringa-Jelsma [a], Joris J. Wijnker [b], Esther M. Zijlstra-Willems [a], Aldo Dekker [a], Norbert Stockhofe-Zurwieden [a], Riks Maas [a], Henk J. Wisselink [a,*]

[a] Central Veterinary Institute of Wageningen UR, P.O. Box 65, NL-8200 AB Lelystad, the Netherlands
[b] Van Hessen BV, P.O. Box 220 NL-2910 AE, Nieuwerkerk a/d IJssel, the Netherlands

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ABSTRACT

Due to possible presence and spread of contagious animal viruses via natural sausage casings the international trade in these food products is subject to veterinary and public health requirements. In order to manage these restrictions we determined the effect of casing preservation on four highly contagious viruses for livestock: foot-and-mouth-disease virus (FMDV), classical swine fever virus (CSFV), swine vesicular disease virus (SVDV) and African swine fever virus (ASFV). We used an *in vitro* 3D collagen matrix model in which cells, infected with the four different viruses were embedded in a bovine collagen type I gel matrix and treated with either saturated salt (NaCl) or phosphate supplemented saturated salt at four different temperatures (4, 12, 20 and 25 °C) during a period of 30 days. The results showed that all viruses were faster inactivated at higher temperatures, but that stability of the various viruses at 4 °C differed. Inactivation of FMDV in the 3D collagen matrix model showed a clear temperature and treatment effect on the reduction of FMDV titres. At 4 and 12 °C phosphate supplemented salt showed a very strong FMDV inactivation during the first hour of incubation. Salt (NaCl) only had a minor effect on FMDV inactivation. Phosphate supplemented salt treatment increased the effect temperature had on inactivation of CSFV. In contrast, the salt (NaCl) treatment only increased CSFV inactivation at the higher temperatures (20 °C and 25 °C). Also SVDV inactivation was increased by phosphate supplemented salt, but salt (NaCl) treatment only resulted in a significant decrease of SVDV titre at a few time points. The ASFV results showed that both salt (NaCl) and phosphate supplemented salt were capable to inactivate ASFV within 48 h. In contrast to the other viruses (FMDV, CSFV and SVDV), ASFV was the most stable virus even at higher temperatures. The results obtained in this *in vitro* model underline the efficacy of a combined treatment using phosphate supplemented salt and storage at 20 °C or higher for a period of 30 days. This treatment may therefore be useful in reducing the animal health risks posed by spread of contagious animal viruses by international trade of natural sausage casings.

1. Introduction

Various animal viral diseases are notifiable to the OIE, the World Organisation for Animal Health, because of their impact on animal welfare and economic damage. Due to possible presence and spread of infectious viruses via products of animal origin, international shipments are subject to veterinary and public health requirements when importing these goods into a specific region or country. These requirements are often defined in a product-specific Import Risk Assessment by individual countries, using the recommendations of the OIE's Terrestrial Animal Health Code (Office International des Epizooties, 2010) or available scientific literature as a point of reference.

Natural sausage casings are sourced, processed and used for sausage production in many different countries and therefore a truly global market exists for this commodity (Wijnker et al., 2008b). Therefore, specific veterinary and public health requirements are applicable to its international trade.

The natural casing consists primarily of the submucosal layer of the intestinal tract (Koolmees et al., 2004; Wijnker et al., 2008b), of which collagen is the most important component (Hiltner et al., 1985; Nishiumi and Sakata, 1999). After cleaning, the natural casings are preserved in salt (NaCl) allowing prolonged temperature-independent storage without bacterial spoilage or quality loss (International Scientific Working Group (ISWG), 2009). In addition, previous studies identified a specific mixture of phosphates as preservative added to salt which improves the usability of natural casings for sausage production (Bakker et al., 1999; Houben et al., 2005; Nakae et al., 2008).

In the past, available data on virus survival in natural casings were limited to a small number of studies, with little information on

* Corresponding author. Tel.: +31 320 238403; fax: +31 320 238961.
E-mail address: henk.wisselink@wur.nl (H.J. Wisselink).

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processing and storage parameters (McKercher et al., 1975, 1978). Only in studies done by Helwig and Keast (1966), Cottral (1969) and Böhm and Krebs (1974) parameters were specified for the effective inactivation of FMDV and CSFV in natural casings that have relevance to current operating procedures of the international casing industry. Recently, additional studies have been performed to determine the amount of surviving FMDV and CSFV using natural casings from experimentally infected animals. Results showed that the standard preservation technique using salt (NaCl) at 20 °C or using phosphate supplemented salt at 4 or 20 °C for a period of 30 days leads to sufficient removal of infectivity of FMDV and CSFV in the treated natural casings (Wijnker et al., 2007; 2008a). Since most of the studies only show data at the end of the inactivation period, more data are needed on inactivation kinetics. Furthermore, additional application of these preservative agents against other viruses and an improved understanding of the temperature effect on virus inactivation during storage would contribute significantly to optimising the risk analysis for these viruses.

To be able to expose infected cells to salt (NaCl) and phosphate supplemented salt in a natural way, we developed a model in which infected cells were embedded in collagen. In recent years, these three-dimensional (3D) cell culture models have been developed to better resemble the *in vivo* situation with regard to cell shape, extra-cellular environment and spatial orientation. Gel–matrix systems belong to the approaches commonly used to produce 3D cell cultures. Cells are embedded in a substrate, such as agarose or matrigel, which may contain a scaffolding of collagen to mimic the extracellular matrix (ECM). Gel–matrix systems have been successfully used for generating *in vitro* bio-artificial tissues from single cells of primary material or of cell lines (Andrei, 2006).

In this study, quantitative data are provided on virus inactivation of four highly contagious viruses for livestock (FMDV, CSFV, SVDV and ASFV) in a natural casings model using infected cells embedded in a bovine collagen type I gel matrix. These quantitative results can be used for replacing some of the current risk mitigating measures to control spread of these viruses via natural casings.

2. Materials and methods

2.1. Virus strains and cell-lines

In this study foot-and-mouth disease strain $O_{1Kaufbeuren}$ (Kanno et al., 2002), classical swine fever virus strain Paderborn 277 (Greiser-Wilke et al., 2000), swine vesicular disease virus strain UK72 (Seechurn et al., 1990) and African swine fever virus strain Malta 78 (Wilkinson et al., 1981) were used. All four virus strains are from disease outbreaks in the field; the strains have not been attenuated and have retained their full virulence capability.

BHK21 cells (ATCC CCL-10) were used for culturing FMDV, SK6 cells for CSFV (Kasza et al., 1972) and IBRS2 cells for SVDV (De Castro, 1964). For testing ASFV the COS cell-line (Hurtado et al., 2010) and porcine alveolar macrophages (PAM) derived from 3-week-old pigs from a high health status pig herd were used (Carrascosa et al., 1982).

2.2. Salt (NaCl) and phosphate supplemented salt

The salt solution (pH 6.1) consisted of saturated salt (NaCl, Mw 58). The phosphate supplemented salt solution (pH 10.3) consisted of 86.5% salt (NaCl, Mw 58), 2.8% sodium phosphate (TSP Na_3PO_4, Mw 164) and 10.7% sodium hydrogen phosphate (DSP, Na_2HPO_4, Mw 142). Saturated solutions of both salts were made in demineralised water.

2.3. Embedding of virus-infected cells in collagen

Prior to embedding in the collagen matrix, cells were infected with the appropriate virus stocks. BHK21 cells for culturing FMDV were grown in Dulbecco's modified essential medium (DMEM) supplemented with glutaMAX® (Gibco-BRL/LifeTechnologies), 10% fetal bovine serum (FBS), non-essential amino acids (Gibco-BRL/LifeTechnologies) and 1% of an antibiotic stock consisting of penicillin (100 U/mL) and streptomycin (100 mg/mL). The cell monolayer was infected at a multiplicity of infection (MOI) of 0.005 in 5 mL medium. After incubation for 1 h at 37 °C in an atmosphere with 5% CO_2, medium was added to a final end-volume of 50 mL, and incubation of the flasks was continued for 17 h.

SK6 cells for culturing CSFV were grown in Earle's minimal essential medium (EMEM), 5% FBS and 1% of an antibiotic stock consisting of penicillin (100 U/mL) and streptomycin (100 mg/mL). The cell monolayer was infected with 50 mL of a 10^{-2} dilution of a CSFV stock containing 6.5 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) and incubated for 4 days at 37 °C in an atmosphere with 5% CO_2.

IBRS2 cells for culturing SVDV were grown in K1000 medium containing 5% FBS, 1% of an antibiotic stock consisting of penicillin (100 U/mL) and streptomycin (100 mg/mL) and 1% fungizone. The cell monolayer was infected at a MOI of 1 in 5 mL medium. After incubation for 1 h at 37 °C in an atmosphere with 5% CO_2, medium was added to a final end-volume of 50 mL, and incubation of the flasks was continued for 21 h.

COS cells for culturing AFSV were grown in DMEM, 10% FBS, 1% of an antibiotic stock consisting of penicillin (100 U/mL) and streptomycin (100 mg/mL) and 1% fungizone. The cell monolayer was infected with 5 mL of a 10^{-1} dilution of Malta 78 stock virus containing 6.32 $\log_{10}TCID_{50}$ and incubated for 1 h at 37 °C in an atmosphere with 5% CO_2. After 1 h, medium was added to a final end-volume of 50 mL, and incubation of the flasks was continued for 4 days.

Infected cells were harvested, counted (Coulter Counter model Z2; Beckman Coulter Nederland B.V., Woerden, The Netherlands) and embedded in the Bovine collagen type I suspension (Gibco-BRL/LifeTechnologies), according to instructions of the manufacturer. In short, the collagen (5 mg/mL), 10× medium and 1 N NaOH were placed on ice. The necessary volume of collagen at a concentration of 3 mg/mL was calculated by the final concentration of collagen times the total volume divided by the initial concentration of collagen. The necessary volume of 10× medium was calculated by dividing the total volume by 10 and the volume of 1 N NaOH was determined by multiplying the total volume of collagen needed with 0.025. The collagen was slowly pipetted into a tube on ice and the medium and NaOH was gently added and mixed well. The resulting mixture achieved a pH of 7–7.5, confirmed by pink coloration of the mixture which disappeared after mixing. Finally the infected cell-suspension was added at a volume calculated by the total volume minus the collagen, medium and NaOH volumes.

The collagen suspension containing infected cells was pipetted into the wells (0.5 mL/well) of a 24-wells plate and incubated for 30 min at 37 °C until a firm gel was formed. The collagen gels in the wells were covered with 1 mL of specific medium containing 2.5% HEPES or salt (NaCl) or phosphate supplemented salt. Each well of a 24-wells plate contained 2.10^6 infected cells per well embedded in collagen (0.5 mL collagen suspension/well). The remains of the embedded infected cell-suspensions were regarded as day 0, and were, after harvesting by collagenase, aliquoted in appropriate storage-vials and stored at −70 °C upon titration. The 24-wells plates were incubated at four different temperatures (4, 12, 20 and 25 °C) and during different days which was for FMDV: 0, 1, 2, 3, 4, 5, 6, 7, 15, 21 and 30 days; for CSFV: 0, 7, 15, 21 and 30 days; for SVDV: 0, 2, 4, 7, 15, 21 and 30 days and for ASFV: 0, 2, 4, 7, 15, 21 and 30 days. All incubations were under standard cell culture conditions with respect to CO_2 and relative humidity levels. To control for dehydration, once or twice per week the level of medium in the wells was visually inspected and if necessary additional medium was added. All tests were performed in duplicate.

2.4. Collagen harvesting

For the isolation of infected cells, the collagen wells were treated with a collagenase solution to liquefy the collagen and harvest the cell suspension. Briefly, the collagenase solution was prepared at a concentration of 1000×Units/mL (~100 μg/mL) in phosphate-buffered saline (PBS) and pre-warmed at 37 °C.

The medium or salt mixtures overlays were aspirated from the collagen wells and after a rinse with PBS the collagen wells were incubated with 0.5 mL of pre-warmed collagenase per well. The collagen wells were incubated at 37 °C until cells were floating; this process was monitored under a microscope.

Once liquefied, 1 mL medium containing serum was added to each well and cell-suspensions were collected and transferred to a suitable storage container. The collected cell suspensions were stored at −80 °C until analysis by virus titration.

2.5. Virus titration

The FMDV endpoint titration was performed by mixing tenfold dilutions of the FMDV samples with BHK21 cells in 96-wells microtitre culture plates and incubating the plates for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation the growth medium was discarded and the plates were dipped in a citric acid solution to kill FMDV, and the monolayers were stained with amido-black (0.1% amido-black in 1 M acetic acid, 0.09 M sodium acetate, 10% glycerol). The read-out of the plates was performed macroscopically; coloured wells were scored as negative and non-coloured wells (cpe) were scored as positive.

CSFV samples were mixed with SK6 cells and incubated to confluence in 4 days in 24-wells microtitre culture plates at 37 °C in a humidified atmosphere with 5% CO_2, and then examined for the presence of virus by the immunoperoxidase-monolayer-assay (IPMA) using CSFV specific monoclonal antibodies (Wensvoort et al., 1986). The read-out of the plates was performed macroscopically; coloured wells were scored as positive.

The SVDV plaque titration was performed by incubation of 200 μL of 10 fold dilutions of the SVDV samples on IBRS-2 monolayers grown in a 6-well microtitre plate. After 1 h incubation at 37 °C in a humidified atmosphere containing 5% CO_2 the wells were covered with 2.5 mL medium containing 1% methylcellulose. The plates were incubated another 3 days. After incubation the medium was discarded and the plates were washed with water, and monolayers were stained with amido-black. Plaques were counted macroscopically.

For ASFV the IPMA procedure was quite similar to the CSFV IPMA (Wensvoort et al., 1986), making use of pig anti-ASFV hyper immune serum (HIS) and anti-swine HRPO conjugate. Detection was performed on PAM monolayers in 24-wells plates and read-out of the plates was performed microscopically scoring coloured PAM cells as positive.

2.6. Statistical analysis

For each virus a linear regression analysis was performed. The input data for regression analysis did not include the data reaching the detection limit. An exception was made for data reaching the detection limit immediately after day 0. The calculated negative reciprocal of the slope of the trend line was the amount of days of incubation required to achieve a decimal reduction (*D*-value) for each of the samples at 4, 12, 20 and 25 °C.

To determine statistically significant differences between the salt treatments versus control treatment at each time point a two-way analysis of variance (ANOVA, Bonferroni) was performed on virus titres using Graph Pad Prism (GraphPad Software, Inc., San Diego, California). A *P*-value of less than or equal to 0.05 was considered statistically significant and less or equal to 0.001 highly significant.

3. Results

3.1. Foot-and-mouth disease virus

The influence of treatment temperature and two different salt preservation methods on virus titres was determined over 30 days in a 3D collagen matrix model. For FMDV the influence of treatment temperature was clearly seen with the non-treated FMDV samples with increasing inactivation rates at increasing temperatures. The detection limit of 1.8 $\log_{10}$ $TCID_{50}$/mL was reached after 21, 7, 3 and 2 days of incubation at 4, 12, 20 and 25 °C respectively and a reduction of 3.2 $\log_{10}$ $TCID_{50}$ (Fig. 1a).

Incubation with salt (NaCl) at the different treatment temperatures showed almost the same curves as for the non-treated FMDV samples. The only significant ($P<0.05$) differences between the control and salt (NaCl) treated samples were at 4 °C on days 6, 7 and 15, with at the latter date higher titres with salt (NaCl) than in the control samples. At 12 °C only after 24 h, the virus reduction was significant higher ($P<0.05$) compared to the control treatment (Fig. 1a).

In contrast to the salt (NaCl) and non-treated samples it was found that the phosphate supplemented salt treatment showed biphasic inactivation curves, with initially rapid virus inactivation of about 2.5 $\log_{10}$ $TCID_{50}$ within 24 h of incubation at all temperatures, followed by a period in which FMDV titres remained constant at the same plateau (Fig. 1a). Reduction of FMDV titres by phosphate supplemented salt treatment was significantly higher ($P<0.001$) compared to the control treatment during the first 7, 3, 2 and 1 days of incubation at 4, 12, 20 and 25 °C respectively. At storage temperatures of 20 and 25 °C, FMDV titres were higher in phosphate supplemented salt samples compared to the non treated and salt treated samples for five and four days respectively, although these differences were not significant. Because of the biphasic curves no linear regression analysis was performed.

3.2. Classical swine fever virus

Similar to the FMDV results, the influence of treatment temperature on CSFV titres was also observed with the non-treated CSFV samples with increasing inactivation rates at increasing temperatures. However, the detection limit of 1.4 $\log_{10}$ $TCID_{50}$/mL was only reached on day 21 at 25 °C after a reduction of 5.8 $\log_{10}$ $TCID_{50}$, indicating that the survival times for non-treated CSFV at different temperatures were much longer than for non-treated FMDV samples (Fig. 1b).

At temperatures 4 and 12 °C no significant additional inactivation effect of the salt (NaCl) treatment over the control treatment was observed. The results of the salt (NaCl) treatment were comparable with the non-treated samples, as also indicated by the *D*-values (Table 1). However at 20 °C and 25 °C treatment with salt (NaCl) showed significant ($P<0.001$) more virus reduction (Fig. 1b).

Highly significant ($P<0.001$) virus inactivation by phosphate supplemented salt was observed at all temperatures for the entire period of incubation (30 days), except on days 21 and 30 at 25 °C when the non-treated samples had reached the detection limit (Fig. 1b). As indicated in Table 1 the phosphate supplemented salt shows the lowest values for the *D*-values, confirming this inactivation method as most effective for inactivating classical swine fever virus.

3.3. Swine vesicular disease virus

SVDV inactivation was observed in control and the two salt treated samples at increasing temperatures in the SVDV 3D collagen model (Fig. 1c). However inactivation below the detection limit of 1.4 $\log_{10}$

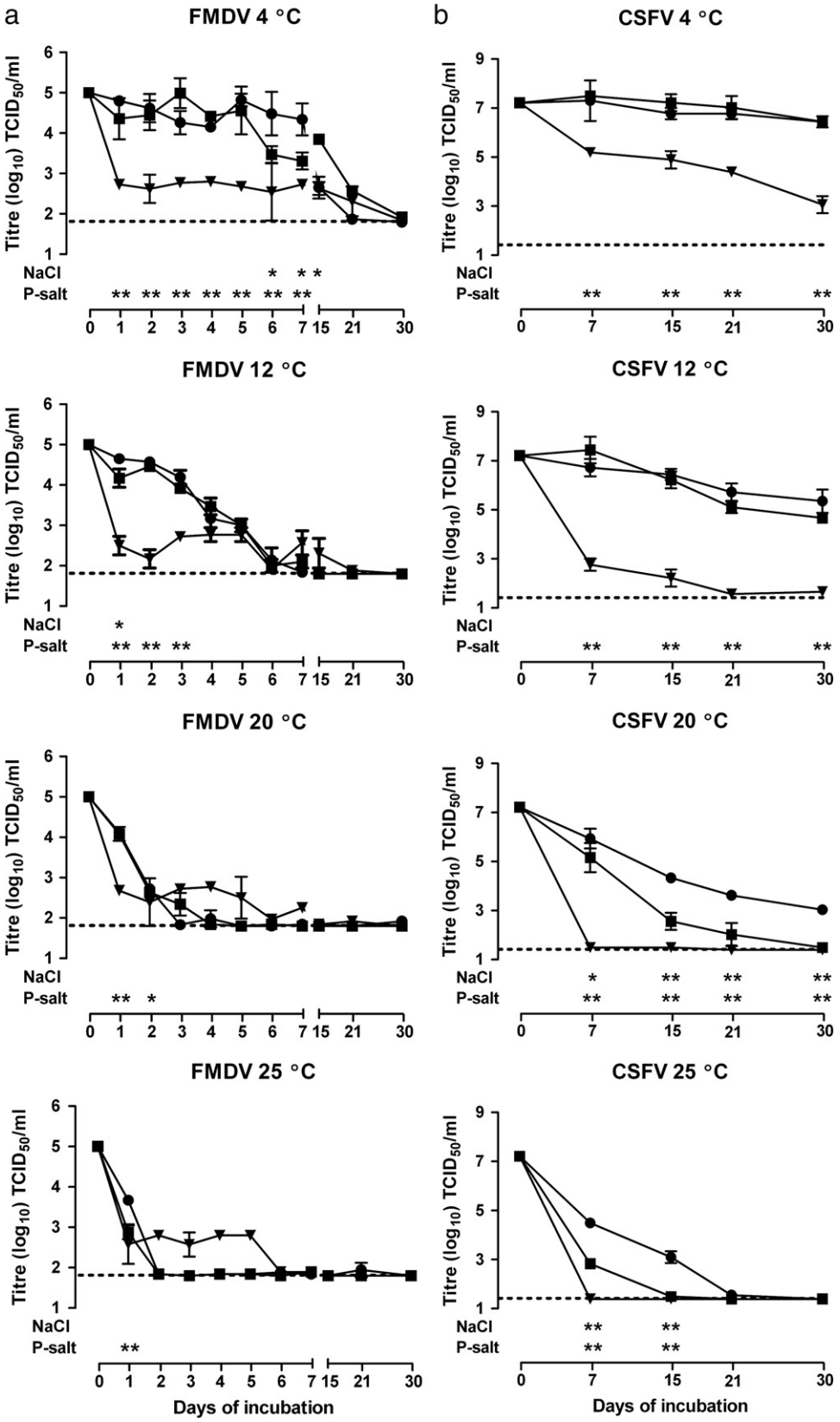

Fig. 1. Mean virus titres and standard deviations in $\log_{10}$ $TCID_{50}$/ml (50% tissue culture infectious dose) or in $\log_{10}$ pfu/mL (plaque forming units) in virus infected cells, embedded in bovine collagen type I after no treatment (●), treatment with NaCl (■) and treatment with phosphate supplemented salt (▼) at different time points and temperatures. Differences between the treatment with NaCl or phosphate supplemented salt (P-salt) versus control treatment at each time point were significant when $P<0.05$ (*) and highly significant when $P<0.001$ (**). The detection limit of virus titrations is represented by dotted lines and is for foot-and-mouth disease virus 1.8 $TCID_{50}$/mL (Fig. 1a), classical swine fever virus 1.4 $TCID_{50}$/mL (Fig. 1b), swine vesicular disease virus 1.4 pfu/mL (Fig. 1c) and African swine fever virus 1.4 $TCID_{50}$/mL (Fig. 1d).

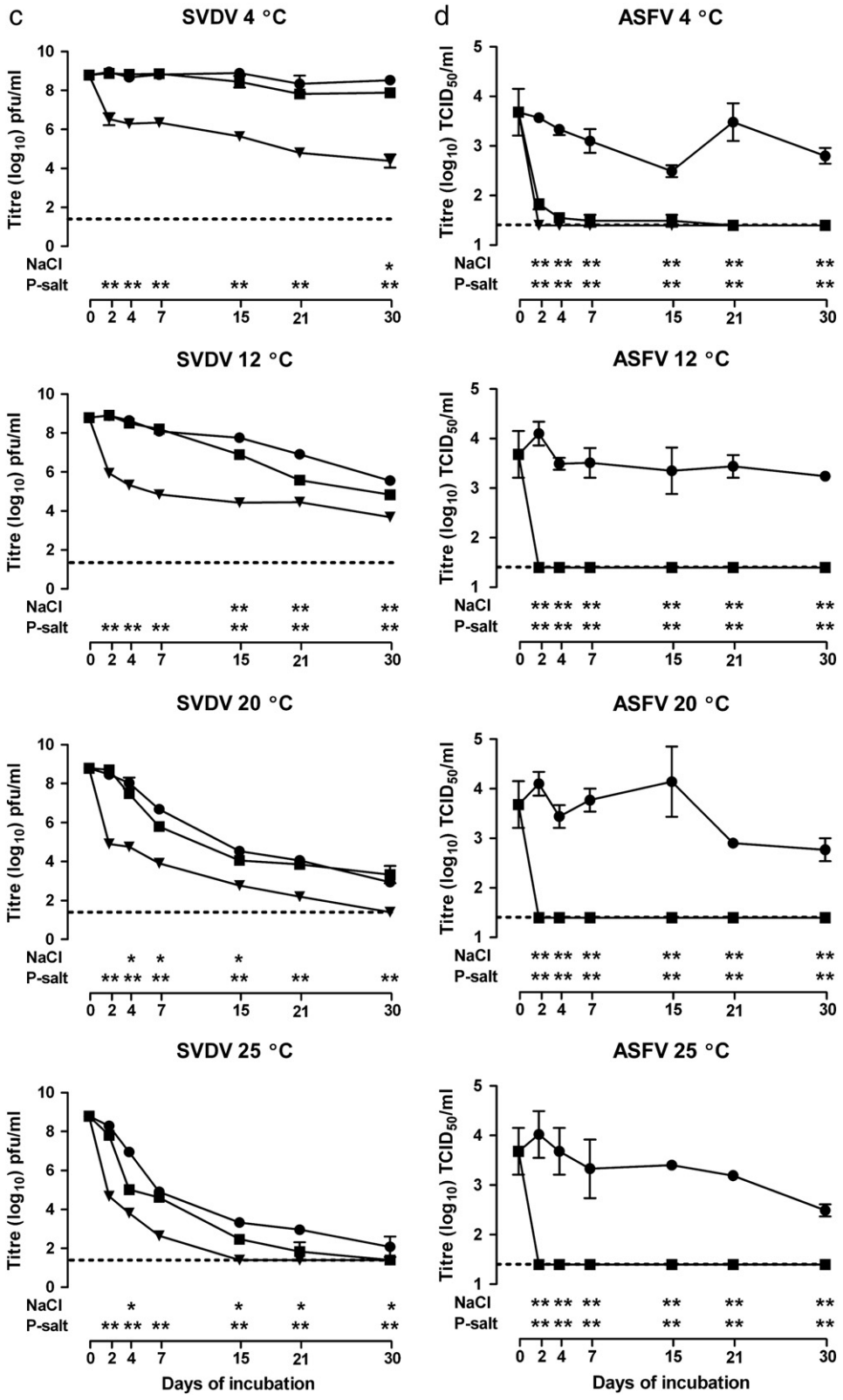

Fig. 1 (*continued*).

pfu /mL and a 7.4 $\log_{10}$ pfu reduction was not reached on the final day at the highest temperature for the non-treated SVDV samples. Treatment with salt (NaCl) leads at all temperatures to a significant ($P<0.05$) higher SVDV reduction compared to the non-treated samples at most time points. Inactivation of SVDV was most effective by phosphate supplemented salt. Highly significant ($P<0.001$) virus inactivation by phosphate supplemented salt was observed at all temperatures for the entire period of incubation. The detection limit was only reached after 15 days of incubation at 25 °C.

Table 1
D-values of four viruses embedded in a 3D collagen matrix after 30 days of treatment by various inactivation methods.

Virus	Temperature	*D*-value (days/R^2) of various treatments[a]		
		Untreated	Salt (NaCl)	P-salt[b]
FMDV[c]	4 °C	NA[d]	NA	NA
	12 °C	NA	NA	NA
	20 °C	NA	NA	NA
	25 °C	NA	NA	NA
CSFV[e]	4 °C	35/0.88	36/0.67	8/0.92
	12 °C	16/0.97	10/0.90	6/0.66
	20 °C	7/0.96	5/0.90	3/0.72
	25 °C	4/0.97	3/0.89	*1*/NA[f]
SVDV[g]	4 °C	76/0.45	26/0.87	9/0.75
	12 °C	9/0.97	7/0.98	9/0.58
	20 °C	5/0.95	5/0.86	4/0.69
	25 °C	4/0.87	3/0.86	1/0.82
AFSV[h]	4 °C	44/0.34	10/0.37	*1*/NA
	12 °C	54/0.53	*1*/NA	*1*/NA
	20 °C	29/0.50	*1*/NA	*1*/NA
	25 °C	25/0.84	*1*/NA	*1*/NA

[a] *D*-value is the time required in days to reduce the viral population by a factor of 10^1. R^2 is the coefficient of correlation of the regression analysis for calculating the *D*-value.
[b] P-salt is phosphate supplemented salt.
[c] Foot-and-mouth disease virus.
[d] Not applicable because *D*-values could not be determined.
[e] Classical swine fever virus.
[f] Input data for regression analysis did not include the ones reaching the detection limit. An exception was made for data reaching the detection limit immediately after day 0. These data are in Italic.
[g] Swine vesicular disease virus.
[h] African swine fever virus.

The *D*-values (Table 1) at 4 °C are the lowest for the phosphate supplemented salt treatment, whereas the *D*-values at 12, 20 and 25 °C for the different treatments are comparable.

3.4. ASFV

Both salt (NaCl) and phosphate supplemented salt treatments inactivated ASFV significantly ($P<0.001$) within 48 h at all temperatures (Fig. 1d). At 4 °C, low titres were still observed after salt (NaCl) treatment up to day 15, while in contrast, the use of phosphate supplemented salt resulted in titres reaching the detection limit of 1.4 $\log_{10}$ $TCID_{50}$/mL already on day 2 at all tested temperatures, which means an AFSV titre reduction of at least 2.2 $\log_{10}$ $TCID_{50}$/mL.

4. Discussion

Earlier it was shown that infectivity of natural casings derived from animals infected either with FMDV or CSFV could be removed effectively by storage in salt (NaCl) or phosphate supplemented salt at room temperature during 30 days, whereas casings at 4 °C contained infectivity (Wijnker et al. 2007; 2008a; unpublished data). Here we confirmed these results in an *in vitro* 3D collagen matrix model and found that these treatments were not only effective for FMDV and CSFV but also for SVDV and ASFV. Moreover, using this model we were able to study inactivation of multiple viruses by different treatments in a time and temperature dependent manner and if desired, the model can be easily adapted to other contagious viruses. Use of this model is attractive in comparison with an *in vivo* model for determining infectivity of casings, since it leads to a reduction of the number of animals used for experimental infections, which is attractive for both ethical and economical reasons.

Studies in a 2D environment for the efficacy of salt treatments at different temperatures have a limited prediction power for the *in vivo* situation due to the fact that environment of the virus differs drastically from the physiologically reality. Virus in cell monolayers is fast and easy accessible for treatments and tissue matrix components which could act as barrier are lacking. To overcome these limitations we have used collagen type 1 in a 3D matrix model to embed virus infected cells. The choice for collagen may be justified by the actual structure of the natural casing. It consists mainly of the submucosa layer of the bovine, ovine and pig intestine and collagen type I is in this layer the major component (Hiltner et al., 1985; Nishiumi and Sakata, 1999; Wijnker et al., 2008b). The collagen matrix ensures a 3D environment and mimics in this way also a natural casing. The experiments were performed with 0.5 mL collagen in wells with a diameter of 15.6 mm providing a theoretical thickness of the collagen gel of 2.62 mm. In comparison, cleaned natural casings are less thick with an average thickness of 0.11 mm for sheep, of 0.32 mm for pigs and of 1.15 mm for cattle (Bartenschlager-Blässing, 1979; Koolmees and Houben, 1997; Wijnker et al., 2008b). Based on these facts, the salt solutions have to penetrate at least to the same depth in the collagen gel as in the natural casing to be effective for inactivation of the virus.

To be representative for the *in vivo* situation in natural casings, preferable virus titres in the 3D collagen matrix models should be in the same order of magnitude. For FMDV, titres were determined in casings of pigs, sheep and cattle and appeared to be maximal 3 $\log_{10}$ PFU/mL (Wijnker et al., 2007; unpublished data) which is considerably lower compared to FMDV titres obtained in our 3D model (5.0 $\log_{10}$ $TCID_{50}$/mL). Since for CSFV, SVDV and ASFV no data are available about titres in natural casings we searched as an alternative for data in literature to virus titres in the intestine or faeces. For CSFV it was described that titres could vary between 2.9 and 3.8 $\log_{10}$ $TCID_{50}$/mL in ileum, duodenum and colon of pigs (Kamolsiriprichaiporn et al., 1992) and for SVDV a mean titre was found of 3.4 $\log_{10}$ PFU/gram in Peyer's Patches of pigs, experimentally infected by SVDV in a natural way (Dekker et al., 1995). Although these data are not necessarily a good reflection of titres in casings, titres in the CSFV 3D model (7.2 $\log_{10}$ $TCID_{50}$/mL) and in the SVDV 3D collagen model (8.8 $\log_{10}$ pfu/mL) were considerable higher. For ASFV, faecal excretion after experimental infection is described. However no quantitative data are available making a comparison with the 3D collagen matrix data impossible (Greig and Plowright, 1970).

The results of the 3D collagen matrix model on the inactivation of FMDV showed that there was a clear temperature and treatment effect on the reduction of FMDV titres. The results from this *in vitro* model seemed to be in line with *in vivo* studies on the inactivation of FMDV in porcine, ovine and bovine natural sausage casings since inactivation was also dependent on temperature and treatment (Cottral, 1969; Wijnker et al., 2007). We found in this study that the temperature effect on inactivation of FMDV was greater than the salt treatments effect at increasing storage temperatures. At lower temperatures (4 °C and 12 °C) a significant additional effect in reduction of virus titres ($P<0.001$) in the phosphate salt treatment groups could be observed whereas at higher temperatures (20 °C and 25 °C) no clear effect of either treatment groups could be demonstrated apart from the temperature effect. Taken together, these results underline that temperature is most important for inactivation of FMDV.

The phosphate supplemented salt treatment of FMDV showed biphasic inactivation curves, with initially rapid virus inactivation of about 2.5 $\log_{10}$ $TCID_{50}$ within 24 h of incubation at all temperatures, followed by an inactivation at the same speed as the untreated samples during the follow-up period of 30 days. Biphasic inactivation curves for MFDV are also seen in other studies (Alexandersen et al., 2003; Kamolsiripichaiporn et al., 2007). Especially in the presence of organic material (e.g. hay or straw) residual virus may be remarkably resistant. A possible explanation for the biphasic inactivation in our study might be that a part of the FMDV virus is included in phosphate salt crystals in the collagen during the incubation process leading to differences in the inactivation rate.

The results of the CSFV 3D *in vitro* model showed that the phosphate supplemented salt treatment was immediately effective in reducing the virus at all temperatures. Similar to the FMDV model, the results of the CSFV *in vitro* model were in agreement with *in vivo* obtained results concerning inactivation of CSFV in porcine natural sausage casings, thus proofing the suitability of the *in vitro* 3D collagen model (Depner et al., 1998; Wijnker et al., 2008a).

Testing SVDV with this collagen model showed again that the phosphate supplemented salt was the most effective and quickest method to inactivate SVDV at all temperatures. The ASFV results showed that both salt (NaCl) and phosphate supplemented salt were capable to inactivate ASFV within 48 h. Literature is scarce on the efficacy of salt treatment on survival of SVDV and ASFV in natural casings. In older literature, remaining SVDV infectivity was reported in natural casings of pigs experimentally infected for at least 200 days when stored at 4 °C in a salt (NaCl) solution (McKercher et al., 1975) and in untreated processed natural casings 780 days post infection (McKercher et al., 1978). These authors also found natural casings of pigs to contain ASFV (97 days p.i.), FMDV (250 days p.i.) and CSFV (147 days p.i.) (McKercher et al., 1978; 1980). However, no supporting data for these prolonged survival periods were available as neither reference to the original studies on casings nor information on the processing and storage conditions was provided, making a comparison between these results and our 3D collagen model impossible.

It can be concluded that the *in vitro* 3D collagen matrix model is a valuable model for studying the efficacy of different treatments on inactivation of viruses. Using this model it was found that the temperature dependent inactivation was enhanced by the phosphate salt treatment. Our data confirm the efficacy of a combined treatment using phosphate supplemented salt and storage at 20 °C or higher for a period of 30 days. The quantified results from this study can be used in evaluating different risk mitigating measures to control the animal health risks posed by spread of contagious viruses by international trade of natural sausage casings.

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