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*Original article*

# A method for the infectivity discrimination of enveloped DNA viruses using palladium compounds pre-treatment followed by real-time PCR

**M. Krzyzankova[1], M. Krasna[1], J. Prodelalova[2], P. Vasickova[1]**

[1] Food and Environmental Virology, Department of Microbiology and Antimicrobial Resistance, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
[2] Molecular Epidemiology of Viral Infections, Department of Infectious Diseases and Preventive Medicine, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic

## Abstract

Cultivation-based assays represent the gold standard for the assessment of virus infectivity; however, they are time-consuming and not suitable for every virus type. Pre-treatment with platinum (Pt) compounds followed by real-time PCR has been shown to discriminate between infectious and non-infectious RNA viruses. This study examined the effect of Pt and palladium (Pd) compounds on enveloped DNA viruses, paying attention to two significant pathogens of livestock – bovine herpesvirus-1 (BoHV-1) and African swine fever virus (ASFV). Native or heat-treated BoHV-1 suspension was incubated with the spectrum of Pt/Pd compounds. Bis(benzonitrile)palladium(II) dichloride (BB-$PdCl_2$) and dichloro(1,5-cyclooctadiene) palladium(II) ($PdCl_2$-COD) produced the highest differences found between native and heat-treated viruses. Optimized pre-treatment conditions (1 mM of Pd compound, 15 min, 4°C) were applied on both virus genera and the heat inactivation profiles were assessed. A significant decrease in the detected quantity of BoHV-1 DNA and ASFV DNA after heat-treatment (60°C and 95°C) and consequent incubation with Pd compounds was observed. BB-$PdCl_2$ and $PdCl_2$-COD could help to distinguish between infectious and non-infectious enveloped DNA viruses such as BoHV-1 or ASFV.

**Keywords:** African swine fever virus, bovine herpesvirus-1, palladium compounds, platinum compounds, molecular methods, viability PCR

Correspondence to: M. Krzyzankova, e-mail: miroslava.krzyzankova@vri.cz

## Introduction

The standard methods for the detection of infectious viruses involve cell cultures (Foddai and Grant 2020). However, virus culture can be time-consuming; the time required for detection varies between 4 and 30 days, depending on the virus (Leland and Ginocchio 2007). Furthermore, detection is problematic in the case of those viruses which cannot be grown in a conventional cell culture. The detection via cell culture of virus pathogens should be confirmed with other immunological or molecular methods (Hamza et al. 2011).

Real-time PCR (qPCR) assay, established in many areas of pathogen diagnostics, is a robust, sensitive and reliable method. Unfortunately, it is not able to distinguish the infectious virus from a damaged virus particle or nucleic acid, which leads to the overestimation of real infectivity. To address the limitations of PCR-based methods, several new approaches have been recently examined. One possibility is the use of nucleic acid binding compounds prior to nucleic acid isolation. These reagents interact only with nucleic acid which is not protected by an intact capsid (Leifels et al. 2020). Following the virus nucleic acid isolation, only nucleic acid originating from intact virus particles is replicated in qPCR.

Propidium monoazide (PMA) and ethidium monoazide (EMA) are favored for viability PCR tests. The disadvantage associated with their use is the need for photoactivation in the staining protocol, and the need to work in a darkroom. As an alternative to PMA and EMA, the properties of platinum (Pt) and palladium (Pd) compounds were studied in non-enveloped (Fraisse et al. 2018, Randazzo et al. 2018, Canh et al. 2019) and in enveloped RNA viruses (Puente et al. 2020, Cuevas-Ferrando et al. 2022). Among the mentioned studies, Pt compounds such as platinum tetrachloride ($PtCl_4$) (Fraisse et al. 2018, Puente et al. 2020, Chen et al. 2020, Cuevas-Ferrando et al. 2022) and cisplatin (CDDP) (Canh et al. 2019, 2021a) demonstrated their potential to discriminate between infectious and non-infectious noroviruses, hepatitis A virus, Aichi virus and coronaviruses. This approach has been also tested in real samples including stool (Fraisse et al. 2018), porcine serum (Puente et al. 2020), water (Canh et al. 2019, 2021a) and fomites (Cuevas-Ferrando et al. 2022). As for Pd compounds, their ability to discriminate between live and dead bacteria in milk has been described (Soejima and Iwatsuki 2016).

Most of these studies have been performed on RNA viruses. However, many important virus pathogens causing severe economic losses in livestock farming belong to enveloped DNA viruses, e.g. Bovine herpesvirus-1 (BoHV-1) and African swine fever virus (ASFV).

This research studied the ability of selected Pt and Pd compounds to determine infectious DNA virus particles using qPCR. Compounds causing the largest difference in quantity of genomic copies (GC) between native and heat-treated viruses were selected and the pre-treatment procedure was improved. Since work with ASFV requires biosafety level-3 laboratory conditions, the main part of the experiments was carried out on BoHV-1. Heat-inactivation profiles of BoHV-1 and ASFV were assessed and compared to infectivity assay. The practical significance of the need for the infectivity determination lies in the possibility of fast and more accurate detection of these pathogens in real samples (e.g. cadavers or in the environment) and especially in determining the true risk for their transmission and spread of a potential outbreak.

## Materials and Methods

### Virus strains, cell lines and infectivity assay

BoHV-1 strain HB185 (CAPM V-402) was provided from the Collection of Animal Pathogenic Microorganisms (Veterinary Research Institute, Brno, Czech Republic). The median tissue culture infective dose/ml ($TCID_{50}$/ml) of BoHV-1 stock suspension was $10^8$ $TCID_{50}$/ml. Stock virus suspension was prepared by propagation of HB185 in susceptible MDBK cells (epithelial cell line derived from bovine kidney, *Bos taurus*), cultivated in Dulbecco's Modified Eagle Medium High Glucose (DMEM, Biosera, Nuaille, France) supplemented with gamma-irradiated fetal bovine serum (3% (v/v), FBS, HyClone Laboratories, Cramlington, UK).

Stocks of ASFV strain Ba71V were obtained from the European Union Reference Laboratory for African swine fever (EURL-ASF). The concentration of ASFV stock solution was $10^6$ $TCID_{50}$/ml. ASFV Ba71V strain was propagated on VERO cells, cultivated in DMEM supplemented with 10% (v/v) FBS.

For the Pt/Pd pre-treatment procedure, stock solution of BoHV-1 was diluted $10^2$ – to $10^4$ – fold in a dilution solution (DS) (PBS, pH 7.2, supplemented with 50 µg/ml bovine serum albumin; (BSA; Thermofisher Scientific, Waltham, MA, USA), and a stock solution of ASFV was diluted $10^1$ – to $10^3$ – fold in DS.

For the determination of the infectious titer, a confluent monolayer of cells (MDBK or VERO) was inoculated with virus suspension (BoHV-1 or ASFV). After one hour of virus adsorption, DMEM with FBS (2%) was added and infected cells were cultivated at 37°C under 5% $CO_2$. An extensive cytopathic effect was observed in the cell culture at four (BoHV-1) or six

Table 1. Overview of examined platinum (Pt) and palladium (Pd) compounds with the tested concentrations.

| Abbreviation | Name | Tested concentration |
|---|---|---|
| CisDEP | dichloro(ethylenediamine) platinum (II) | 1000 µM |
| CDDP | cis-diammineplatinum(II) dichloride | 1000 µM |
| $Pt(PPh_3)_4$ | tetrakis(triphenylphosphine)platinum(0) | 1000 µM |
| $H_2PtCl_6$ | chloroplatinic acid hexahydrate | 1000 µM |
| $PtCl_4$ | platinum (IV) chloride | 2.5 mM, 1000 µM, 100 µM, 10 µM |
| $PdCl_2COD$ | dichloro (1,5-cyclooctadiene) palladium(II) | 2.5 mM, 1000 µM, 100 µM, 10 µM |
| BB-$PdCl_2$ | bis(benzonitrile) palladium(II) dichloride | 2.5 mM, 1000 µM, 100 µM, 10 µM |
| transPd | trans-diamminedichloropalladium(II) | 1000 µM |
| $Pd(OAc)_2$ | palladium(II)acetate | 1000 µM |

(ASFV) days post infection. The virus infectivity titer ($log_{10}$ $TCID_{50}$) was calculated using the Spearman--Kärber method (Finney 1964).

### Tested Pt/Pd compounds

The overview of tested Pt and Pd compounds, with associated abbreviations, is shown in Table 1. Pt compounds CisDEP, $Pt(PPh_3)_4$, $PtCl_4$, CDDP, Pd compounds BB-$PdCl_2$, $PdCl_2COD$, $Pd(OAc)_2$ (Sigma-Aldrich, St.Louis, MO, USA) and transPd (Alfa Aesar, Haverhill, MA, USA) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). $H_2PtCl_6$ (Sigma-Aldrich) was dissolved in ultrapure water (Top-Bio, Vestec, Czech Republic). All substances were aliquoted and stored at -20°C for later use. Prior to use, stock solutions were dissolved in ultrapure water.

### Determination of appropriate Pt/Pd compound

A spectrum of Pt/Pd compounds was first tested with BoHV-1 suspension. Stock virus suspension ($10^8$ $TCID_{50}$/ml of BoHV-1) was diluted 100-fold in DS and aliquots were heat-treated (5 min at 95°C, 5 min on ice). 5 µl of the given 20 mM Pt/Pd compound was then added to 95 µl of virus suspension, mixed and incubated for 30 min at 4°C. Each experiment was performed in sets of three using the following controls: virus suspension without heat-treatment and without Pt/Pd compound (--) served as a positive control, heat-treated suspension without Pt/Pd compound (+-) demonstrated the impact of the temperature on the virus detection and suspension without heat-treatment and with Pt/Pd compound (-+) indicated the possible impact of these compounds on native viruses.

### Improvement of the method

To enhance the difference between native and heat-treated virus suspension after Pd compound pre-treatment, the method parameters, including the use of detergents, heating conditions and incubation time, were optimized.

The effect of detergents on the pre-treatment procedure was investigated on BoHV-1. The working suspension ($10^5$ BoHV-1 virus particles/100 µl DS) was heat-treated for 5 min at 95°C, supplemented with 0.5% Triton X-100 or 0.1% Sodium deoxycholate (SD), pre-treated with 1 mM BB-$PdCl_2$ or 1 mM $PdCl_2$-COD, and incubated for 30 min at 4°C. The experiment contained appropriate controls (infectious particles without Pd compound and without detergent; infectious particles without Pd compound with detergent, and heat-treated particles without Pd compound with detergent).

The heat-treatments of diluted suspension and of concentrated suspension ($10^8$ $TCID_{50}$/ml BoHV-1; stock solution) were then compared. The suspension was 100-fold diluted, aliquoted into 95 µl samples and incubated for 5 min at 95°C (heat-treated virus) in a dry bath (Dry Block Heating Thermostat, Biosan, Riga, Latvia). After incubation, samples were immediately placed on ice for 5 min. The concentrated virus suspension was incubated for 5 min at 95°C, and was then placed on ice for 5 min, 100-fold diluted in DS and aliquoted. Native, concentrated virus suspension (maintained at 4°C), diluted analogically in DS, served as a control. Afterward, samples were either pre-treated with 1 mM BB-$PdCl_2$ or left untreated.

Finally, the two incubation times for Pd compounds, 15 min and 30 min at 4°C were compared.

### Effects of Pt/Pd compound pre-treatment on purified DNA

The effect of Pt/Pd compounds $PtCl_4$, BB-$PdCl_2$ and $PdCl_2$-COD was investigated using the purified BoHV-1 DNA. 5 µl of Pt/Pd compound was added to bring the final concentrations to between 10 µM and 2.5 mM. This was applied in a tube containing 95 µl of BoHV-1 DNA (~$5*10^3$ GC/µl); the suspension was

mixed and incubated for 30 min at 4°C. Each experiment included a positive control: a purified nucleic acid sample with no added Pt/Pd compound.

### Heat inactivation profile of BoHV-1 and ASFV

For studying the heat inactivation profile of Pd compounds BB-$PdCl_2$ and $PdCl_2$-COD, BoHV-1 suspension at $10^{6.3}$ $TCID_{50}$/ml and ASFV suspension at $10^{6.6}$ $TCID_{50}$/ml were used. These were incubated at 4°C, 37°C, 50°C, 60°C and 95°C for 10 min and 60 min. The 60 min incubation at 95°C was not performed for ASFV. An aliquot of the undiluted virus suspension was used to determine its infectivity on cell culture. Each sample was then diluted $10^3$-fold and split into three aliquots. One aliquot was analyzed with control qPCR (qPCR without addition of Pd compound); two additional aliquots were pre-treated using 1 mM BB-$PdCl_2$ and 1 mM $PdCl_2$COD respectively, with subsequent qPCR (BB-$PdCl_2$-qPCR; $PdCl_2$COD-qPCR). This stage of the evaluation was performed in biological duplicates.

### DNA isolation

The virus nucleic acid was isolated from 100 µl viral stocks (in the case of BoHV-1 10-fold diluted in DS). Analogously, after each pre-treatment procedure, extraction of virus nucleic acids was performed from 100 µl of virus suspension. The DNA was purified using QIAamp® MinElute® Virus Spin Kit (Qiagen, Hilden, Germany) and eluted in 40 µl of PCR water (Top-Bio).

### Quantification of pre-treated and native virus suspension

Oligonucleotides and the probe previously described (Chandranaik et al. 2013) were used for the detection of BoHV-1 DNA. qPCR reaction included internal amplification control (IAC), to monitor false-negative results (Vojkovska et al. 2015). The total volume of 20 µl of reaction mixture contained 10 µl LightCycler 480 Probes Master Mix (Roche, Basel, Switzerland), 7 pmol of the IAC F and IAC R primers, 7 pmol of BoHV-1 F and BoHV-1 R primers, 2 pmol of BoHV-1 probe, 1 pmol of IAC specific probe, 0.1 U of Uracil DNA Glycosylase (UNG, Roche), $5*10^2$ copies of IAC plasmid DNA and 5 µl of isolated DNA.

Detection and quantification of ASFV DNA was performed as described previously (King et al. 2003) and the qPCR reaction additionally included IAC (Vojkovska et al. 2015). The total volume of 20 µl of reaction mixture contained: 10 µl Light-Cycler 480 Probes Master Mix (Roche), 10 pmol of the IAC F and IAC R primers, 10 pmol of ASFV F and ASFV R primers, 2 pmol of ASFV probe, 1 pmol of IAC probe, 0.1 U of UNG, $5*10^2$ copies of IAC plasmid DNA and 5 µl of isolated DNA.

Each sample was analyzed in duplicate. For the purpose of quantification, each qPCR run contained standard plasmid DNA in a decimal dilution ranging in concentration from $10^1$ to $10^6$ GC/µl of BoHV-1 or ASFV. The reaction was performed using a LightCycler 480 instrument (Roche) under the following conditions: Initial denaturation at 95°C for 7 min, followed by 45 cycles at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 10 seconds. The analysis of results (quantification) was carried out using the "Fit point analysis" option of the LightCycler 480 Software release 1.5.1 (version 1.5.1.62).

### Statistical analysis

The experiments with Pt/Pd compound pre-treatment and optimization experiments were performed in triplicate. The heat inactivation profile was performed in biological duplicates for all temperatures and incubation times. Statistical significance was assessed with the Student t-test, and the level of the significance was determined at $p<0.05$ (*) and 0.01 (**).

## Results

### Determination of optimal Pt/Pd compound

The greatest differences between heat-treated and native (non-heat-treated) viruses were observed for the BoHV-1 virus suspension incubated with 1 mM Pd compounds $PdCl_2$COD (0.539 $\log_{10}$; $p<0.01$), BB-$PdCl_2$ (0.611 $\log_{10}$; $p<0.01$) and $Pd(OAc)_2$ (0.468 $\log_{10}$; $p<0.01$) (Fig. 1). A very slight, yet significant, effect was recorded also for the treatment with 1 mM $PtCl_4$ (0.145 $\log_{10}$; $p<0.05$) and 1 mM cisDEP (0.07 $\log_{10}$; $p<0.01$). Other Pt and Pd compounds did not show any significant effect between native and heat-treated viruses. The effect of $PtCl_4$ was the highest among the Pt compounds, and thus it was used in further experiments. The effect of 1 mM cisDEP was lower than 0.1 $\log_{10}$, and thus it was excluded from further experiments. $Pd(OAc)_2$ had higher reactivity to the native virus suspension (-2 $\log_{10}$ decrease, results not shown), and was excluded from further experiments. For further experiments Pd-$Cl_2$COD, BB-$PdCl_2$ and $PtCl_4$ were selected.

### Improvement of Pt/Pd pre-treatment

Both tested detergents (0.5% Triton X-100, 0.1% SD) enhanced the reduction of BoHV-1 GC in heat-treated virus suspension; however, a similar and even higher

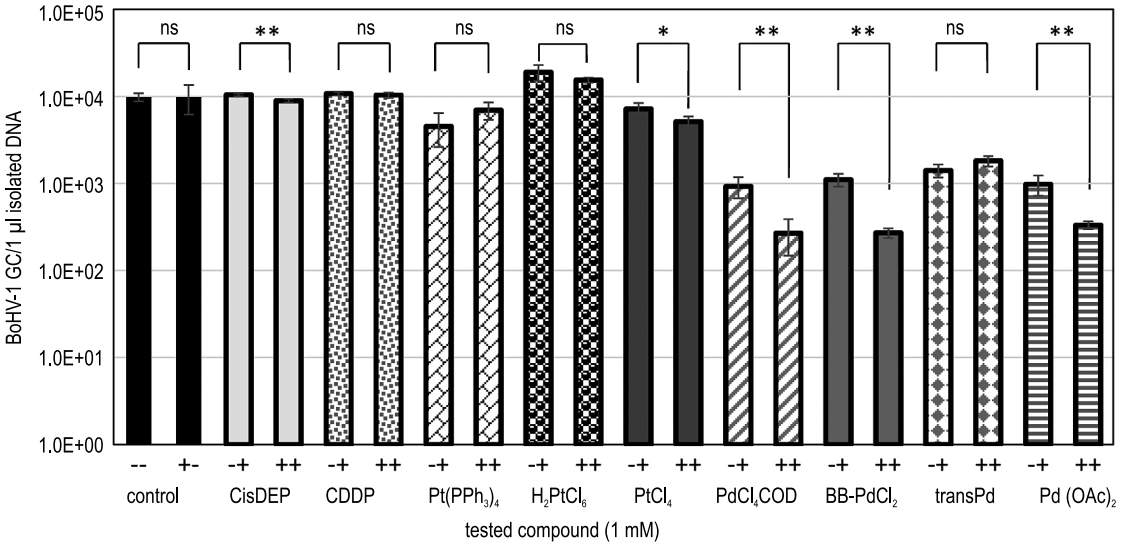


Fig. 1. Screening of platinum (Pt) and palladium (Pd) compounds on bovine herpesvirus 1 (BoHV-1) suspension, log scale. BoHV-1 suspensions with native virus (--) or heat-inactivated (95°C, 5 min) virus (+-) were treated with 1 mM Pt/Pd compound (native virus suspension treated with Pt/Pd compound, labelled as -+, heat-inactivated virus suspension, treated with Pt/Pd compound, labelled as ++) and quantified (as genomic copies; GC in 1 µl of isolated DNA) with real-time polymerase chain reaction (qPCR). Mean values ± SEM (n=3). Control refers to native (--) and heat-inactivated (+-) virus suspension without the addition of Pt/Pd compound. Asterisks indicate significant difference between native and heat-inactivated virus suspension for respective compound. * $p<0.05$, ** $p<0.01$, ns – not significant.

Table 2. Overview of the tested pre-treatment procedures and their effects (in $\log_{10}$ reduction) on native and heat-treated virus suspension, and their use in improved protocol. BB-$PdCl_2$ – bis(benzonitrile) palladium (II) dichloride; $PdCl_2$-COD – dichloro (1,5-cyclooctadiene) palladium (II); SD – sodium deoxycholate.

| Improvement | Compound (1mM) | Effect on native virus suspension | Effect on heat-treated virus suspension | Usage |
|---|---|---|---|---|
| 0.5% Triton X-100 | BB-$PdCl_2$ | -3.1 $\log_{10}$ | -3.1 $\log_{10}$ | no |
| 0.5% Triton X-100 | $PdCl_2$COD | -2.4 $\log_{10}$ | -2.1 $\log_{10}$ | no |
| 0.1% SD | BB-$PdCl_2$ | -2.5 $\log_{10}$ | -2.1 $\log_{10}$ | no |
| 0.1% SD | $PdCl_2$COD | -2.4 $\log_{10}$ | -2.0 $\log_{10}$ | no |
| Heating of concentrated virus suspension | BB-$PdCl_2$ | -1.2 $\log_{10}$ | -2.8 $\log_{10}$ | yes |
| Heating of diluted virus suspension | BB-$PdCl_2$ | -1.2 $\log_{10}$ | -1.7 $\log_{10}$ | no |
| Incubation 15 min | BB-$PdCl_2$ | -0.8 $\log_{10}$ | -2.8 $\log_{10}$ | yes |
| Incubation 15 min | $PdCl_2$COD | -1.1 $\log_{10}$ | -2.5 $\log_{10}$ | yes |
| Incubation 30 min | BB-$PdCl_2$ | -1.2 $\log_{10}$ | -2.6 $\log_{10}$ | no |
| Incubation 30 min | $PdCl_2$COD | -1.2 $\log_{10}$ | -2.9 $\log_{10}$ | no |

reduction of BoHV-1 GC was observed in native virus suspension, treated with detergent (Table 2). Thus, the tested detergents were not used for the Pd compound pre-treatment of BoHV-1.

Comparison of the heat-treatment of concentrated virus suspension and of diluted virus suspension demonstrated a considerably (~1.1 $\log_{10}$) higher reduction of BoHV-1 GC of concentrated and subsequently diluted virus suspension than heat-treatment of diluted virus suspension (Table 2). Heat-treatment of concentrated virus suspension for 5 min at 95°C was used in the following experiments.

Finally, two incubation times, 15 and 30 min at 4°C, were compared. The reduction of BoHV-1 GC between native virus suspension without Pd compound and native virus suspension pre-treated with Pd compound was lower after 15 min incubation (0.8 $\log_{10}$ and 1.1 $\log_{10}$ for BB-$PdCl_2$ and $PdCl_2$COD, respectively) than after 30 min incubation (1.2 $\log_{10}$, both Pd compounds; Table 2), and thus subsequent experiments were incubated for 15 min at 4°C.

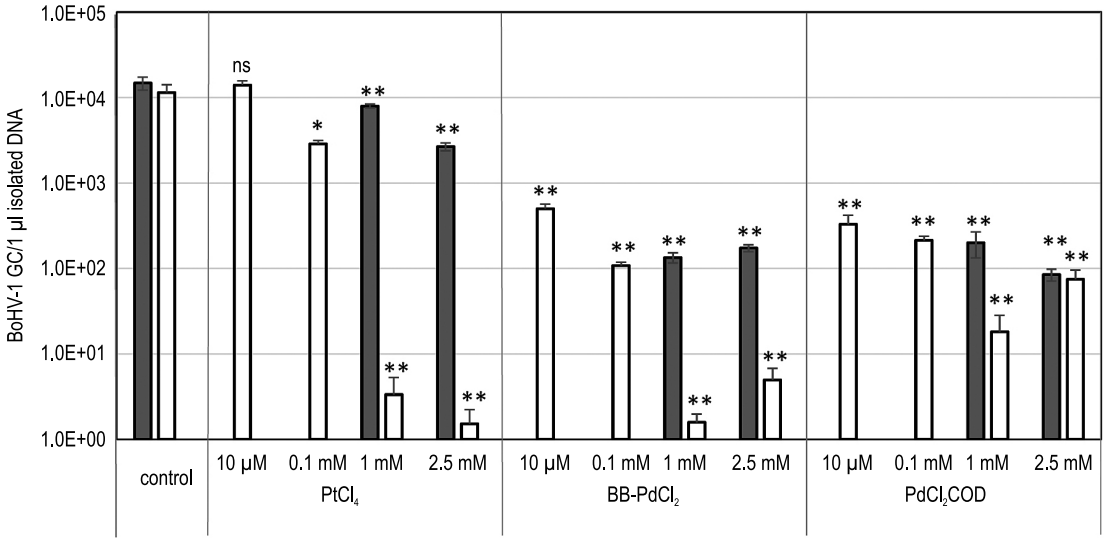


Fig. 2. Comparison of the effect of different concentrations of platinum (IV) chloride ($PtCl_4$), bis(benzonitrile) palladium(II) dichloride (BB-$PdCl_2$) and dichloro (1,5-cyclooctadiene) palladium(II) ($PdCl_2COD$) on purified bovine herpesvirus-1 (BoHV-1) DNA (empty bars) and on equivalent amount of BoHV-1 heat-treated virus suspension (95°C, 5 min; filled bars), quantified with qPCR (genomic copies; GC in 1 µl of isolated DNA). Control refers to heat–treated virus suspension and purified BoHV-1 DNA without Pt/Pd compound. Mean values ± SEM (n=3). Asterisks indicate significant difference from respective control. * $p<0.05$, ** $p<0.01$, ns – not significant.

### Effect of Pt/Pd compounds on purified BoHV-1 DNA and virus suspension

Figure 2 presents the effect of different concentrations of $PtCl_4$, BB-$PdCl_2$ and $PdCl_2COD$ on purified BoHV-1 DNA and virus suspension. As for purified DNA, the highest reduction of BoHV-1 GC was observed in the treatment with 2.5 mM $PtCl_4$ -3.87 $\log_{10}$. For both Pd compounds, the highest reduction of BoHV-1 GC was detected for the treatment with 1 mM concentration, -3.80 $\log_{10}$ and -2.80 $\log_{10}$ for BB-$PdCl_2$ and $PdCl_2COD$, respectively.

However, the effect of $PtCl_4$ on heat-treated virus suspension was only - 0.27 $\log_{10}$ and - 0.74 $\log_{10}$ reduction of BoHV-1 GC for 1 mM and 2.5 mM, respectively. The effect of Pd compounds on heat-treated virus suspension was -2.04 $\log_{10}$ and -1.87 $\log_{10}$ reduction of BoHV-1 GC for 1 mM BB-$PdCl_2$ and 1 mM $PdCl_2COD$, respectively. Based on these results, 1 mM $PdCl_2COD$ and 1 mM BB-$PdCl_2$ were used for further experiments.

The effect of 1 mM BB-$PdCl_2$ and 1 mM $PdCl_2COD$ on serial ten-fold dilutions of BoHV-1 virus suspension ($10^6$, $10^5$ and $10^4$ $TCID_{50}$/ml) is presented in Table 3. The extent of GC reduction between native and heat-treated virus suspension treated with 1 mM BB-$PdCl_2$ and 1 mM $PdCl_2COD$ ranged from -1.71 ± 0.29 to -2.81 ± 0.72 and -1.35 ± 0.07 to -1.77 ± 0.12, respectively.

### Effect of Pd compounds on purified ASFV DNA and virus suspension

1 mM BB-$PdCl_2$ and 1 mM $PdCl_2COD$ caused complete reduction of the qPCR signal of purified ASFV DNA (~5 $\log_{10}$) and of heat-treated 100-fold diluted ASFV suspension ($10^4$ $TCID_{50}$/ml; Table 3). However, the inhibition effect of cell culture medium appeared in 10-fold diluted ASFV virus suspension ($10^5$ $TCID_{50}$/ml), where only differences of -0.86 ± 0.05 and -0.66 ± 0.25 $\log_{10}$ for 1 mM BB-$PdCl_2$ and 1 mM $PdCl_2COD$, respectively, were observed between native and heat-treated virus (Table 3).

### Heat inactivation profile of BoHV-1 and ASFV

The performances of qPCR, BB-$PdCl_2$-qPCR and $PdCl_2COD$-qPCR in discriminating between native and heat-treated BoHV-1 and ASFV (10 and 60 min) at different temperatures compared to virus infectivity assay are presented in Fig. 3.

After heat-treatment for 10 min the reductions of BoHV-1 GC (compared to values from qPCR) were 0.89 ± 0.05, 1.17 ± 0.29 and 2.20 ± 0.90 $\log_{10}$ for 1 mM BB-$PdCl_2$ and 1.06 ± 0.03, 1.76 ± 0.29 and 2.39 ± 0.16 $\log_{10}$ for 1 mM $PdCl_2COD$, when incubated at 37°C, 50°C and 60°C, respectively (Fig. 3A). The highest reduction (3.15 ± 0.54 $\log_{10}$ and 2.56 ± 0.77 $\log_{10}$ for BB-$PdCl_2$ and $PdCl_2COD$, respectively) of qPCR signal was achieved for both Pd compounds, when heated at 95°C.

After heat-treatment for 60 min the reductions of BoHV-1 GC were 1.02 ± 0.06, 1.72 ± 0.13 and 2.17 ± 0.17 $\log_{10}$ for 1 mM BB-$PdCl_2$ and 1.59 ± 0.16, 1.67 ± 0.19 and 2.45 ± 0.33 $\log_{10}$ for 1 mM $PdCl_2COD$ when heated for 60 min at 37°C, 50°C and 60°C, respectively (Fig. 3B). The highest reduction (2.60 ± 0.85

Table 3. Impact of virus concentration on the efficiency of the treatment with palladium (Pd) compounds. Bovine herpes virus 1 (BoHV-1) and African swine fever virus (ASFV) suspensions were serially ten-fold diluted in dilution solution before treatment with 1 mM bis(benzonitrile) palladium (II) dichloride (BB-$PdCl_2$) and 1 mM dichloro (1,5-cyclooctadiene) palladium(II) ($PdCl_2$-COD). The effect of Pd compounds on purified DNA is shown for one concentration of DNA.

| Native BoHV-1 titer ($TCID_{50}$/ ml) | Compound | Native BoHV-1 DNA reduction ($log_{10}$) after treatment with Pd compounds[1] | Heat-treated BoHV-1 DNA reduction ($log_{10}$) after treatment with Pd compounds[2] |
|---|---|---|---|
| 1E +6 | BB-$PdCl_2$ | −0.969 ± 0.105 | −1.910 ± 0.154 |
| | $PdCl_2$ COD | −1.271 ± 0.100 | −1.349 ± 0.075 |
| 1E +5 | BB-$PdCl_2$ | −0.788 ± 0.178 | −1.708 ± 0.288 |
| | $PdCl_2$ COD | −0.650 ± 0.063 | −1.617 ± 0.129 |
| 1E +4 | BB-$PdCl_2$ | −0.728 ± 0.125 | −2.805 ± 0.715[3] |
| | $PdCl_2$ COD | −0.962 ± 0.151 | −1.771 ± 0.125[3] |
| BoHV-1 DNA 1E +6 | | | BoHV-1 DNA reduction ($log_{10}$) |
| | BB-$PdCl_2$ | – | −3.799 ± 0.203 |
| | $PdCl_2$ COD | – | −2.803 ± 0.304 |
| Native ASFV titer ($TCID_{50}$/ml) | | Native ASFV DNA reduction ($log_{10}$) after treatment with Pd compounds[1] | Heat-treated ASFV DNA reduction ($log_{10}$) after treatment with Pd compounds[2] |
| 1E +5 | BB-$PdCl_2$ | −1.039 ± 0.271 | −0.859 ± 0.048 |
| | $PdCl_2$ COD | −0.944 ± 0.176 | −0.655 ± 0.249 |
| 1E +4 | BB-$PdCl_2$ | −0.520 ± 0.078 | −3.214 ± 0.078[4] |
| | $PdCl_2$ COD | −0.618 ± 0.121 | −3.115 ± 0.121[4] |
| ASFV DNA 1E +6 | | | ASFV DNA reduction ($log_{10}$) |
| | BB-$PdCl_2$ | – | −5.039 ± 0.056[4] |
| | $PdCl_2$ COD | – | −5.039 ± 0.056[4] |

[1] – represents the effect of Pd compound on native virus suspension, [2] – represents the difference between native virus suspension with Pd compound and heat-treated virus suspension with Pd compound [3] – three of six qPCR values were negative, [4] – all qPCR values were negative

and 3.52 ± 0.43 $log_{10}$ for BB-$PdCl_2$ and $PdCl_2$COD, respectively) of the qPCR signal was achieved for both Pd compounds, when heated at 95°C.

Infectivity determined using cell culture demonstrated that BoHV-1 was inactivated by 0.28 and >4.79 $log_{10}$ $TCID_{50}$/ml when heated for 10 min at 50°C and 60°C, respectively (Fig. 3A). The extent of inactivation for 60 min incubation was 3.70 and >4.50 $log_{10}$ $TCID_{50}$/ml at 50°C and 60°C, respectively (Fig. 3B).

After heat-treatment for 10 min the reductions of ASFV GC (compared to values from qPCR) were 0.16 ± 0.18, 0.64 ± 0.03 and 0.73 ± 0.07 $log_{10}$ for 1 mM BB-$PdCl_2$ and 0.25 ± 0.01, 0.81 ± 0.09 and 1.13 ± 0.21 $log_{10}$ for 1 mM $PdCl_2$COD, when heated at 37°C, 50°C and 60°C, respectively (Fig. 3C). Complete (7.65 $log_{10}$) elimination of the qPCR signal was achieved for both Pd compounds, when heated at 95°C.

After heat-treatment for 60 min the reductions of ASFV GC were 0.76 ± 0.37, 0.71 ± 0.11 and 1.06 ± 0.05 $log_{10}$ for 1 mM BB-$PdCl_2$ and 0.57 ± 0.10, 1.25 ± 0.90 and 1.75 ± 0.20 $log_{10}$ for 1 mM $PdCl_2$COD when heated for 60 min at 37°C, 50°C and 60°C, respectively (Fig. 3D). Differences in ASFV GC and BoHV-1 GC between control qPCR and BB-$PdCl_2$-qPCR or $PdCl_2$COD-qPCR increased in a time- and temperature-dependent manner. Differences were highly significant for temperatures equal to or higher than 60°C and for 10 min of incubation at 50°C ($p<0.01$) (Fig. 3).

Infectivity determined using cell culture showed that ASFV was inactivated by 3.5 and >4.75 $log_{10}$ $TCID_{50}$/ml when heated for 10 min at 50°C and 60°C, respectively (Fig. 3C). The extent of inactivation for 60 min incubation was 3.88 and >4.75 $log_{10}$ $TCID_{50}$/ml at 50°C and 60°C, respectively (Fig. 3D).

## Discussion

ASFV is a contagious virus disease affecting domestic swine (*Sus scrofa* f. *domestica*) and wild boars (*Sus scrofa*). Infection is associated with high mortality and devastating outbreaks worldwide, followed by sub-

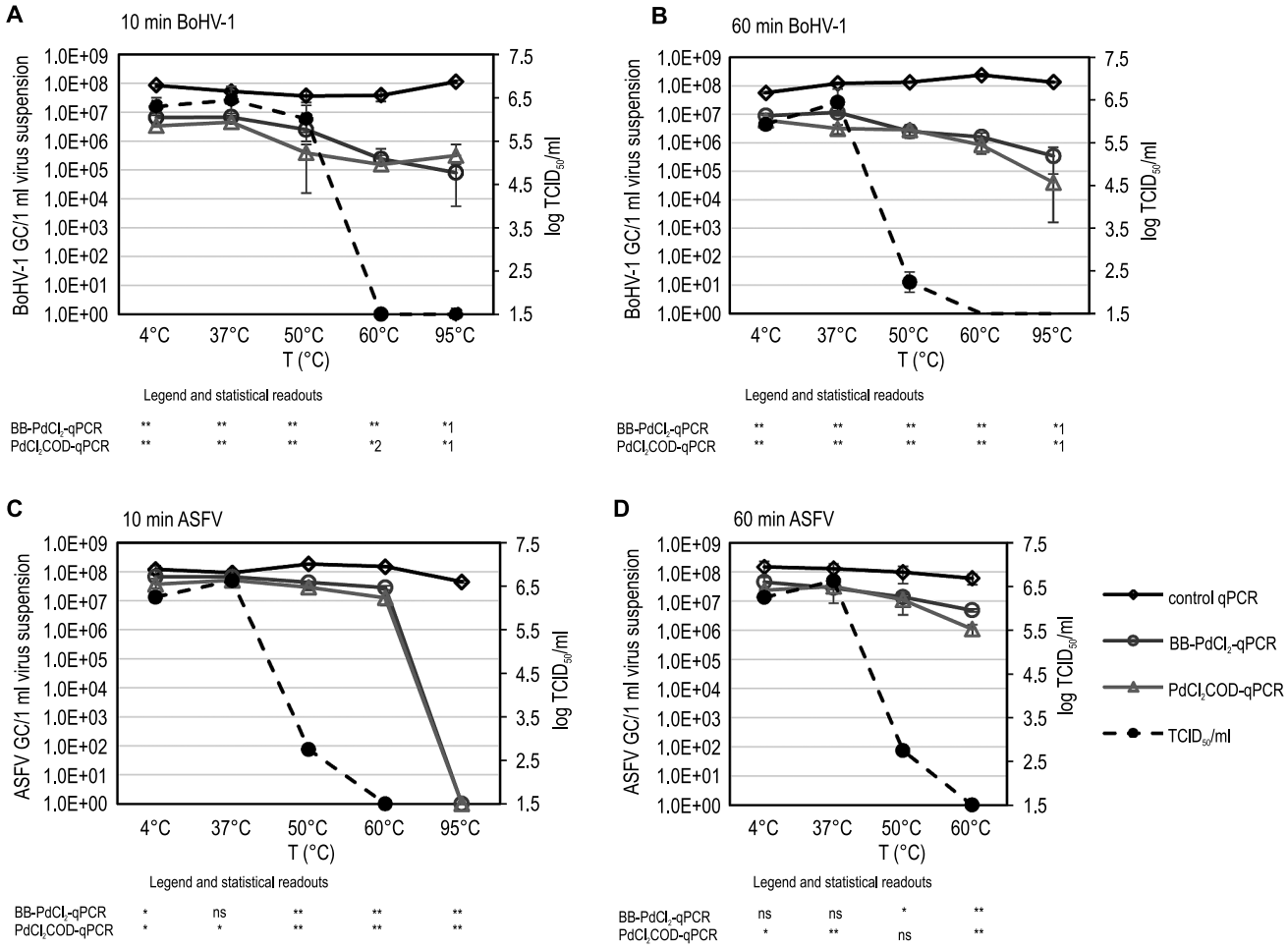


Fig. 3. Performance of control real-time polymerase chain reaction (qPCR without treatment; squares), bis(benzonitrile) palladium(II) dichloride qPCR (BB-$PdCl_2$-qPCR; open circles), dichloro (1,5-cyclooctadiene) palladium(II) qPCR ($PdCl_2$-COD-qPCR; triangles) to discriminate between native and heat-treated bovine herpesvirus-1 (BoHV-1) incubated at different temperatures for 10 min (A), 60 min (B), and African swine fever virus (ASFV) for 10 min (C) and 60 min (D), compared to infectivity assay (solid circles, dashed line). GC – genomic copies; mean values ± SEM (n=2). Asterisks in frames below the respective graphs indicate significant difference of BB-$PdCl_2$-qPCR and $PdCl_2$COD qPCR from control. * $p<0.05$, ** $p<0.01$, ns – not significant. [1]- one of four qPCR values were negative, [2]- two of four qPCR values were negative.

stantial economic losses in affected areas. Until now, no effective treatment for the disease has been identified (Brookes et al. 2021) and despite extensive work on vaccine development, there are as yet no effective and safe vaccines (Sang et al. 2020). The virus is relatively resistant to high temperature and is stable in pH ranging between 4-10, especially in protein-rich environments, where it can persist for months (Sanchez-Vizcaino et al. 2012), and as a matrix-free stock solution for up 30 days (Dee et al. 2018). Its long persistence in the environment and in feed or feed ingredients (Dee et al. 2018) can represent potential sources of infection. Therefore, early detection, and especially rapid and reliable determination of infectious/non-infectious sites/sources of infection, would help to minimize production losses.

In recent years, several studies have appeared that test the ability of Pt compounds to detect infectious virus particles (Fraisse et al. 2018, Randazzo et al. 2018, Canh et al. 2019, Chen et al. 2020, Puente et al. 2020). The ability of Pd compounds to discriminate between infectious and non-infectious agents has been demonstrated on bacteria (Soejima and Iwatsuki 2016, Cechova et al. 2022).

Initial studies performed with an identical spectrum of Pd and Pt compounds as described previously for noroviruses (Fraisse et al. 2018) demonstrated the highest effect between a native and heat-treated virus suspension for Pd compounds, BB-$PdCl_2$ and $PdCl_2$-COD in particular. The difference between Pd-pre-treated native viruses and Pd-pre-treated heat-inactivated viruses was after optimization in the range of 1.3 - 1.9 $\log_{10}$. From the group of Pt compounds the highest effect was observed for 2.5 mM $PtCl_4$; nevertheless, this represented a decrease of only ~0.3 $\log_{10}$ between native and heat-treated BoHV-1 (not shown). These

results, obtained on a model of enveloped DNA viruses, were different from those reported for RNA viruses (Fraisse et al. 2018, Randazzo et al. 2018, Puente et al. 2020). Fraisse (2018) described $PtCl_4$ as the substance with the highest effect on norovirus RNA and virus suspension, with no effect for any Pd compounds. $PtCl_4$ has been extensively used in further studies (Randazzo et al. 2018, Puente et al. 2020, Chen et al. 2020). In addition, the Pt compound CDDP was also tested for the discrimination between infectious and non-infectious viruses (Canh et al. 2019) and was successfully used in combination with SD for the determination of infectious viruses in surface and tap water samples (Canh et al. 2021a).

Several strategies to enhance the efficacy of Pt/Pd pre-treatment have been evaluated. Application of detergents diminished the difference between heat-treated and native viruses. Conflicting data have been reported for the effect of detergents on pre-treatment with Pt compounds. In agreement with the present results, other authors observed the diminishing effect of SD detergent between native and heat-treated enveloped RNA viruses as well (Canh et al. 2021b). No effect of detergents was observed for the pre-treatment of noroviruses (Fraisse et al. 2018); however, Triton X-100 was effective for hepatitis E virus (Randazzo et al. 2018), porcine epidemic diarrhoea virus in serum (Puente et al. 2020), and SD for the detection of Aichi virus in wastewater (Canh et al. 2019).

As for affinity to nucleic acid, very good reactivity of $PtCl_4$ to BoHV-1 DNA (3.7 $\log_{10}$ decrease) has been observed, in agreement with Fraisse (2018). However, for BoHV-1 virus suspension the reactivity was not confirmed. In contrast, Pd compounds BB-$PdCl_2$ and $PdCl_2$-COD caused a decrease of 3.8 and 2.8 $\log_{10}$ for BB-$PdCl_2$ and $PdCl_2$-COD respectively for BoHV-1 DNA, and absolute elimination of the qPCR signal for ASFV DNA.

The effect of Pd compounds on native virus suspension was observed for BoHV-1 and ASFV (Table 3). A decrease of approximately 1 $\log_{10}$ shows how much virus nucleic acid (from damaged virus particles without infectious potential) is present in the suspension before heat-treatment. The difference between the amount of infectious virus particles determined via infectivity assay ($TCID_{50}$) and the amount detected with qPCR can be up to two orders of magnitude. The effect of Pd compounds on BoHV-1 virus suspension was stable in all three serial dilutions of BoHV-1 (corresponding to a dilution of the original virus suspension of $10^2$, $10^3$ and $10^4$-fold). However, for ASFV (10- and $10^2$-fold diluted virus suspension), a reduced effect of Pd compounds in 10-fold diluted virus suspension was observed. This effect may be due to unspecific binding of Pd compounds to other substances present in the cultivation medium, which can still contain, in 10-fold dilution, at least 1% of various proteins. It was previously shown that Pt compounds interact with free thiol groups of biologically relevant proteins (Brauckmann et al. 2013). Several studies also describe the ability of Pd compounds to bind to proteins (Omondi et al. 2020). This can result in a lowered difference between native and heat-treated virus suspension. In addition, cultivation medium contains, besides virus nucleic acids, nucleic acids from cell culture (intact cells or cells which have undergone a cytopathic effect). Pd compounds can bind to these nucleic acids as well. All these compounds may contribute to a reduced effect of Pd compounds in 10-fold diluted virus suspension.

The concentrations of Pd compounds required for the efficient determination between heat-treated and native viruses were considerably higher (20-40 ×) than those described for bacteria. While concentrations of 25 µM, 50 µM of $PdCl_2$-COD and 30 µM BB-$PdCl_2$ were effective for the detection of viable bacteria (Soejima and Iwatsuki 2016, Cechova et al. 2022), for DNA viruses in the present work a concentration of 1 mM was used. This is in accordance with previous results documented for Pt compounds, where the effective concentrations of $PtCl_4$ for viruses were considerably higher than for bacteria (Soejima and Iwatsuki 2016, Fraisse et al. 2018, Randazzo et al. 2018, Puente et al. 2020, Cuevas-Ferrando et al. 2022).

Differences in $\log_{10}$ of BoHV-1 GC between qPCR and BB-$PdCl_2$-qPCR and $PdCl_2$COD-qPCR increased in a time- and temperature- dependent manner and were significantly different from control qPCR for all temperatures tested. Pre-treatment with $PdCl_2$-COD showed higher differences to control qPCR than pre-treatment with BB-$PdCl_2$ and better pattern matching with $TCID_{50}$ assay for the 60 min incubation. Although with increased incubation temperature and time negative qPCR results appeared, complete elimination of the qPCR signal was not achieved. This is in accordance with the results obtained for purified BoHV-1 DNA, where the complete elimination of qPCR led to maximal reduction of 3.8 $\log_{10}$ and 2.8 $\log_{10}$ for the treatment with 1 mM BB-$PdCl_2$ and 1 mM $PdCl_2$COD, respectively (Table 3).

As for ASFV, results obtained from different temperatures and incubation times showed that, although the complete inactivation of ASFV occurred after 10 min at 60°C, capsid damage evoked by such treatment was not so strong that Pd compounds could penetrate into all the non-infectious virus particles. Results of other studies performed in the temperature range between 50 and 60°C show similar data (Fraisse et al. 2018, Randazzo et al. 2018). However, treatment

with both tested Pd compounds at 95°C for 10 min completely reduced the qPCR signal for heat-treated ASFV suspension.

The aim of this study was to develop a method for the determination of infectious virus particles via Pd-compound pre-treatment prior to qPCR. Based on the present results, the method can be used in cell culture supernatants. The applicability of this method has also been shown in meat samples (Krzyzankova et al. 2023).

The present study demonstrated that from the panel of tested Pt/Pd compounds BB-$PdCl_2$ and $PdCl_2$COD had the ability to bind to purified BoHV-1 DNA and ASFV DNA. Pre-treatment with 1 mM BB-$PdCl_2$ or 1 mM $PdCl_2$COD can reduce qPCR signals in heat-treated and, to some extent, in native virus suspension. The reduction of the qPCR signal in native BoHV-1 and ASFV virus suspensions can be a consequence of the presence of non-infectious virus particles in the virus suspension. The effect of BB-$PdCl_2$ and $PdCl_2$COD at temperatures between 50°C - 60°C did not fully reflect the viability of BoHV-1 and ASFV, but this effect was enhanced with a longer incubation time and higher temperature. ASFV virus suspension was more sensitive to the treatment with Pd compounds than BoHV-1 virus suspension.

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