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In vitro virucidal activity of chemical compounds against porcine transmissible gastroenteritis virus and porcine respiratory coronavirus

Marta Antas[1*] and Monika Olech[2]

Abstract

Porcine transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) are enveloped, single-stranded RNA viruses belonging to the genus *Alphacoronavirus* in the family *Coronaviridae*. In the absence of effective treatments, these viruses may lead to significant economic losses in the swine industry. To date, there is no or limited information about the efficiency of disinfectants against TGEV and PRCV. Therefore, in this study, we investigated the virucidal activity of eleven chemical compounds against TGEV and PRCV using a quantitative suspension test method based on the European Standard EN 14675. The results revealed that caustic soda (3%, 2%, 1%), potassium peroxymonosulfate (2%, 1%, 0.5%), and 80% ethanol exhibited effective virucidal activity (reduction ≥ 4 log10TCID50/ml) under low- and high-level soiling conditions against both TGEV and PRCV. Ethanol (60% and 40%) showed virucidal efficacy against PRCV under low- and high soiling conditions but against TGEV only under low soiling conditions. Sodium hypochlorite (1.5%, 1%, 0.5%) was effective only against TGEV in low-level soiling conditions but was ineffective against PRCV. Furthermore, acetic acid (3.5%, 2.5%, 1.5%), hydrogen peroxide (0.5%, 1%, 2%), and phenol (1%, 1.5%, 2%) did not result in a ≥ 4 log10TCID50/ml reduction in the PRCV and TGEV viral titers under both tested conditions, so these compounds were found to be ineffective. Benzalkonium chloride (0.5%, 1%, 1.5%), formaldehyde (0.125%, 0.275%, 0.5%), and glutaraldehyde (0.1%, 0.5%, 1%) were found to be cytotoxic, limiting the detection of viral infectivity reduction to less than 4 log10TCID50/ml. Our study also revealed that caustic soda and potassium peroxysulfate were the most stable disinfectants and that organic matter notably reduced the activity of sodium hypochlorite. To the best of our knowledge, this is the first report on in vitro testing of chemicals that can help prevent the spread and transmission of PRCV and TGEV.

Keywords Porcine transmissible gastroenteritis virus, Porcine respiratory coronavirus, TGEV, PRCV, Coronaviruses, Disinfection, Virucidal effect

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Introduction

Porcine transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) are enveloped, single-stranded RNA viruses belonging to the genus *Alphacoronavirus* in the family *Coronaviridae*. TGEV causes transmissible gastroenteritis (TGE), a disease notifiable by the World Organization for Animal Health (WOAH), which is characterized by diarrhea, vomiting, and dehydration with high morbidity and nearly 100% mortality, especially in piglets less than two weeks old [1, 2]. PRCV is a natural mutant of TGEV with a large deletion (621–681 nt) in the 5' spike (S) gene and small deletions in the 3/3a and 3 – 1/3b genes. Deletion in the S gene is thought to be associated with differences in tissue tropism between TGEV and PRCV. TGEV replicates mostly in small intestine epithelial cells, whereas PRCV replicates almost exclusively in respiratory epithelial cells and causes no or mild respiratory infection symptoms [1, 3–5]. However, PRCV may contribute to respiratory disease syndrome in pigs infected with other respiratory pathogens [6, 7]. The transmission of TGEV occurs mainly through the oral–fecal route, whereas PRCV spreads mainly by direct contact with infected animals or by aerosols. Both viruses can also be transmitted by any surface which may be contaminated with manure (mechanical transmission). In addition, people may carry the virus on their hands and clothing, as well as on their shoes [8].

Currently, the prevalence of TGEV has declined, which is assumed to be related to the spread of PRCV, which causes immunological cross-protection against TGEV [2, 9]. However, PRCV does not completely protect against TGEV infection. TGEV continues to be reported in various parts of the world, even in herds with simultaneous TGEV and PRCV infection, suggesting that this virus continues to threaten the swine industry. In some parts of the world, especially in low biosecurity countries such as China, TGEV is more prevalent, and virulent TGEV strains have recently been reported [10–13]. Currently, effective vaccines and drugs against TGEV/PRCV are not available. Both viruses can persist in the environment for a long time. On smooth surfaces such as stainless steel and plastic, both viruses can survive for up to 28 days if stored at 4 °C and low humidity [14, 15]. Therefore, disinfection and proper use of effective disinfectants play important roles in preventing and controlling the spread of TGEV and PRCV.

Disinfectants are chemical substances used to destroy or inhibit the growth of microorganisms, such as bacteria, viruses, and fungi, on inanimate objects and surfaces. Substances that inactivate viruses by disrupting their structural or functional components, making them non-infectious, are called virucidal compounds [16–19]. Common types of virucidal compounds include alcohols, aldehydes, oxidizing agents, chlorine-based disinfectants, phenolics and quaternary ammonium compounds [19]. These compounds play a crucial role in veterinary medicine for disinfecting surfaces, equipment, and environments, which helps prevent the spread of viral infections among animals and from animals to humans. Since viruses can cause significant illness, death, and economic loss in both companion and farm animals, the use of virucidal disinfectants is essential for maintaining animal health, biosecurity, and public safety [20–23]. Suscetpibility of viruses to chemical desinfectants varies depending on their structure [24]. Several chemical compounds are generally accepted as inactivating enveloped viruses [21, 22]. However, very limited data are available regarding the efficiency of chemical compounds against TGEV/PCRV [25, 26]. Furthermore, to the best of the authors' knowledge, no studies have evaluated the viricidal activity of disinfectants against TGEV/PRCV according to European standards. In general, publications evaluating the virucidal activity of disinfectants used in veterinary medicine according to European standards are scarce [21, 23]. Therefore, to fill this gap, in this study, we evaluated the virucidal efficacy of eleven commonly used chemical compounds (ethanol, 2-propanol, formaldehyde, sodium hypochlorite, acetic acid, hydrogen peroxide, caustic soda, phenol, glutaraldehyde, benzalkonium chloride, and potassium peroxysulfate) against TGEV and PRCV according to the modified protocol of the European standard EN 14675 on the basis of a quantitative suspension testing method [27]. The results of this study will undoubtedly provide valuable information on the use of proper chemical disinfectants in the control of TGEV and PRCV.

Materials and methods

Tested disinfectants

We used the following concentrations of tested substances: 80%, 60% and 40% ethanol (POCH, Gliwice, Poland, CAS:64–17-5); 80%, 60% and 40% 2-propanol (LGC Promochem GmbH, Wesel, Germany, CAS: 67–63-0); 0.5%, 0.25% and 0,125% formaldehyde (POCH, Gliwice, Poland, CAS: 50–00-0); 1%, 2% and 3% caustic soda (NaOH) (POCH, Gliwice, Poland, CAS: 1310–73-2); 0.5%, 1% and 1.5% sodium hypochlorite (containing minimum 100 g/l active chlorine, POCH, Gliwice, Poland, CAS: 7681–52-9%); 0.5%, 1% and 2% hydrogen peroxide (POCH, Gliwice, Poland, CAS: 7722–84-1); 0.1%, 0.5% and 1% glutaraldehyde (25%, Carl Roth, Karlsruhe, Germany, CAS: 111–30-8); 0.5%, 1% and 1.5% of benzalkonium chloride (Pol-Aura, Olsztyn, Poland, CAS: 63,449–41-2); 1%, 1%, 5% and 2% phenol (Chempur, Piekary Śląskie, Poland, CAS: 108–95-2); 1.5%, 2.5% and 3.5% of acetic acid (POCH, Gliwice, Poland, CAS: 64–19-7) and 0.5%, 1% and 2% of potassium peroxymonosulfate

(Envolab, Długomiłowice, Poland, CAS: 70,693–62-8) (Supplementary Table 1). All the tested chemical compounds were prepared immediately before use by dilution in hard water (pH 7). Three concentrations of each chemical compound were tested. The concentrations used were selected on the basis of the literature data [21, 25, 26, 28]. All samples were tested in the presence of interfering substances: clean (low-level soiling, 3.0 g/L bovine albumin (BSA)) and dirty (high-level soiling, 10 g/L bovine albumin (BSA) plus 10 g/L yeast extract) conditions. The hard water and interfering substances were prepared in accordance with PN-EN 14675:2015 European Standard [27].

Cells and viruses

The swine testis (ST) cell line (CRL-1746) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in minimum essential medium (MEM) supplemented with Earle's salts, L-glutamine, and sodium bicarbonate (Sigma–Aldrich, St. Louis, MO, USA). The medium was additionally supplemented with 10% (v/v) fetal bovine serum (FBS) (ATCC, 30–2020 or Gibco, Billings, MT, USA), 1% antibiotic antimycotic solution (Sigma–Aldrich, St. Louis, MI, USA), 1% (v/v) MEM nonessential amino acids (100x) (Life Technologies Corporation, Gibco, New York, USA), and 1.0 mM sodium pyruvate (PAN Biotech, Aidenbach, Germany). Cells were subculturedin a humidified 5% CO_2 incubator at 37 °C. The cells were maintained and used as monolayers in disposable tissue culture flasks and 96-well microtiter plates as needed. The virucidal activity was evaluated using PRCV and TGEV obtained from the ATCC (VR-3379™) and (VR-1740™), respectively. Viruses were used at titers of at least $10^{6.5}$ tissue culture infective doses per milliliter (TCID50/ml).

Virus stock preparation

To propagate TGEV and PRCV viruses, ST cell cultures with 90–100% confluence were washed twice with sterile phosphate-buffered saline (PBS) suplemented a 1% antibiotic antimycotic mixture and incubated with 1 ml of the viral strain diluted in virus growth medium containing 5 µg/ml trypsin (Gibco, Life Technologies Corporation, Grand Island, NY, USA) for 1 h in a T25 flask at 37 °C under 5% CO_2. Then, 5 ml of virus growth medium supplemented with 1 g/ml trypsin was added. When 70–100% cytopathic effect (CPE) was observed, the cells were subjected to three freeze–thaw cycles. The mixture was centrifuged at 3,000 × g for 10 min at 4 °C to remove cell debris, and the obtained supernatant was aliquoted and stored at −80 °C until use. Virus titration was performed in a 96-well plate containing a confluent monolayer of ST cells, with tenfold serial dilutions of virus prepared in triplicate for each dilution. CPE was observed daily and, after 3 days, expressed as a 50% tissue culture infective dose ($TCID_{50}$/ml) according to the Spearman–Kärber method [29]. The TGEV and PRCV viruses used in this study were not passaged more than five times from the original ATCC strain, which is stated to be in compliance with ATCC guidelines.

Virucidal assay methodology

The protocol of the European Committee for Standardization (EN 14675, 2006) was adapted to determine the virucidal activity of disinfectants used in veterinary medicine against TGEV and PRCV (Fig. 1). Briefly, the virus suspension was mixed with the interfering substance (low or high) in a 1:1 ratioand incubated at 10 ± 1 °C for 2 min ± 10 s. Then, eight parts of each dilution of each chemical compound were added to the two parts of virus suspension in the solution containing the interfering substance and incubated at 10 ± 1 °C for 30 min ± 10 s. The test mixtures were then tranferred ontocrushed ice and serially diluted (in triplicate) tenfold in MEM without FBS but containing 1 µg/ml 2.5% trypsin (10x) in 96-well plates. The dilutions were further transferred into the wells of plates with containing a monolayer of ST cells (ST cells were previously washed with PBS containing an antibiotic–antimycotic mixture and incubated with medium without FBS but supplemented with 5 µg/ml 2.5% trypsin (10x) for 3 h). In the following step the plates were incubated for 3 days at 37 ± 2 °C in air containing 5% CO_2 and examined daily for the appearance of a CPE, which was microscopically observed and calculated using the Spearman–Kärber method. Virucidal activity was calculated by subtracting the logarithmic titer of each chemical compound dilution from the logarithmic titer of the virus control (hard water was used instead of the disinfectant). All reagents used in the test were prepared in accordance withthe PN-EN 14675:2015 European Standard. The chemical compound was considered effective if the viral titer was reduced by at least 4 log10TCID50/ml indicating 99.99% loss of infectivity level.

Cytotoxicity

The cytotoxicity of the chemical compound was assessed via morphological observations of the cells. When cytotoxicity was so high that a reduction in residual infectivity titers in the 4 lg range could not be observed, Microspin S-400 HR columns (GE Healthcare, Fairfield, CT, USA) were used to remove cytotoxic products from the tested mixture. To asess the loss of virus titer, the titer of the initial unfiltered viral control and the viral control after filtration were compared. Both the standard procedure and the procedure involving the special technique of ultrafiltration with Microspin S-400 HR columns were performed in the same manner as the virus control, where

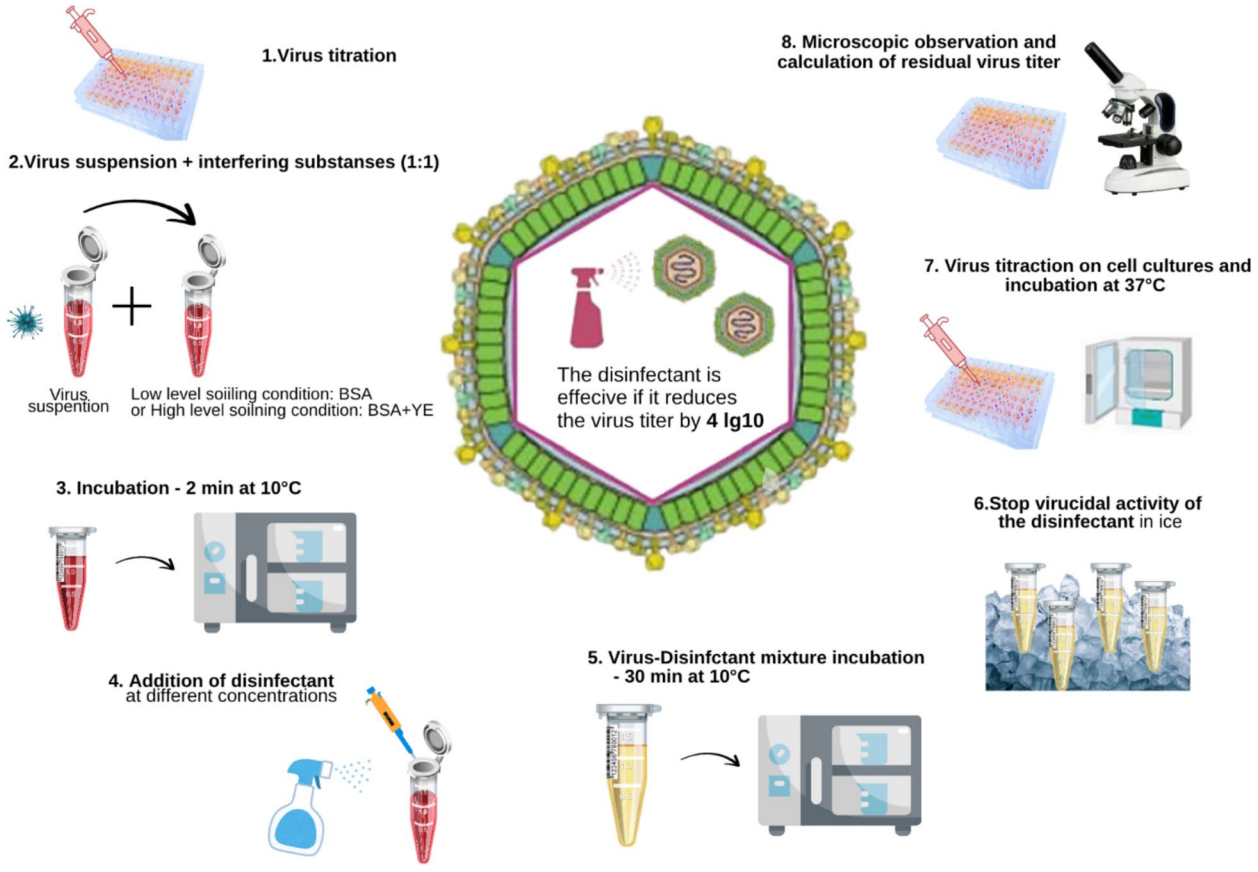

Fig. 1 Quantitative suspension test for virucidal activity against TGEV/PRCV according to the UNI EN 14675:2015 standard. BSA: bovine serum albumin; YE: yeast extract. The virucidal activity testing scheme was modified based on the figure published by Beato et al. [21]

hard water was used instead of the product tested. The 95% confidence limit for the assay results should be ± 0.5 log10TCID50/ml or less, as specified in the guidelines.

Statistical analysis

The virus titers were expressed as the mean log10TCID50/ml with standard deviation (SD) from three replicates. Differences between mean titers were analyzed via two-way ANOVA followed by Tukey's post hoc test. A p value of < 0.05 was considered statistically significant. All the statistical analyses and graphical representations of the obtained results were performed using the Prism 9.0 software package (GraphPad Software, Inc., USA).

Results

Results against TGEV

A summary of the results is presented in Table 1. From the eleven chemical compounds tested, virucidal efficacy against TGEV under high- and low-level soiling conditions was demonstrated in three products: ethanol at a concentration of 80%, caustic soda at concentrations of 3%, 2%, and 1%, and potassium peroxysulfate at concentrations of 2%, 1%, and 0.5%. Ethanol at concentrations of 60% and 40%, 2-propanol at concentrations of 90%, 60% and 40%, and sodium hypochlorite at concentrations of 1.5%, 1%, and 0.5% demonstrated virucidal effectiveness only under low-level soiling conditions. Statistical analysis using the ANOVA test followed by post hoc Tukey's test confirmed that the log10TCID50/ml reduction mean values of all the chemical compounds that showed virucidal effectiveness (reduction results ≥ 4 log10TCID50/ml) were statistically significant ($P < 0.001$).

Ethanol at concentrations of 60% and 40%, 2-propanol at concentrations of 80%, 60%, and 40%, and sodium hypochlorite at a concentration of 1.5% in the presence of organic matter reduced TGEV titers above 3 log10TCID50/ml but below the required 4 log10TCID50/ml reduction.

Three tested products, acetic acid (at concentrations of 3.5%, 2.5%, and 1.5%), hydrogen peroxide (at concentrations of 2%, 1%, and 0.5%), and phenol (at concentrations of 2%, 1.5%, and 1%), did not show antiviral effectiveness against TGEV. The mean log10TCID50/ml reduction did not exceed 2.5. Three of the tested chemical compounds, glutaraldehyde (1%, 0.5%, 0.1%), formaldehyde (0.5%, 0.275%, 1.125%), and benzalconium chloride (1.5%, 1%, 0.5%), showed cytotoxic effects on ST cells, making it impossible to assess the reduction in virus titer by the

Table 1 Titers reduction obtained for TGEV treated with different disinfectants (30 min contact time at 10 °C with low- and high-level soiling). The reduction is the difference between the titer values obtained for the control and the tested concentrations of disinfectant. Here, the reduction is presented as the mean ± standard deviation of three runs

Disinfectant	Tested concentration	Titer reduction (± SD) ($TCID_{50}$/ml)	
		Low level soiling (3.0 g/l BSA)	High level soiling (10 g/l BSA + 10 g/l YE)
ethanol	80%	**4.5 (± 0.50)**	**4.83 (± 0.29)**
	60%	**4.5 (± 0.50)**	3.83 (± 0.29)
	40%	**4.5 (± 0.50)**	3.50 (± 0.50)
2-propanol	80%	**4.5 (± 0.50)**	3.82 (± 0.29)
	60%	**4.5 (± 0.50)**	3.83 (± 0.29)
	40%	**4.33 (± 0.29)**	3.83 (± 0.29)
Sodium hypochlorite	1.5%	**4.67 (± 0.29)**	3.67 (± 0.58)
	1%	**4.67 (± 0.29)**	1.33 (± 0.58)
	0.5%	**4.67 (± 0.29)**	0.50 (± 0.87)
Acetic acid	3.5%	1.5 (± 0.50)	0 (± 0.87)
	2.5%	1.33 (± 0.58)	0 (± 0.87)
	1.5%	1.17 (± 0.29)	0 (± 0.58)
Hydrogen peroxide	2%	2.5 (± 0.00)	0.5 (± 1.00)
	1%	1.5 (± 0.50)	0.17 (± 0.58)
	0.5%	1.17 (± 0.29)	0.0 (± 0.29)
Caustic soda	3%	**4.20 (± 0.17)**	**4.33 (± 0.29)**
	2%	**4.20 (± 0.17)**	**4.33 (± 0.29)**
	1%	**4.20 (± 0.17)**	**4.33 (± 0.29)**
Phenol	2%	2.17 (± 0.29)	2.5 (± 0.50)
	1.5%	1.67 (± 0.76)	1.33 (± 0.76)
	1%	0.17 (± 0.76)	0.0 (± 0.58)
Potassium peroxymonosulfate	2%	**4.67 (± 0.29)**	**4.67 (± 0.58)**
	1%	**4.67 (± 0.29)**	**4.67 (± 0.58)**
	0.5%	**4.67 (± 0.29)**	**4.67 (± 0.58)**
Glutaraldehyde[C]	1%	2.5 (± 0.50)*	2.33 (± 0.58)*
	0.5%	2.5 (± 0.50)*	2.33 (± 0.58)*
	0.1%	3.5 (± 0.50)*	3.33 (± 0.58)*
Formaldehyde[C]	0.5%	1.33 (± 0.29)*	2.33 (± 0.29)*
	0.275%	2.33 (± 0.29)*	2.33 (± 0.29)*
	0.125%	2.17 (± 0.29)*	2.00 (± 0.00)*
Benzalkonium chloride[C]	1.5%	1.5 (± 0.50)*	2.33 (± 0.58)*
	1%	1.5 (± 0.50)*	2.33 (± 0.58)*
	0.5%	1.5 (± 0.50)*	2.33 (± 0.58)*

BSA Bovine serum albumin, *YE* Yeast extract

[C]A cytotoxic effect was observed. The results are presented after cytotoxicity reduction

*Due to cytotoxicity, the detection limit did not allow detection of greater virus reduction

required 4 logarithms. To reduce cytotoxicity, these three chemicals at each concentration were filtered through Miscrospin S-400 HR columns, and the collected filtrate was reused for testing. Microfiltration resulted in a slight decrease (≤ 0.5 log10TCID50/ml) in the initial virus titer. Unfortunately, microfiltration only slightly reduced (≥ 1 log10TCID50/ml) the cytotoxicity of all three compounds, not allowing an evaluation of their efficacy. The log10TCID50/ml values before and after ultrafiltration using Microspin S-400 HR columns are presented in Fig. 2.

Results against the PRCV

A summary of the results is presented in Table 2. Among the eleven tested chemical compounds, the virucidal activity against PRCV under high- and low-level soiling conditions was demonstrated in four products: ethanol at concentrations of 80%, 60%, and 40%; 2-propanol at concentrations of 80%, 60%, and 40%; caustic soda at concentrations of 3%, 2%, and 1%; and potassium peroxymonosulfate at concentrations of 2%, 1%, and 0.5%. Statistical analysis confirmed that the mean log10TCID50/ml reduction factors of all mentioned chemical compounds were statistically significant ($P < 0.001$).

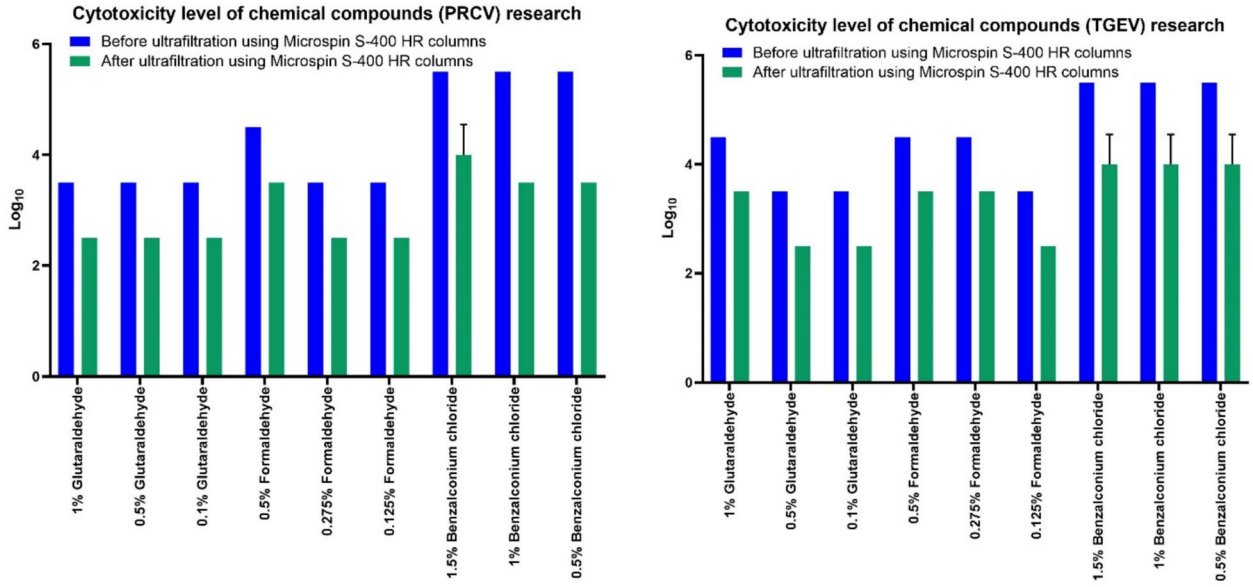

Fig. 2 Results of log10TCID50/ml values before and after ultrafiltration using Microspin S-400 HR columns in TGEV and PRCV research

Four tested products, sodium hypochlorite (at concentrations of 1.5%, 1%, and 0.5%), acetic acid (at concentrations of 3.5%, 2.5%, and 1.5%), hydrogen peroxide (at concentrations of 2%, 1%, and 0.5%), and phenol (at concentrations of 2%, 1.5%, and 1%), did not show virucidal effectiveness against PRCV. However, a fairly high PRCV titer reduction was observed for sodium hypochlorite tested at concentrations of 1.5%, 1%, and 0.5% under low-level soiling conditions (3.67 (± 0.29)) and for 1.5% phenol under low (3.00 (± 0.5)) and high (2.83 (± 0.29)) soiling conditions (Table 2). Glutaraldehyde at concentrations of 1%, 0.5%, and 0.1%, formaldehyde at concentrations of 0.5%, 0.275%, and 1.125%, and benzalconium chloride at concentrations of 1.5%, 1%, and 0.5% showed cytotoxic effects on ST cells. Despite the reduction in the level of cytotoxicity, it was not possible to obtain titer reduction results ≥ 4 log10TCID50/ml in the purified samples. Thus, it was not possible to estimate the efficacy of these three compounds. The log10TCID50/ml values before and after ultrafiltration using Microspin S-400 HR columns are presented in Fig. 2.

Comparison of TGEV and PRCV results

Caustic soda at concentrations of 3%, 2%, and 1%; potassium peroxymonosulfate at concentrations of 2%, 1%, and 0.5%; and ethanol at a concentration of 80% showed virucidal activity (reduction ≥ 4 log10TCID50/ml) in low- and high-level soiling conditions against both TGEV and PRCV. Ethanol at concentrations of 60% and 40% was effective against TGEV and PRCV only under low-level soiling conditions. The virucidal activity of these ethanol concentrations under high-level soiling conditions was only effective against the PRCV. The virucidal activity against TGEV under these conditions was high (≥ 3.5), but unfortunately, it did not reach the minimum value of 4 log10TCID50/ml. 2-propanol at the tested concentrations of 80%, 60%, and 40% was effective against both PRCV and TGEV only under low-level soiling conditions. Under high soiling conditions, 2-propanol tested at all concentrations was effective only against the PRCV. For TGEV, the reduction was minimally less than 4 log10TCID50/ml, with a value of 3.82 (± 0.29). Sodium hypochlorite at concentrations of 1.5%, 1%, and 0.5% was effective only against TGEV in low level soiling conditions. Against PRCV under low-level soiling conditions, the reduction reached 3.67 (± 0.29) log10TCID50/ml. A similar reduction was also obtained for 1.5% sodium hypochlorite under high soiling conditions for TGEV.

Acetic acid at concentrations of 3.5%, 2.5%, and 1.5%; hydrogen peroxide at concentrations of 2%, 1%, and 0.5%; and phenol at concentrations of 2%, 1.5%, and 1% didn't show virucidal effectiveness against both TGEV and PRCV under both conditions tested. A summary of the results is presented in Figs. 3 and 4.

All tested concentrations of glutaraldehyde, formaldehyde, and benzalconium chloride showed cytotoxic effects on ST cells, both when PRCV and TGEV were tested. Despite the purification technique used, it was not possible to obtain titer reduction results above ≥ 4 log10TCID50/ml in the purified samples. Minimally greater virus titer reductions were observed for PRCV than TGEV, particularly when glutaraldehyde was tested.

In addition, two-way ANOVA was used to compare two variables, chemical compounds and virus type (PRCV vs TGEV) and chemical compounds and conditions (high vs low soiling conditions), and their influence

Table 2 Titers reduction obtained for PRCV treated with different disinfectants (30 min contact time at 10 °C with low- and high-level soiling). The reduction is the difference between the titer values obtained for the control and the tested concentrations of disinfectant. Here, the reduction is presented as the mean ± standard deviation of three runs

Disinfectant	Tested concentration	Titer reduction (± SD) ($TCID_{50}$/ml)	
		Low level soiling (3.0 g/l BSA)	High level soiling (10 g/l BSA + 10 g/l YE)
ethanol	80%	**4.67 (± 0.29)**	**5.00 (± 0.00)**
	60%	**4.67 (± 0.29)**	**4.50 (± 0.00)**
	40%	**4.67 (± 0.29)**	**4.50 (± 0.00)**
2-propanol	80%	**4.67 (± 0.29)**	**5.00 (± 0.00)**
	60%	**4.67 (± 0.29)**	**4.50 (± 0.00)**
	40%	**4.67 (± 0.29)**	**4.50 (± 0.00)**
Sodium hypochlorite	1.5%	3.67 (± 0.29)	2.17 (± 0.76)
	1%	3.67 (± 0.29)	2.33 (± 0.29)
	0.5%	3.67 (± 0.29)	1.00 (± 0.00)
Acetic acid	3.5%	0.67 (± 0.76)	0.33 (± 0.76)
	2.5%	0.17 (± 0.76)	0.33 (± 0.29)
	1.5%	0.17 (± 0.76)	0.33 (± 0.29)
Hydrogen peroxide	2%	1.5 (± 0.87)	1.17 (± 0.76)
	1%	0.67 (± 0.29)	0.83 (± 0.29)
	0.5%	0.67 (± 0.29)	0.0 (± 0.50)
Caustic soda	3%	**4.20 (± 0.17)**	**4.33 (± 0.29)**
	2%	**4.20 (± 0.17)**	**4.33 (± 0.29)**
	1%	**4.67 (± 0.29)**	**4.67 (± 0.29)**
Phenol	2%	1.00 (± 0.50)	1.00 (± 0.00)
	1.5%	3.00 (± 0.50)	2.83 (± 0.29)
	1%	1.83 (± 0.58)	0.83 (± 0.58)
Potassium peroxymonosulfate	2%	**4.33 (± 0.29)**	**4.00 (± 0.00)**
	1%	**4.33 (± 0.29)**	**4.00 (± 0.00)**
	0.5%	**5.33 (± 0.29)**	**5.00 (± 0.00)**
Glutaraldehyde[C]	1%	3.00 (± 0.00)*	3.33 (± 0.29)*
	0.5%	3.00 (± 0.00)*	3.33 (± 0.29)*
	0.1%	3.00 (± 0.00)*	3.33 (± 0.29)*
Formaldehyde[C]	0.5%	2.00 (± 0.00)*	2.33 (± 0.29)*
	0.275%	2.00 (± 0.00)*	2.33 (± 0.29)*
	0.125%	1.83 (± 0.76)*	2.50 (± 0.50)*
Benzalkonium chloride[C]	1.5%	2.00 (± 0.00)*	2.33 (± 0.29)*
	1%	2.00 (± 0.00)*	2.33 (± 0.29)*
	0.5%	2.00 (± 0.00)*	2.33 (± 0.29)*

BSA Bovine serum albumin, *YE* Yeast extract

[C]A cytotoxic effect was observed. The results are presented after cytotoxicity reduction

*Due to cytotoxicity, the detection limit did not allow detection of greater virus reduction

on log10TCID50/ml virus reduction. The results revealed that most of the tested chemical compounds and their concentrations had the same effect on the PRCV and TGEV. Only the reductions in infectivity caused by 80% 2-propanol ($p < 0.001$), 40% ethanol ($p < 0.01$), 2% and 1.5% phenol ($p < 0.05$), 0.5% and 1% glutaraldehyde ($p < 0.05$) and 1.5% sodium hypochlorite ($p < 0.01$) under low soiling conditions, and by 1.5% ($p < 0.05$) and 1% phenol ($p < 0.01$) under high soiling conditions were significantly different between TGEV and PRCV (Fig. 5).

Statistical analyses comparing the virucidal activity of compounds depending on the conditions (low vs. high levels of soiling) showed that sodium hypochlorite is particularly sensitive to the presence of organic compounds. Statistical differences were found for 0.5% and 1% sodium hypochlorite ($p < 0.001$) when TGEV was tested and for 0.5% ($p < 0.001$), 1% ($p < 0.05$), and 1.5% ($p < 0.01$) sodium hypochlorite when the PRCV was tested (Figs. 6 and 7). In addition, significant differences were observed for 2% hydrogen peroxide ($p < 0.01$), 3.5% acetic acid ($p < 0.05$), and 0.5% formaldehyde ($p < 0.01$), but only for TGEV. The differences in the activity of these compounds at low and high levels of soiling were not statistically significant when the PRCV was tested.

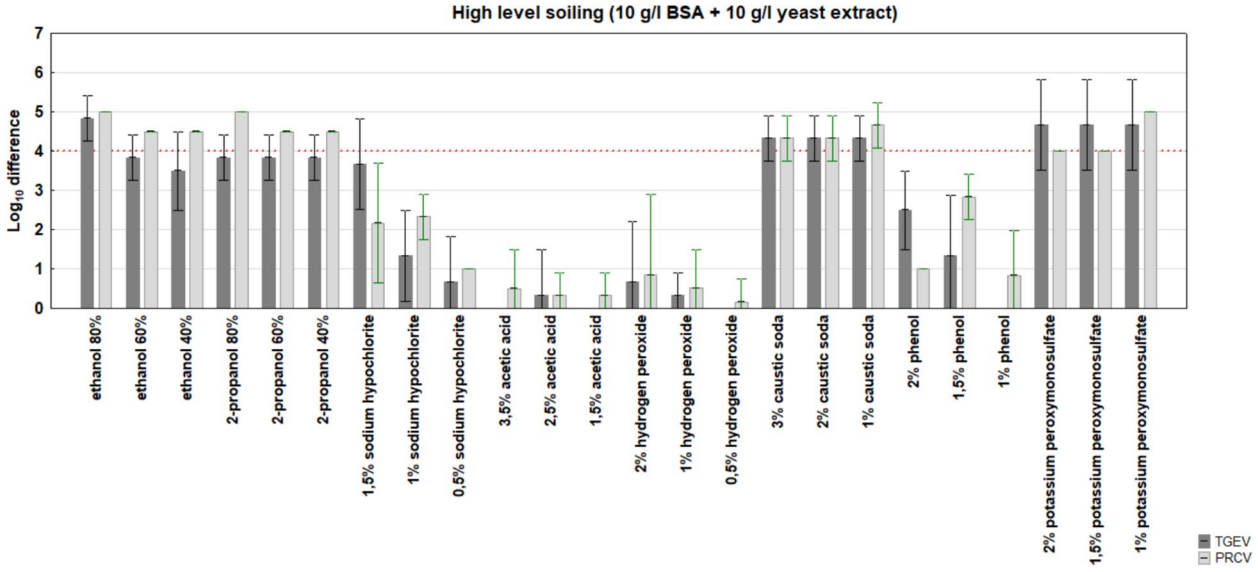

Fig. 3 Virucidal efficacy of the tested chemical compounds under high-level soiling conditions. The values shown are the mean ± standard deviations of three independent replicates. The red dotted line represents the virucidal effect threshold (≥ 4 log10TCID50/ml). The light gray color represents the results for the PRCV, and the dark gray color represents the results for the TGEV

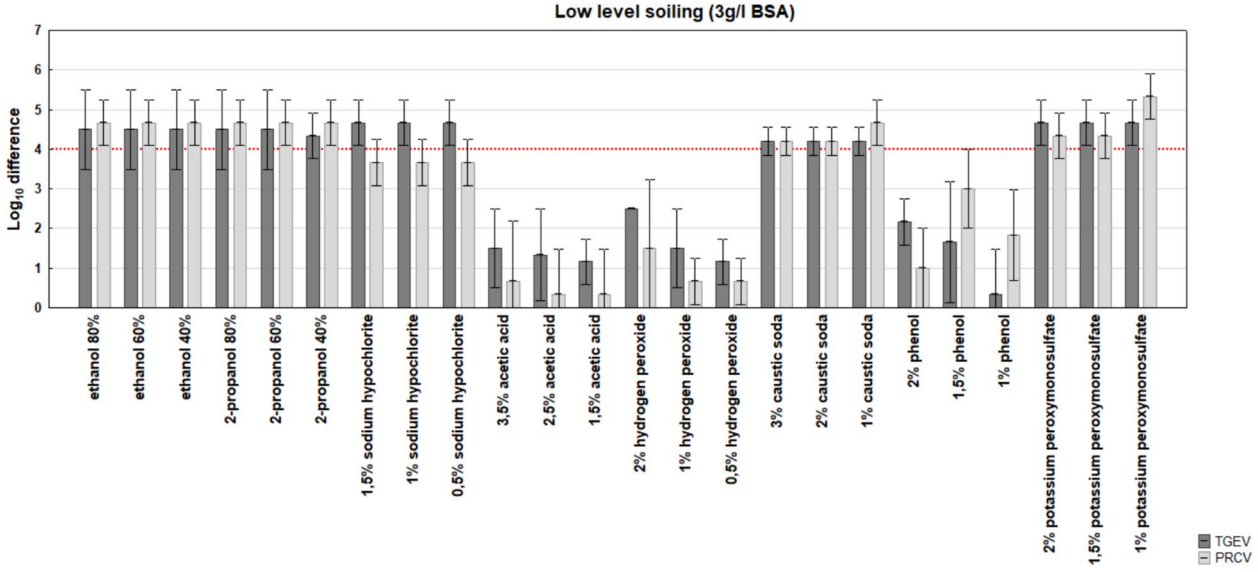

Fig. 4 Virucidal efficacy of the tested chemical compounds under low-level soiling conditions. The values shown are the mean ± standard deviations of three independent replicates. The red dotted line represents the virucidal effect threshold (≥ 4 log10TCID50/ml). The light gray color represents the results for the PRCV, and the dark gray color represents the results for the TGEV

Discussion

There are only two reports on the virucidal activity of chemicals against TGEV [30, 31]. However, there are no reports on the effectiveness of chemical compounds against PRCV. The lack of effective treatments underscores the importance of identifying such agents against TGEV/PRCV. Therefore, in this study, we tested eleven of the most popular chemicals representing alcohols, oxidants, acids, aldehydes, phenols, and quaternary compounds, against TGEV and PRCV. Since special attention should be given to the use of products with proven efficiency against pathogens, we tested the virucidal activity of these chemical compounds in an in vitro suspension test based on the EN 14675 European standard. The modification of the method involved only adapting the assay to TGEV/PRCV using the appropriate medium, cell line, and virus propagation methods. It is worth noting that in this study tests were conducted under both low- and high-soiling conditions, which reflect the actual conditions in which the disinfectants will be used. In

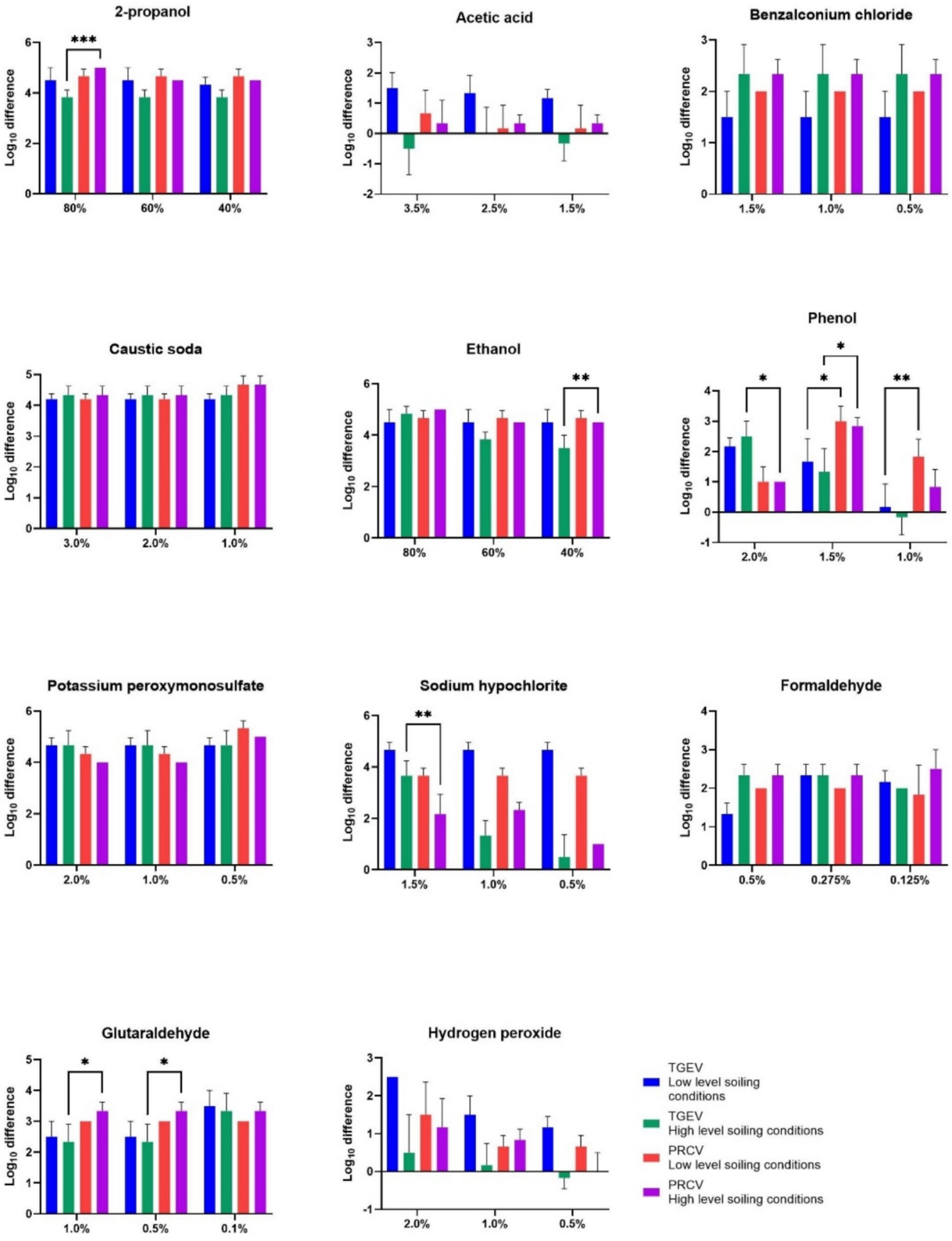

Fig. 5 Statistical comparison of the virucidal activity of the chemical compounds for TGEV and PRCV. The data are presented as the mean ± SD of three independent assays performed in triplicate. Asterisks represent statistical significance: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; nonsignificant reduction is not marked

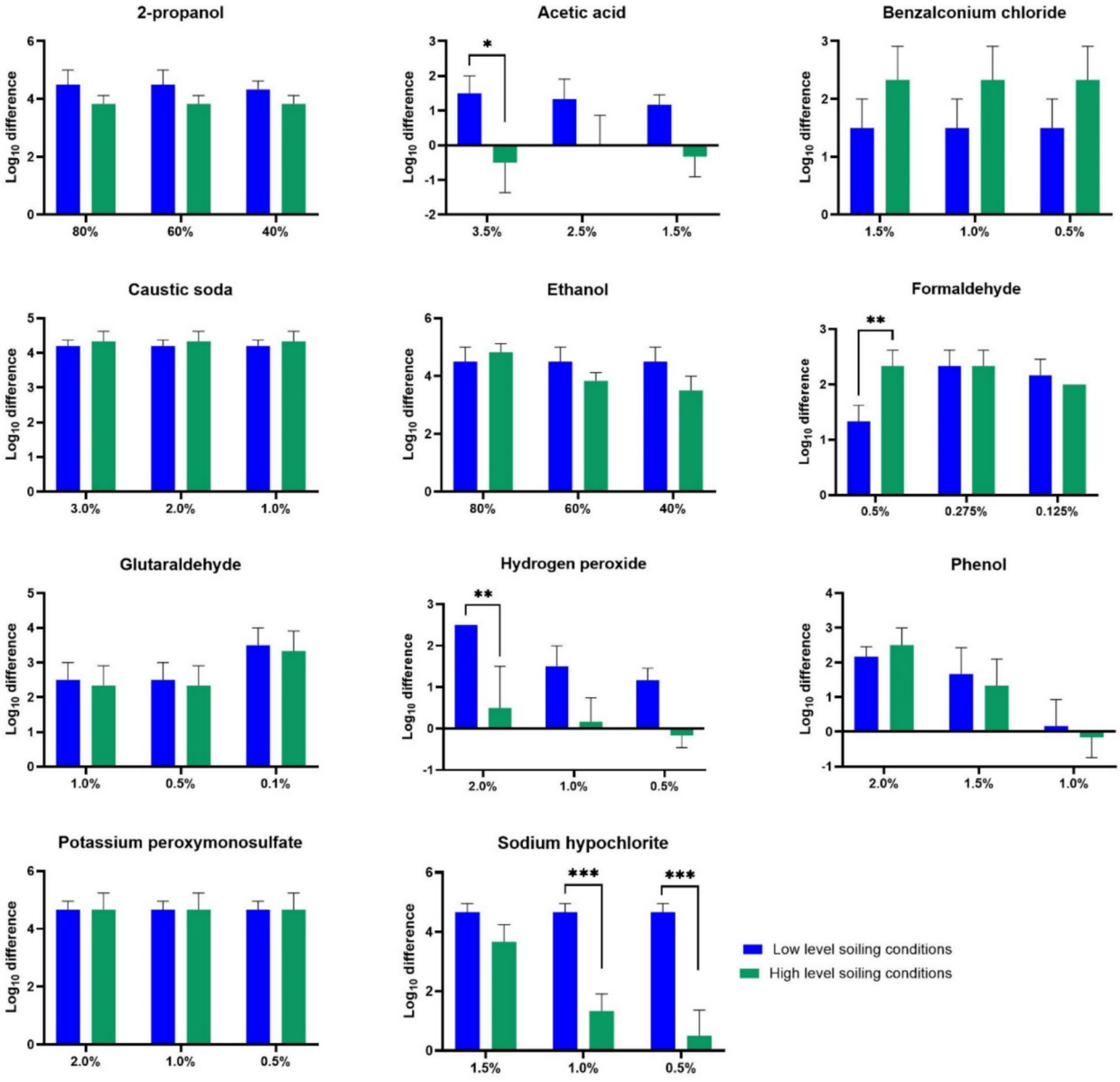

Fig. 6 Statistical comparison of the virucidal activity of the chemical compounds in low- and high-level soiling against TGEV. The data are presented as the mean ± SD of three independent assays performed in triplicate. Asterisks represent statistical significance: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; nonsignificant reduction is not marked

veterinary practice, the high level soiling (dirty condition) is particularly important because it reflects the realities of farm environments, where disinfectants must work effectively in the presence of organic materials such as feces, urine, and other secretions. The rigorous testing under these conditions ensures that the disinfectants used are not only effective in laboratory tests but also in the challenging field conditions and prevent the spread of infectious diseases on farms.

Our results revealed that 5 and 4 of the 11 chemical compounds effectively inactivated TGEV and PRCV, respectively. These compounds include ethanol, 2-propanol, sodium hypochlorite, caustic soda, and potassium peroxymonosulfate. However, these chemical compounds were effective at specific concentrations and under different soiling conditions.

2-propanol and ethanol are the main alcohols capable of inactivating a broad spectrum of viruses, bacteria, and fungi. The inactivation mechanism of these alcohols is rather nonspecific and involves disruption of the cell membrane or virus lipid envelope and denaturation of proteins [22, 32, 33]. These alcohols have shown virucidal properties mainly against enveloped viruses (hepatitis B virus, herpes virus, human immunodeficiency virus (HIV), and coronaviruses), whereas they were rather ineffective against nonenveloped viruses [34–38].

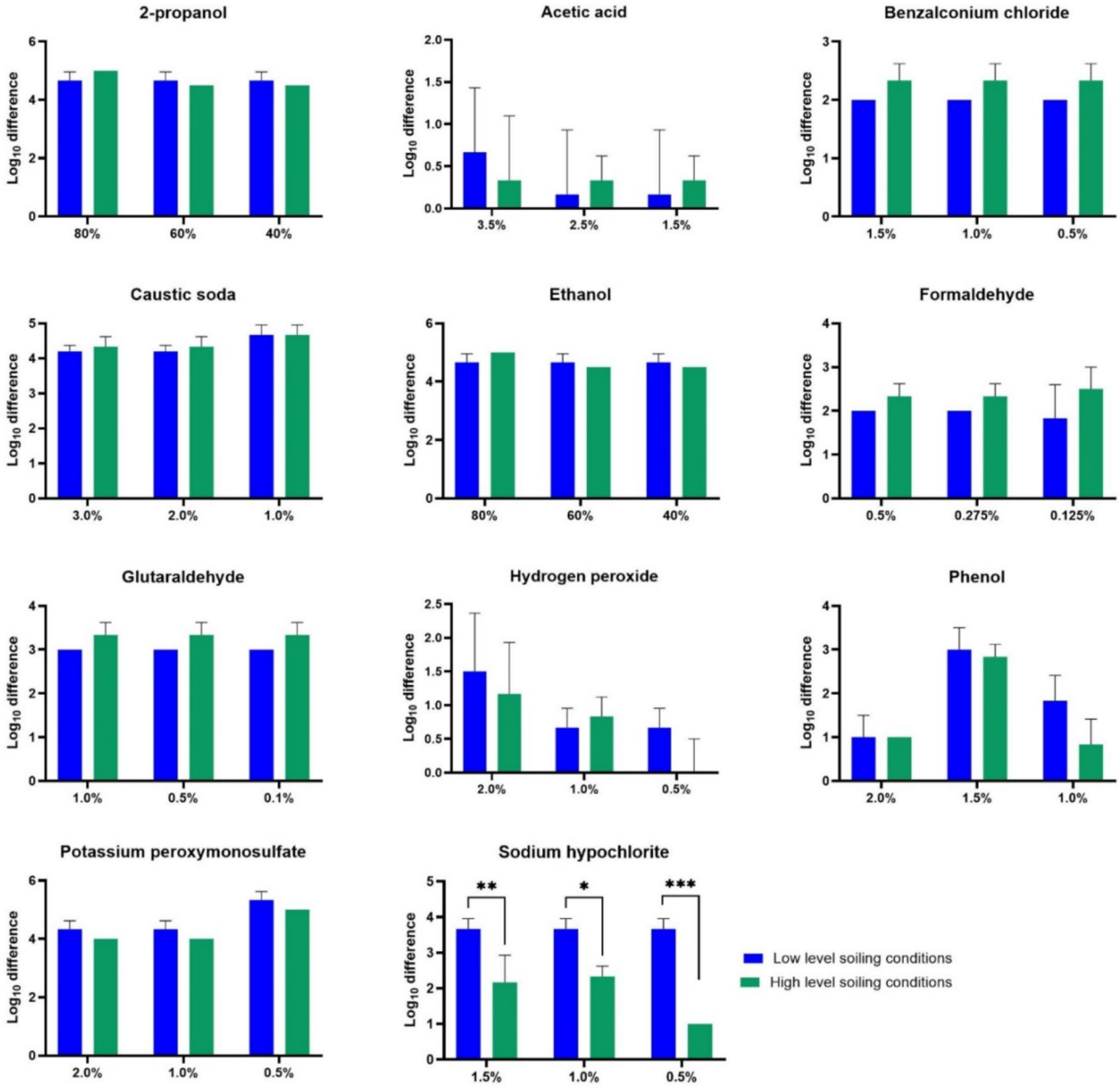

Fig. 7 Statistical comparison of the virucidal activity of the chemical compounds in low- and high-level soiling against the PRCV. The data are presented as the mean ± SD of three independent assays performed in triplicate. Asterisks represent statistical significance: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; nonsignificant reduction is not marked

A number of studies have shown that concentrations of ethanol and 2-propanol ranging from 30 to 90% were active against many coronaviruses, including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome-related coronavirus (MERS-CoV), murine hepatitis virus (MHV), canine coronavirus (CCoV) and bovine coronavirus (BCoV) [30, 31, 39–47]. Kratzel et al. [37] and Huang et al. [47] revealed that > 30% ethanol concentrations was effective to completely inactivte SARS-CoV-2 within 30 and 15 s, respectively [37, 47]. However, the efficiency of alcohols is optimal in the 60%–90% range. At this concentration, ethyl alcohol is a potent virucidal agent that effectively inactivates all lipophilic viruses, including vaccinia, herpes and influenza viruses, as well as many hydrophilic viruses such as enteroviruses, adenoviruses, rotaviruses and rhinoviruses [48]. Brown et al. showed that 70% ethanol reduced TGEV titers by 4.5 log10TCID50/ml in a suspension assay when the virus-disinfectant mixture was incubated for 5 min at room temperature without organic load [26]. However, Hulkower et al. revealed that 70% ethanol reduced the titer of infectious TGEV by 3.19 (2.97–3.40) log10TCID50/ml on surfaces after 20 min of exposure in the presence of 15% beef extract

[25]. Our results revealed that only 80% ethanol resulted in a ≥ 4 log10TCID50/ml reduction in TGEV titer under low- and high-level soiling conditions. In addition, 40% and 60% ethanol showed virucidal activity (reduction ≥ 4 log10TCID50/ml) against TGEV only under low-level soiling conditions, whereas under high-level soiling conditions reduced TGEV titers above 3.5 log10TCID50/ml. However, it is very difficult to compare these data since the viral infectivity models and protocols used for disinfectant testing were different. It is also known that carrier tests are more demanding, and it is more difficult to obtain ≥ 4 log10TCID50/mL (99.99%) reduction in viral titer. Our results also revealed that even 40% ethanol reduced PRCV titers above 4 log10TCID50/ml under high- and low-level soiling conditions. 2-propanol reduced the PRCV/TGEV titers above 3.82 log10TCID50/ml. These findings confirm that alcohol-based disinfectants are effective against coronaviruses, including TGEV and PRCV.

Sodium hypochlorite (NaOCl) is a strong chlorine-oxidizing agent and is widely used as a disinfectant because of its broad biocidal activity, fast action, and low cost. This compound acts against viruses mainly by disrupting the viral capsid and nucleic acid [32]. According hierarchy paradigm sodium hypochlorite should inactivate not only enveloped viruses but also less susceptible, non-enveloped viruses [34]. Several publications have investigated the effects of NaOCl against coronaviruses (mainly SARS-CoV-2) with different results depending on whether the tests were carried out in suspension or on the surface and in the presence or absence of organic matter. Watanabe et al. revealed that 1% sodium hypochlorite inactivated SARS-CoV-2 below the detection limit after 0.5 min of exposure under clean conditions without interfering substances when a suspension test according to EN14476 was performed [49]. Brown et al. revealed that sodium hypochlorite diluted 1/32 reduced TGEV titer above 4 log10TCID50/ml after 5 min at room temperature without organic load [26]. Al-Khleif revealed that sodium hypochlorite was effective against SARS-CoV-2 at 0.25% (0.0325% active chlorine) after 15 min at 20 °C without an organic load and at 1% (0.13% active chlorine) after 60 min of contact time at 20 °C with BSA and yeast extract as interfering substances [50]. Furthermore, Morris and Esseili revealed that in a suspension test, sodium hypochlorite resulted in a > 3 log10TCID50/ml reduction in the SARS-CoV-2 titer at 50 ppm with a 1-min contact time and in the absence of an organic load. However, in the presence of organic loading, 200 ppm was required to obtain a > 3 log10TCID50/ml reduction in the virus titer [51]. Kapes et al. [34] showed that SARS-CoV-2 and two other enveloped viruses, bovine viral diarrhea virus and vaccinia virus, were completely inactivated by 100 ppm sodium hypochlorite after 1 min of contact time and with 5% FBS as an organic load [34]. In contrast, NaOCl at 200 ppm was not effective against SARS-CoV-2 when the carrier method was used. In this case, 1000 ppm sodium hypochlorite for 10 min was effective [51]. On a stainless steel carrier, 1500 ppm sodium hypochlorite was ineffective in inactivating ASF in the presence of blood and feces [52], and 0.06% (600 mg/L) sodium hypochlorite minimally (0.35 log10TCID50/ml) inactivated TGEV after 1 min of exposure on stainless steel in the presence of 15% beef extra [25]. Sodium hypochlorite of 100 ppm effectively inactivated the HIV-1 virus within 30 s on a clean surface. In contrast, 500 ppm and 1–2 min were required to inactivate the HIV-1 virus in the presence of 80% serum and about 10,000 ppm (1%) of sodium hypochlorite was required to inactivate this virus in the presence of 80% blood [53]. The results of our study also confirmed that the activity of sodium hypochlorite is reduced in the presence of organic matter. Therefore, a cleaning process before sodium hypochlorite disinfection is important to ensure an effective disinfection process [54–56]. In addition, the results of the above studies clearly indicate that only rigorous testing under high-level soiling conditions ensures that the disinfectants used are effective in challenging field conditions.

Although alcohols and sodium hypochlorite are effective in eliminating coronaviruses, other disinfectants, such as caustic soda (NaOH) and potassium peroxymonosulfate, exhibit stronger antiviral properties. Our study showed that only caustic soda and potassium peroxysulfate exhibited virucidal activity at every concentration tested against both TGEV and PRCV under clean and dirty conditions (> 4 log10TCID50/ml reduction), indicating that these compounds retain their disinfectant properties in the presence of organic material. Caustic soda is a strongly alkaline compound, whereas potassium peroxymonosulphate is an oxidizing disinfectant. Both are commonly used as disinfectants in the food and livestock industries because of their broad spectrum of action [52, 57–62]. Caustic soda has been shown to be effective in inactivating viruses with a lipid envelope, such as pseudorabies virus and HIV, as well as viruses without an envelope, such as minute virus of mice and porcine circovirus type 2 [56–58]. Concentrations of 2 and 3% caustic soda were effective in reducing ASFV titers by more than four log10TCID50/ml after 30 min of exposure at 10 °C under low and high contamination conditions. In contrast, potassium peroxysulfate at dilutions of 0.5% and 1% was effective in reducing ASFV titers under both soiling conditions [28]. Potassium peroxysulfate was also shown to be able to inactivate enveloped viruses such as avian influenza virus (AIV) and Newcastle disease virus (NDV) on surface carriers at

concentrations of 1×, 0.5×, 0.25× and 0.125× after 30 s exposure time [63].

In this work, it was not possible to estimate the virucidal activity of glutaraldehyde, formaldehyde, and benzalkonium chloride because they exhibited cytotoxic effects on ST cells at all dilutions tested. Despite the cytotoxicity reduction technique used, it was not possible to obtain titer reduction results above ≥ 4 log10TCID50/ml in the purified samples. The reduction in TGEV/PRCV titers for these compounds ranged from 1.33 to 3.33 log10TCID50/ml and was slightly greater for PRCV than for TGEV. However, the observed cytotoxicity of these compounds was not surprising, as it had already been demonstrated by many authors [28, 40, 64–67]. According to the European standard EN 14675, when cytotoxicity is so high that a reduction in virus titer within 4 log10TCID50/ml cannot be observed, a molecular sieving with a molecular sievefilter (i.e. Sephadex™ LH 206) or ultrafiltration with Minicon®6) (Millipore) or with ready-to-use columns, i.e. MicroSpinTM S 400 HR6) (GE Healthcare) should be used. In this study Microspin S-400 HR columns were used to remove cytotoxic products from the tested mixture. The other techniques were not tested, which is a limitation of the publication.

Our study showed the lack of virucidal efficacy (reduction ≥ 4 log10TCID50/ml) of acetic acid, hydrogen peroxide, and phenol at all concentrations tested against both PRCV and TGEV. Acetic acid was effective against enveloped viruses, such as vaccinia virus, African swine fever virus, influenza virus, and SARS-CoV [28, 52, 68–71]. Krug et al. found that for ASFV dried on nonporous surfaces, a 10-min treatment with ≥ 1% citric acid appeared to reduce virus titers by 4 log10TCID50/ml, while for ASFV dried on porous surfaces, exposure to a higher concentration of citric acid (2%) for a longer time (30 min) was required to effectively reduce ASV titers [72, 73]. Juszkiewicz et al. showed that only higher concentrations of acetic acid (2% and 3%) reduced ASV titers by 4 log10TCID50/ml under low-level soiling conditions, but only 3% acetic acid reduced ASV titers by 4 log10TCID50/ml under high-level soiling conditions [28]. Acetic acid significantly reduced the SARS-CoV-2 titer at pH 2 but did not significantly reduce the titer at pH 4 or 6. Chin et al. also reported that SARS-CoV-2 was extremely stable over a wide pH range (between 3 to 10) at room temperature for 1 h [43]. Darnell et al. showed that SARS-CoV-2 was relatively stable at a pH in the range of 5 to 9 at temperatures of 4°C-37°C, but pH below 3 and above 12 effectively inactivated the virus [74]. As demonstrated by Lai et al. SARS-CoV-2 could survive at pH 8 to 9 for 1 to 5 days, but only a few hours at pH 6 [75].

The lack of virucidal activity of acetic acid at concentrations of 1.5%, 2.5%, and 3.5% against PRCV and TGEV in our paper may be due to the fact that coronaviruses are generally more stable at slightly more acidic pH values than at alkaline pH values [74]. There are no reports on the pH sensitivity of PRCV, but it has been reported that TGEV is rather stable from pH 5 to 8 at 4 °C. It was also shown that changes in pH had no effect on the steps of adsorption, penetration, and envelope removal in the TGEV replication cycle [76]. Moreover, TGEV causes intestinal diseases such as diarrhea, suggesting that this coronavirus is resistant to stomach acid. Therefore, coronaviruses are potentially more resistant to low pH than other enveloped viruses [77].

In our study, the highest TGEV and PRCV titer reductions caused by phenol (1.5% –2%) were 2.5 (± 0.5) and 3.0 (± 0.5) log10TCID50/ml, respectively. These results are quite consistent with previous reports that showed moderate effects of phenols against coronaviruses [78]. For example, the combination of 9.09% O-phenylphenol and 7.66% P-tertiary amylphenol moderately reduced the titer of TGEV on steel carriers (2.03 log10TCID50/ml) [25]. Chloroxylenol at a concentration of 0.24% was unable to effectively reduce the titer of OC43 coronavirus within 10 min and at 20°C [79]. Moreover, moderate reductions in the titers of canine coronavirus and mouse hepatitis virus were achieved with cresol (methiphenol) [80]. In addition, phenol at a dilution of 1:256 did not reduce PEDV RNA load in cell culture in the presence of a 10% (v/v) fecal slurry [81]. Moreover, Juszkiewicz et al. showed that only 1% phenol caused a four log10TCID50/ml reduction in the ASFV titer under low- and high-soiling conditions [28].

Hydrogen peroxide (H_2O_2) is an oxidizing agent that is believed to be effective against a broad spectrum of pathogens, including bacteria, yeasts, and viruses. However, many papers indicate that diluted aqueous solutions of hydrogen peroxide are ineffective disinfectants. Research results indicate that hydrogen peroxide has virucidal activity but at relatively high concentrations. It is believed that only a concentration of ≥ 3% hydrogen peroxide is an effective disinfectant [82]. A 6% hydrogen peroxide solution is approved by the Centers for Disease Control and Prevention (CDC), the U.S. Environmental Protection Agency (EPA), and the Occupational Safety and Health Administration (OSHA) to combat COVID-19. Lee et al. [40] reported that SARS-CoV-2 was completely inactivated by hydrogen peroxide but only at a high concentration (~ 150 mM, 0.5%) after 10 min of exposure, while Mileto et al. reported that a 3% w/w hydrogen peroxide solution acidified to pH 2.5 inactivated the SARS-CoV-2 virus by more than 4 log10TCID50/ml within 5 min. However, hydrogen peroxide solutions without additives showed little virucidal activity (1.1 log10TCID50/ml reduction in 5 min), confirming that the pH-modifying component is essential for obtaining an H2O2-based disinfectant active

against SARS-CoV-2 [40, 83]. Goyal et al. reported that human SARS-CoV-2 was inactivated in a carrier test by hydrogen peroxide vapor at a concentration of 35% [84]. Urushidani et al. reported that SARS-CoV-2 was more resistant to the virucidal effects of aerosolized hydrogen peroxide than influenza A virus; therefore, higher disinfectant concentrations or longer contact times were required to inactivate the SARS-CoV-2 virus than the influenza A virus [85]. Gabbert et al. showed that the hydrogen peroxide product had low (less than 2 log10TCID50/ml) efficacy in reducing ASF virus titers [86]. A 13% hydrogen peroxide solution inactivated the enveloped Herpes Simplex virus and non-enveloped polio virus by more than 5 log10TCID50/ml within 5 min [87]. Moreover, 0.5% accelerated hydrogen peroxide was effective (reduction > 4 log10TCID50/ml) at 20°C for 1 min and in the presence of 5% serum against both enveloped and non-enveloped viruses, including coronavirus 229E, HIV, rotavirus and polio virus [88]. The lack of virucidal activity of hydrogen peroxide in our work may be due to the fact that only 2% and lower concentrations of this compound were studied. Two percent hydrogen peroxide resulted in 2.5 and 1.5 log10TCID50/ml reductions in TGEV and PRCV virus titers, respectively, under clean conditions. Thus, we suppose that higher concentrations of hydrogen peroxide could be effective disinfectants against TGEV/PRCV.

In conclusion, our results revealed that ethanol, 2-propanol, sodium hypochlorite, caustic soda, and potassium peroxymonosulfate were the most effective chemicals against TGEV and PRCV. In contrast, acetic acid, hydrogen peroxide, and phenol at all the tested concentrations failed to show virucidal efficacy against both TGEV and PRCV under both conditions tested. Benzalkonium chloride (0.5%, 1%, and 1.5%), formaldehyde (0.125%, 0.275%, and 0.5%) and glutaraldehyde (0.1%, 0.5%, and 1%) were found to be cytotoxic, limiting the detection of viral infectivity reduction to less than 4 log10TCID50/ml. Our study also revealed that caustic soda and potassium peroxysulfate were the most stable disinfectants and that organic matter notably reduced the activity of sodium hypochlorite. To the best of our knowledge, this study is the first using a quantitative suspension test method based on the European Standard EN 14675 to evaluate the activity of chemical compounds against TGEV and PRCV. Future studies on the efficacy of disinfectants against TGEV/PRCV under different conditions and dilutions would be beneficial. In particular, the virucidal activity of disinfectants should be tested under conditions of cold and organic contamination that reflect winter field conditions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-05119-7.

Supplementary Material 1

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Not applicable.

Authors' contributions
Conceptualization: MO. Data curation: MO and MA. Formal analysis: MO and MA. Investigation: MO and MA. Methodology: MO and MA. Resources: MO and MA. Writing—original draft: MO and MA. Writing—review and editing: MO and MA. Supervision: MO. All the authors read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are available from the corresponding author on reasonable request.
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Declarations

Ethics approval and consent to participate
This study did not involve any animal experiments.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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