Comparative inactivation of *Aujeszky's disease virus*, *Porcine teschovirus* and *Vesicular stomatitis virus* by chemical disinfectants

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ABSTRACT: We tested the germicide activity of 1% Chloramin BM, 1% Incidin Plus, 1% Lysoformin 3000, 0.2% Mikasept KP, and 2% Sekusept Forte against viruses in suspension (suspension test) and dried onto a surface (carrier test). The agents of the porcine encephalomyelitis (*Porcine teschovirus*, strains CAPM V-86, CAPM V-37), Aujeszky's disease (strains CAPM V-166, CAPM V-327) and vesicular stomatitis (strains CAPM V-499, CAPM V-331) were used as model viruses. After 30 min contact time in both the suspension and carrier tests, the *Porcine teschovirus* was 4 lg inactivated only by Mikasept, which was thus the only disinfectant to meet the standard. The other disinfectants decreased the viral titre insufficiently. Under the same conditions, *Aujeszky's disease virus* was inactivated by at least 4 lg by all the tested disinfectants except for Chloramin BM which decreased the titre of CAPM V-166 only by 3.75 lg in the carrier test. For the inactivation of *Vesicular stomatitis virus* Chloramin BM and Mikasept KP were tested. Both the disinfectants reliably decreased the viral titre in both the suspension and carrier tests. Our results show that the inactivation of a surface-bound virus is more difficult than its inactivation in suspension. We confirm the high resistance of non-enveloped viruses (*Porcine teschovirus*) to chemical inactivation.

Keywords: virucidal activity; suspension test; carrier test

Viruses usually manifest a relatively high ability to survive on contaminated surfaces because of their minimal metabolic activity and stable structure. The success of decontamination of surfaces (e.g. in microbiological laboratories) depends on the type of active substances and the concentrations of disinfectants, the contact time, the type and number of microorganisms and surrounding conditions, e.g., viral particles embedded in organic or cellular debris are better protected against the influence of environment (Assar and Block, 2001).

Commercially available chemical germicides with several effective substances, such as alcohols, halogens, phenolic compounds, aldehydes and surface-active agents, are commonly used for the decontamination of surfaces. The disinfectants usually inactivate viruses by disrupting their surface structures and attachment proteins. Viruses covered with a lipid membrane (enveloped viruses) are usually much more susceptible to chemical disinfection than non-enveloped viruses (Sattar and Springthorpe, 2001).

Most animal viruses are nowadays cultivated and quantitated in *in vitro* cultures of mammalian cells, where viral replication often leads to degenerative changes referred to as cytopathic effects (CPE). These visual changes in the cultures of infected cells, examined under a light microscope and compared to a negative control, form a basis of many methods for the determination of the virucidal activity of germicides. The virucidal activity of chemical germicides can be tested either in suspension

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(EN 14675, 2006) or in carrier tests (Gaustad et al., 1974; Sattar et al., 2003). The suspension tests are generally easier to perform and they are suitable for the evaluation of the basic activity of disinfectants (Sattar and Springthorpe, 2001). Since pathogens are normally found adsorbed to surfaces and embedded in organic debris, however, the results of carrier tests are more relevant for predicting the activity of chemical germicides in practice (Sattar et al., 2003; Ferrier et al., 2004).

Since the late 1970s, standard methods for the determination of the virucidal activity of disinfectants have been developed in different countries. In 1990 the European Committee for Standardization (CEN) set up a Technical Committee for chemical disinfectants and antiseptics (CEN/TC 216) whose methods have replaced heterogenous national standards used before in individual EU countries (Cremieux et al., 2001).

All applications in the European standard for evaluation of virucidal activity of disinfectants in suspension in the veterinary area (EN 14675) use a single viral species as a model. However, there are large differences in resistance against chemicals between and within different viral groups (Prince and Prince, 2001). The aim of our work was to test selected disinfectants commonly used in laboratories and to evaluate their virucidal activity against *Aujeszky's disease virus*, *Porcine teschovirus* and *Vesicular stomatitis virus*.

MATERIAL AND METHODS

Viral strains

Six different viral strains were used in this study. These included: *Porcine teschovirus* – PTV (strains: Roznava, CAPM V-86 and Talfan, CAPM V-37), *Aujeszky's disease virus* – AV (strains: Aujeszky, CAPM V-166 and V-8/73, CAPM V-327) and *Vesicular stomatitis virus* – VSV (strains: Indiana, CAPM V-331 and Hazlehurst, CAPM V-499). PTV and AV were propagated in a porcine kidney cell line (PK 15). VSV was propagated in an African green monkey kidney cell line (VERO). Cell cultures showing extensive cytopathic effects were frozen, thawed and cell debris were separated by centrifugation (500 g, 10 min). The stocks of viral suspensions were aliquoted into 1.5 ml tubes and stored at –80°C.

Disinfectants

The tested disinfectants together with their characterization are listed in Table 1. The tested solutions were prepared in sterile distilled water. For the suspension test, the concentration of the disinfectant was prepared to be 1.25 times greater than the desired test concentration as it was diluted to 80% during the test.

Cytotoxicity caused by disinfectants

To detect any possible structural alteration of cells caused by the disinfectants themselves (not by the virus), we prepared ten-fold dilutions of disinfectants and inoculated 250 µl each into four wells of microtitre plates containing a cell monolayer (PK 15 and VERO). Any microscopic changes in the cells were recorded when checking the tests for CPE after five days of cultivation at 37°C. The level of cytotoxicity was quantified in lg $TCID_{50}$ by the Spearman-Karber method (Finney, 1964).

Table 1. Disinfectants used for the evaluation of virucidal activity

Disinfectant	Chloramin BM	Incidin Plus	Lysoformin 3000	Mikasept KP	Sekusept Forte
Manufacturer	Bochemie, Czech Republic	Ecolab, USA	Lysoform, Germany	Mika, Czech Republic	Ecolab, USA
Active substances (%)	chloramine B – trihydrate 72.5%, active chlorine 19%, sodium dodecylbenzenesulphonate < 3%	glukoprotamine 26%	glutaraldehyde 9.5%, glyoxal 7.5%, didecyldimetylammonium chloride 9.6%	peracetic acid 14%, hydrogen peroxide 21%, acetic acid, water	glutaraldehyde 3.75%, formaldehyde 11.1%, alkylbenzyldimethylammonium chloride 2.7%, glyoxal 12%
Concentration used in the study (%)	1	1	1	0.2	2

For testing, only those concentrations of disinfectants showing cytotoxicity which still allowed the determination of at least 4 lg reductions of titres of viral controls were used.

Interfering substance solution

An interfering substance was used to simulate the organic soiling that can reduce the virucidal activity of disinfectants. The solution was prepared according to the standard (EN 14675, 2006) for high level soiling. The final concentration in the test procedure was 10 g/l yeast extract and 10 g/l bovine albumin.

Suspension test

This method followed the EN 14675 norm with minor modifications. Test conditions were slightly adapted for laboratory conditions, e.g., all reagents were equilibrated to 20°C instead of the 10°C standardly used in veterinary area.

The suspension test was performed in a volume of 10 ml which consisted of 1 ml viral suspension, 1 ml interfering substance and 8 ml disinfectant. In viral controls the disinfectant was replaced with sterile distilled water. After mixing all the components, the tubes were placed in an incubator (20°C) for 30 min. At the end of the exposure time, 200 µl of each test mixture was pipetted into 1.8 ml cell culture medium placed on crushed ice to stop the effect of the disinfectant. Ten-fold dilutions up to 10^{-8} were prepared in the tubes containing cell culture medium at 0°C and each dilution was assayed for its viral titre.

Carrier test

A solution of 900 µl viral suspension and 100 µl interfering substance was prepared; 30 µl of this mixture was placed into a well of a cell culture plate and allowed to dry for one hour at room temperature in a biosafety cabinet. A 27 µl drop of disinfectant solution was placed on the spot of the dried virus, so that the solution covered the virus entirely. In viral controls, distilled water was applied instead of the disinfectant. The cell culture plate was incubated for 30 min at 20°C. After this exposure time, 243 µl of iced (0°C) cell culture medium was added into the well to stop the effect of the disinfectant and the virus was resuspended by scraping. Ten-fold dilutions up to 10^{-8} were prepared in the tubes containing 0°C cell culture medium and each dilution was assayed for the viral titre.

Viral titration and calculation of virucidal activity

Titres of viral controls and titres of viruses after treatment with disinfectants in suspension and carrier tests were determined as follows. Each dilution (250 µl) of tested suspension was transferred into four wells of microtitre plates containing the cell culture. Another four wells were inoculated with 250 µl of cell culture medium as uninfected negative controls. The microtitre plates were incubated for one hour at 37°C. Then 250 µl of the cell culture medium was added into each well. After five days, the visual changes in the cultures of infected cells (CPE) were examined against the negative control. The viral infectivity titre (lg $TCID_{50}$) was calculated using the Spearman-Karber method (Finney, 1964). The value of virucidal activity quantified the difference between the infectivity titre of viral control and the viral titre after disinfectant treatment (lg $TCID_{50}$ of water/virus suspension minus lg $TCID_{50}$ of disinfectant/virus suspension). Those disinfectants which decreased the viral infectivity titre at least by 4 lg (i.e. 99.99%) were considered efficient.

RESULTS

Firstly, the cytotoxicity of the disinfectants for the cell cultures was determined. Since the titres of the viruses used in this study ranged between 6.75 and 7.5 lg $TCID_{50}$, the cytotoxicity would have to be lower than 2.8 lg to allow determination of at least a 4 lg reduction in viral titre due to the action of disinfectants required by the standard (EN 14675). In the case of PK 15, the cell line used for propagation of PTV and AV, this was achieved when Chloramin BM, Incidin Plus and Lysoformin 3000 were used at 1% concentration, Mikasept KP at 0.2% and Sekusept Forte at 2% concentration. In the case of Lysoformin 3000 and Sekusept Forte these concentrations were already below the concentration recommended by the manufacturer. The

Table 2. Cytotoxicity of the disinfectants

Disinfectant	Chloramin BM	Incidin Plus	Lysoformin 3000		Mikasept KP	Sekusept Forte	
Concentration	1%	1%	1.5%	1%	0.2%	3%	2%
Cytotoxicity for PK 15 (lg $TCID_{50}$)	2.0*	2.75*	3.5	2.75*	2.5*	3.5	2.5*
Cytotoxicity for VERO (lg $TCID_{50}$)	2.5*	3.5	–	3.5	≤ 0.5*	–	3.5

*tolerable values of cytotoxicity

VERO cell line was more sensitive to the action of disinfectants than PK 15. Because of the cytotoxicity of Incidin Plus, Lysoformin 3000 and Sekusept Forte for the VERO cell line, the virucidal activity of these disinfectants against VSV (propagated in VERO) could not be tested (Table 2). Therefore, for the inactivation of VSV, only Chloramin BM (1%) and Mikasept KP (0.2%) were tested.

The results of the suspension tests are summarized in Table 3. Incidin Plus, Lysoformin 3000 and Sekusept Forte were tested only against AV and PTV. In all the cases it could be concluded that these disinfectants were efficient against AV but not against PTV. After a 30 min contact time under the conditions of high level soiling, Incidin Plus reduced the PTV titre on average by 1.375 lg, while the AV strains were effectively inactivated by Incidin, on average by more than 4.25 lg. The reduction of PTV by Lysoformin 3000 was only 2.125 lg; Lysoformin, however, effectively inactivated titres of AV by more than 4.125 lg. Sekusept Forte inactivated the titres of PTV insufficiently by 1.625 lg and reduced the titres of AV strains effectively by more than 4.5 lg.

Chloramin BM and Mikasept KP could be tested for their virucidal activity against all the viruses. Chloramin BM inactivated the titres of PTV by 2.5 and 3.5 lg, depending on the viral strain. According to the standard which requires a 4 lg reduction, the inactivation of the PTV titre must be considered as insufficient. When Chloramin was tested against both the enveloped viruses, the titre of AV was decreased on average by 4.75 lg and the titre of VSV by more than 4.5 lg. Chloramin can, therefore, be

Table 3. Results of the suspension tests

Virus	1% Chloramin BM		1% Incidin Plus		1% Lysoformin 3000		0.2% Mikasept KP		2% Sekusept Forte	
	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result
Porcine teschovirus CAPM V-86	2.5	–	1.0	–	2.0	–	≥ 4.75	+	1.25	–
Porcine teschovirus CAPM V-37	3.5	–	1.75	–	2.25	–	≥ 4.5	+	2.0	–
Aujeszky's disease virus CAPM V-166	5.0	+	≥ 4.5	+	≥ 4.25	+	≥ 4.5	+	≥ 4.5	+
Aujeszky's disease virus CAPM V-327	4.5	+	≥ 4.0	+	≥ 4.0	+	≥ 4.25	+	≥ 4.5	+
Vesicular stomatitis virus CAPM V-499	≥ 4.75	+	N		N		≥ 5.75	+	N	
Vesicular stomatitis virus CAPM V-331	≥ 4.25	+	N		N		≥ 5.25	+	N	

+ = sufficient (reduction of lg $TCID_{50}$ ≥ 4); – = insufficient (reduction of lg $TCID_{50}$ < 4); N = not tested

Table 4. Results of the carrier tests

Virus	1% Chloramin BM		1% Incidin Plus		1% Lysoformin 3000		0.2% Mikasept KP		2% Sekusept Forte	
	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result	reduction of lg $TCID_{50}$	result
Porcine teschovirus CAPM V-86	2.25	–	0.75	–	1.0	–	4.5	+	1.25	–
Porcine teschovirus CAPM V-37	2.75	–	1.25	–	1.0	–	≥ 5.5	+	1.0	–
Aujeszky's disease virus CAPM V-166	4.0	+	5.0	+	≥ 5.75	+	≥ 6.0	+	5.5	+
Aujeszky's disease virus CAPM V-327	3.75	–	4.5	+	≥ 5.0	+	≥ 5.5	+	≥ 5.25	+
Vesicular stomatitis virus CAPM V-499	4.25	+	N		N		4.25	+	N	
Vesicular stomatitis virus CAPM V-331	4.75	+	N		N		5.0	+	N	

+ = sufficient (reduction of lg $TCID_{50} \geq 4$); – = insufficient (reduction of lg $TCID_{50} < 4$); N = not tested

considered as suitable for the disinfection of AV and VSV but not PTV. Mikasept KP reduced all the viruses sufficiently, PTV on average by more than 4.625 lg, AV by more than 4.375 lg, and VSV by more than 5.5 lg.

The results of the carrier tests are summarized in Table 4. Similar to the suspension test, Incidin Plus, Lysoformin 3000 and Sekusept Forte were efficient against AV but not against PTV. After 30 min contact time under conditions of high level soiling, Incidin Plus reduced the titre of PTV by 1.0 lg, while the AV strains were inactivated by Incidin effectively, on average by more than 4.75 lg. The reduction of PTV by Lysoformin 3000 was only 1.0 lg, Lysoformin, however, effectively reduced the titres of AV by more than 5.375 lg. Sekusept Forte inactivated the infectivity titres of the PTV strains insufficiently by 1.125 lg, whereas the titres of AV were reduced effectively by more than 5.375 lg.

In the carrier test, Chloramin BM inactivated the titres of the PTV strains by 2.25 and 2.75 lg, and thus it was considered as insufficient. The reduction of AV by Chloramin was effective (by 4.0 lg) in the case of strain CAPM V-166, the other strain (CAPM V-327) was reduced insufficiently by 3.75 lg. The VSV strains were inactivated by Chloramin sufficiently on average by 4.5 lg. Mikasept KP reduced all the viruses effectively, PTV on average at least by 5.0 lg, AV by 5.75 lg and VSV by 4.625 lg.

DISCUSSION

In this study, the disinfectants used for the evaluation of virucidal activity were selected to represent a wide spectrum of active substances commonly used for the decontamination of laboratories. The test viruses included enveloped DNA viruses, members of the family Herpesviridae (AV), and enveloped RNA viruses of the family Rhabdoviridae (VSV), which are known for their high susceptibility to chemical inactivation (Wright, 1970; Heckert et al., 1997). PTV was a representative of small non-enveloped RNA viruses of the family Picornaviridae (genus *Teschovirus*) which are quite resistant to chemical disinfections (Derbyshire and Arkell, 1971).

Because of their high cytotoxicity for the VERO cell line, Incidin Plus, Lysoformin 3000 and Sekusept Forte were tested only against AV and PTV. Nevertheless, as members of both enveloped and non-enveloped viruses, a comparison of the effects of the used disinfectants on members of both basic viral groups was still possible. Incidin Plus at 1% concentration was not effective in the

inactivation of PTV, for a sufficient reduction it probably should be used in a higher concentration. Incidin was effective at 1% concentration only for the inactivation of AV.

Because of its high cytotoxicity, Lysoformin 3000 was tested at a lower concentration (1%) than recommended by the manufacturer (1.5%). This concentration reduced the infectivity titre of PTV insufficiently. The consequent concetration of glutaraldehyde in Lysoformin was less then 0.1% (Table 1). A sufficient inactivation of picornaviruses using a 1% clear glutaraldehyde has been reported (Saitanu and Lund, 1975; Bailly et al., 1991; Chambon et al., 1993). The used concentration of Lysoformin was however sufficient for an almost total inactivation of AV.

Sekusept Forte was tested at a lower concentration (2%) than recommended by manufacturer (3%) because of its high cytotoxicity. This concentration was not effective in inactivating titres of PTV strains. The concentration of aldehydes in Sekusept was ca. 0.3% (Table 1). A sufficient inactivation of picornaviruses has been described for 1% clear glutaraldehyde (Saitanu and Lund, 1975; Bailly et al., 1991; Chambon et al., 1993). Sekusept was sufficient for an almost total inactivation of AV.

Chloramin BM at 1% concentration was relatively effective in the inactivation of PTV in comparison with the remaining disinfectants, but still insufficient according to the standard requiring a 4 lg reduction. A better efficacy of similar chlorine disinfectants for the inactivation of PTV has been documented, e.g., 1% sodium hypochlorite completely inactivates a suspension of PTV (Derbyshire and Arkell, 1971). The efficacy of active chlorine has also been proven on other members of the family Picornaviridae, e.g., *Poliovirus* (Engelbrecht et al., 1980). The number of enveloped viruses was reduced sufficiently with the exception of a single strain (CAPM V-327) of AV in the carrier test. According to Wright (1970) sodium hypochlorite (more effective but less stable than chloramin) is effective against VSV even at a 0.645% concentration.

Under the test conditions, 0.2% Mikasept KP was the only reliable disinfectant for the inactivation of PTV; it reduced the infectivity titre of PTV as well as the titres of the both enveloped viruses (AV and VSV) almost totally. A high efficacy of another oxidizing agent, hydrogen peroxide, for the inactivation of members of many viral families including Picornaviridae, Herpesviridae and Rhabdoviridae has been proven, e.g., by Heckert et al. (1997).

Our results confirm that the inactivation of viruses bound onto surfaces (carrier test) is more difficult than the reduction of viral titres in suspensions (Ferrier et al., 2004). In some cases, the value of inactivation in suspension was, however, limited by a high cytotoxicity and did not allow a direct comparison with the carrier test (Table 3 and 4). The results of this study clearly demonstrate a significantly higher susceptibility of enveloped viruses to chemical disinfection. Within the enveloped viruses (AV and VSV), individual disinfectants decreased viral titres similarly. Individual strains within a viral species showed slight differences in resistance. In most cases (82%), one of the two tested strains was more resistant to all the disinfectants. Some studies (Heinz et al., 1989; Chambon et al., 1993) show that molecular differences in the capsid proteins of viral particles account for the differences in sensitivity of different strains of human enteroviruses to disinfectants.

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