American Journal of Infection Control 000 (2023) 1–6

Contents lists available at ScienceDirect

American Journal of Infection Control

journal homepage: www.ajicjournal.org

Major Article

Impact of concentration, temperature and pH on the virucidal activity of alcohols against human adenovirus

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Key Words:
Alcohols
Adenovirus type 5
Temperature
pH-value
Virucidal activity

Background: Adenoviruses belong to the stable nonenveloped viruses playing an important role in healthcare-associated infections mainly causing respiratory infections and epidemic keratoconjunctivitis. Hand disinfection with alcoholic preparations is therefore one of the most important measures to prevent such viral infections in hospitals and other medical settings.
Methods: The inactivation of adenovirus type 5 by ethanol, 1- and 2-propanol, and 2 commercially available hand disinfectants was examined at different concentrations, temperatures, and pH-values.
Results: For ethanol and 1-propanol the maximum virus-inactivating properties after 30 seconds exposure were found at a concentration of 60%-70% and 50%-60%, respectively, whereas with 2-propanol no activity was observed. The virucidal activity of all alcohols and the 2 hand disinfectants examined was increased when raising the temperature from 20°C to 25°C. By increasing the pH value to 9, a strong improvement of the activity of ethanol, 1-propanol and 1 hand disinfectant was observed, whereas pH lowering resulted in decrease of activity.
Conclusions: These results demonstrate the importance of physical parameters in the inactivation of adenoviruses by alcohols and will help to improve measures to reduce adenovirus transmission in healthcare settings.

Human adenoviruses are nonenveloped, double stranded DNA viruses causing healthcare-associated infections like respiratory infections, conjunctivitis, and gastroenteritis. They are transmitted by direct and indirect contact. For example, in hospitals and other healthcare facilities, an outbreak of epidemic keratoconjunctivitis has been reported caused by adenovirus type 8, ie, through medical devices or a contact with infected physicians.[1] Furthermore, it was described that shaking hands of patients with epidemic keratoconjunctivitis caused a transfer of adenovirus to uninfected control patients.[2] Consequently, the activity of various disinfectants used in the hospital has been evaluated against adenovirus type 8 in suspension.[3] Thereby, the choice of 70% 2-propanol for disinfection of pneumotonometer tips showed only limited activity in a medical center ophthalmology clinic.[4]

Importantly, adenoviruses are extremely stable when deposited on environmental surfaces ranging from 7 days to 3 months.[5,6] Therefore, the adenovirus is incorporated as test virus when evaluating the activity of chemical disinfectants against human pathogenic viruses in suspension and on surfaces in national and international guidelines.[7–9] The European Norm EN 14476 describes a standard suspension test as baseline for the evaluation of the virus-inactivating properties of alcohol-based hand disinfectants.[9] In spite of the fact, that the adenovirus type 5 (AdV-5) is an important test virus in this EN 14476 (besides the enveloped vaccinia virus, the nonenveloped poliomyelitis virus type 1 strain LSc 2ab (PV-1) and murine norovirus (MNV)), only limited data are available on the activity of alcohols against adenoviruses. Therefore, it was the aim of this study to extend the basic and translational knowledge of the activity of alcohols used in disinfectants against

Abbreviations: AdV-5, adenovirus type 5; BSA, bovine serum albumin; EN, European norm; EMEM, Eagle minimum essential medium; PV, poliomyelitisvirus type 1; RF, reduction factor; $TCID_{50}$, tissue culture infectious dose 50; TRIS-HCl, Tris-(hydroxymethyl)-aminomethan –hydrochlorid

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Conflict of interest: MR, BB, DP are employed at Dr Brill + Partner GmbH Institute for Hygiene and Microbiology. FHHB is head and shareholder of Dr Brill + Partner GmbH Institute for Hygiene and Microbiology. JS works as a consultant for Dr Brill + Partner GmbH Institute for Hygiene and Microbiology. The other authors declare that they have no competing interests.

https://doi.org/10.1016/j.ajic.2023.01.014
0196-6553/

human adenovirus type 5 to optimize the development of chemical disinfectants in general. Therefore, we analyzed the virus inactivating properties of 3 different alcohols and 2 commercially available disinfection products and additionally tested the influence of concentration, pH values, and temperature on virucidal activity.

METHODS

Test virus

The AdV-5 (ATCC-VR-5) was obtained from PD Dr A. Heim, Hannover Medical School.

Test products

Ethanol, 1-propanol, 2-propanol and 2 commercially available products were provided by B. Braun Medical AG (CH-6204 Sempach). One commercially available product (M1) is based on 52.3% ethanol and 24.6% 1-propanol (v/v) and the other (M2) on 52.0% 2-propanol and 34.4% 1-propanol (v/v). Alcohol concentrations in the test ranged from 50.0%-97.0%. In addition, pH values of the samples were adjusted to pH 3 either with phosphoric acid or lactic acid and to pH 9 with tetrahydroxypropyl ethylenediamine (TE). In addition, the influence of 2 buffer systems was investigated. TRIS HCl buffer was used for pH 9 and sodium citrate buffer for pH 3.

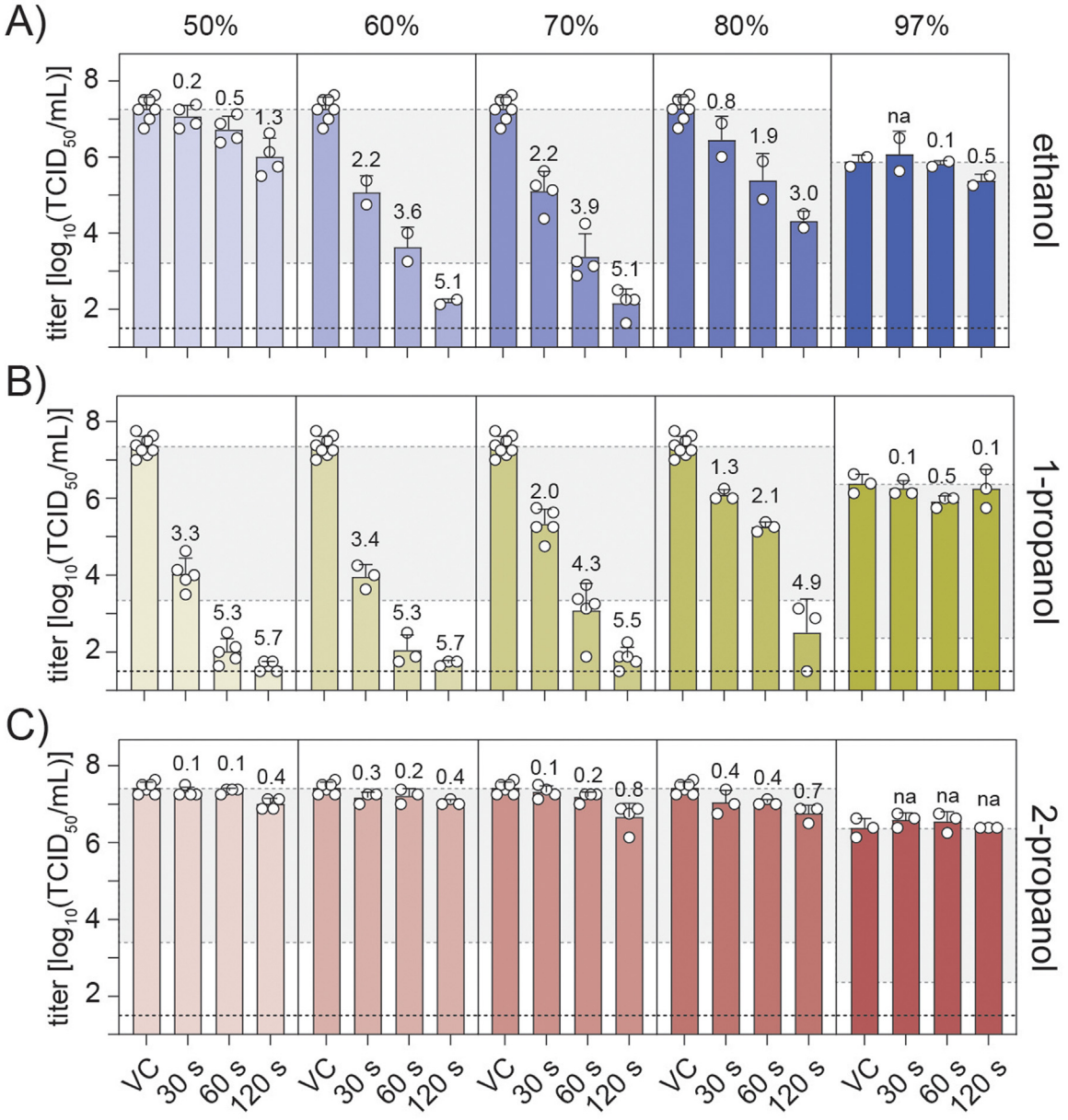

Fig 1. Virucidal activity of ethanol, 1-propanol, 2-propanol against AdV-5 at different concentrations. AdV-5 inactivating activities of ethanol (A), 1-propanol (B), 2-propanol (C) at different concentrations (50%-97%) were measured in a suspension test at 20°C under clean conditions according EN 14476. Titers are given at 30-, 60-, and 120-second exposure time. Bars represent mean values plus standard deviation of individual biological replicates (white dots), activity is indicated as reduction factor above bars compared to virus control (VC). Gray areas mark 4-$\log_{10}$ reduction.

Virus propagation

A-549 cells (human epitheloid lung cell) were used for the replication and titration of adenovirus. These cells were obtained from Vircell, S., Spain. Eagle's Minimum Essential Medium with Hanks' salts (EMEM) was used for culturing A-549 cells. Cells were frozen and thawed after exhibiting a cytopathic effect followed by centrifugation at 1600 g for 10 minutes. The resulting supernatant was aliquoted at −80°C as test virus suspension.

Virucidal testing

Suspension tests were conducted in accordance with EN 14476 starting at 20°C.[9] Eight parts by volume of the different test product concentrations (prepared as 1.25× solutions) were mixed with 1 part by volume of the test virus suspension and 1 part by volume of interfering substance (clean conditions, 0.3 g/l BSA) resulting in test concentrations of 50%-80%. In some cases, a modified procedure with a 97% test concentration according to EN 14476 was carried out, in which 9.7 parts of the test sample was mixed with 0.2 parts 1.5 g/l BSA as well as 0.1 parts of virus suspension [EN 14476]. Furthermore, suspension tests were additionally performed at temperatures between 20°C and 30°C. Thirty, 60, and 120 seconds were chosen as exposure times in all experiments. Immediately at the end of the chosen exposure time activity of the alcohols and the test products was stopped by serial dilution. Virus controls with water instead of a test product were included after the longest exposure time (120 seconds). Infectivity was examined in a microprocedure by endpoint dilution titration and the infective dose ($TCID_{50}$) was determined according to the method of Spearman and Kärber. Titer reduction was calculated as the difference between the $\log_{10}$ virus titers of the virus control and the residual $\log_{10}$ virus titers after the respective exposure time and is presented as reduction factor (RF). The EN 14476 requires a 4 $\log_{10}$ reduction (inactivation of ≥99.99%) of virus titer for demonstrating sufficient virucidal activity.[9]

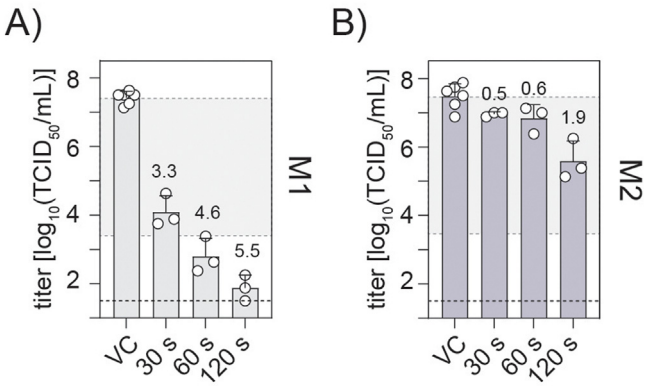

Fig 2. Virucidal activity of 2 commercially available hand disinfectants against AdV-5. AdV-5 inactivating activities of product M1 (52.3% ethanol and 24.6% 1-propanol (v/v)) (A) and M2 (52.0% 2-propanol and 34.4% 1-propanol (v/v)) (B) were measured in a suspension test at 20°C under clean conditions according EN 14476. Titers are given at 30-, 60-, and 120-second exposure time. Bars represent mean values plus standard deviation of individual biological replicates (white dots), activity is indicated as reduction factor above bars compared to virus control (VC). Gray areas mark 4-$\log_{10}$ reduction.

RESULTS

Influence of alcohol concentrations on the virucidal activity against AdV-5

The first test parameter for the virucidal activity of alcohols was the concentration. Ethanol at 60% and 70% led to a reduction of AdV-5 titers of 5.1 $\log_{10}$ steps for both concentrations after 120 seconds

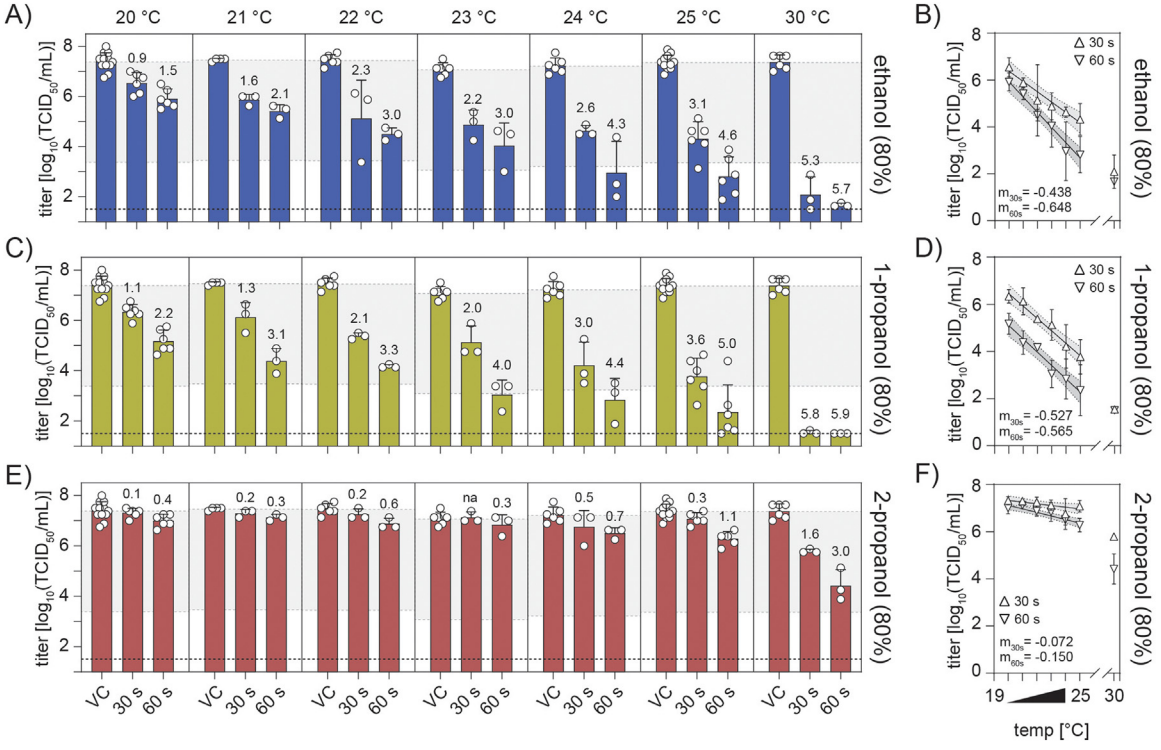

Fig 3. Virucidal activity of ethanol (80%), 1-propanol (80%), 2-propanol (80%) against AdV-5 at different temperatures. AdV-5 inactivating activities of 80% ethanol (A, B), 80% 1-propanol (C, D), 80% 2-propanol (E, F) at different temperatures (20°C-25°C and 30°C) were measured in a suspension test under clean conditions. Titers are given at 30-, and 60-second exposure time. Bars represent mean values plus standard deviation of individual biological replicates (white dots), activity is indicated as reduction factor above bars compared to virus control (VC). Gray areas mark 4-$\log_{10}$ reduction (A, C, E). Slopes (m) in a linear regression analysis (B, D, F) indicate distinct temperature dependent reduction of viral titers.

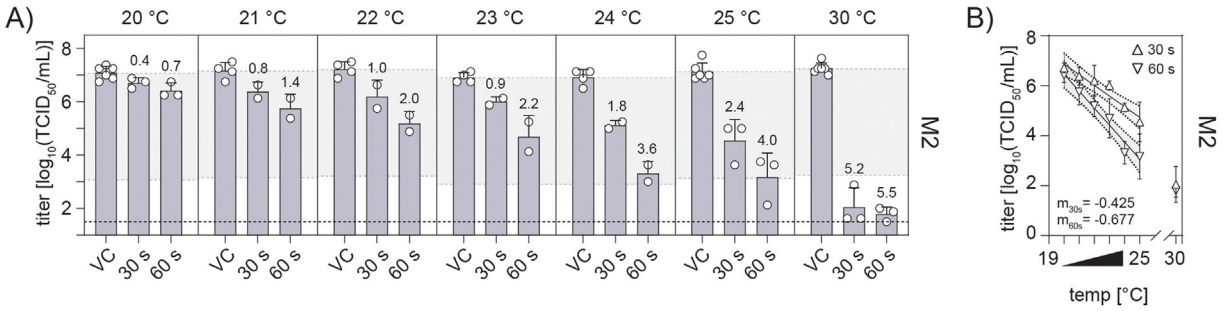

Fig 4. Virucidal activity of one commercially available hand disinfectants against AdV-5 at different temperatures. AdV-5 inactivating activity of M2 (52.0% 2-propanol and 34.4% 1-propanol (v/v)) at different temperatures (20°C-25°C and 30°C) was measured in a suspension test under clean conditions. Titers are given at 30-, and 60-second exposure time. Bars represent mean values plus standard deviation of individual biological replicates (white dots), activity is indicated as reduction factor above bars compared to virus control (VC). Gray areas mark 4-$\log_{10}$ reduction (A). Slopes (m) in a linear regression analysis (B) indicate temperature dependent reduction of viral titers.

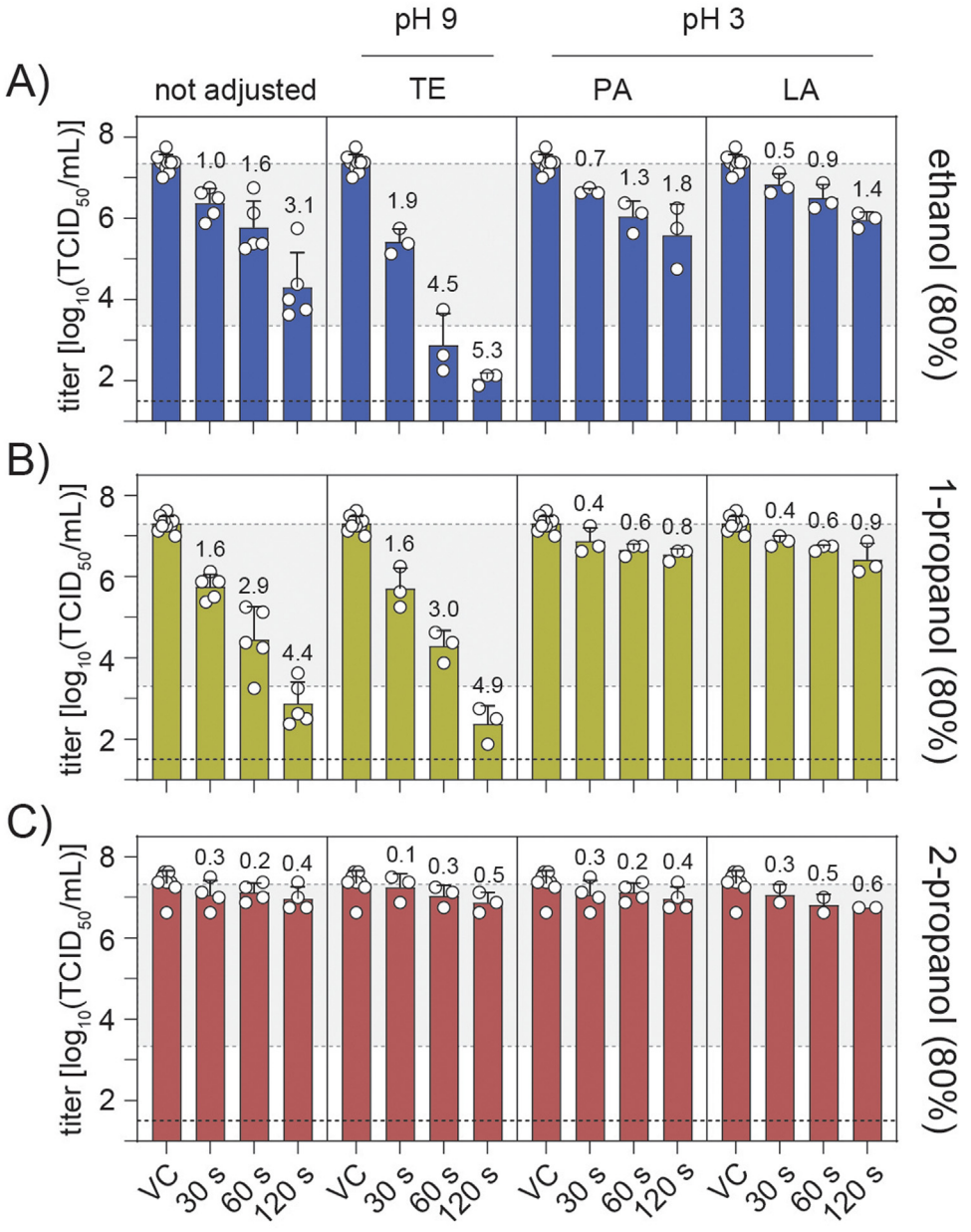

Fig 5. Virucidal activity of ethanol (80%), 1-propanol (80%), 2-propanol (80%) against AdV-5 at different pH values. AdV-5 inactivating activities of 80% ethanol (A), 80% 1-propanol (B), 80% 2-propanol (C) at different pH were measured in a suspension test under clean conditions. The pH value was either not adjusted, pH 9 adjusted with tetrahydroxypropyl ethylenediamine (TE), or pH 3 adjusted with phosphoric acid (PA) or LA (lactic acid). Titers are given at 30-, 60-, and 120-second exposure time. Bars represent mean values plus standard deviation of individual biological replicates (white dots), activity is indicated as reduction factor above bars compared to virus control (VC). Gray areas mark 4-$\log_{10}$ reduction.

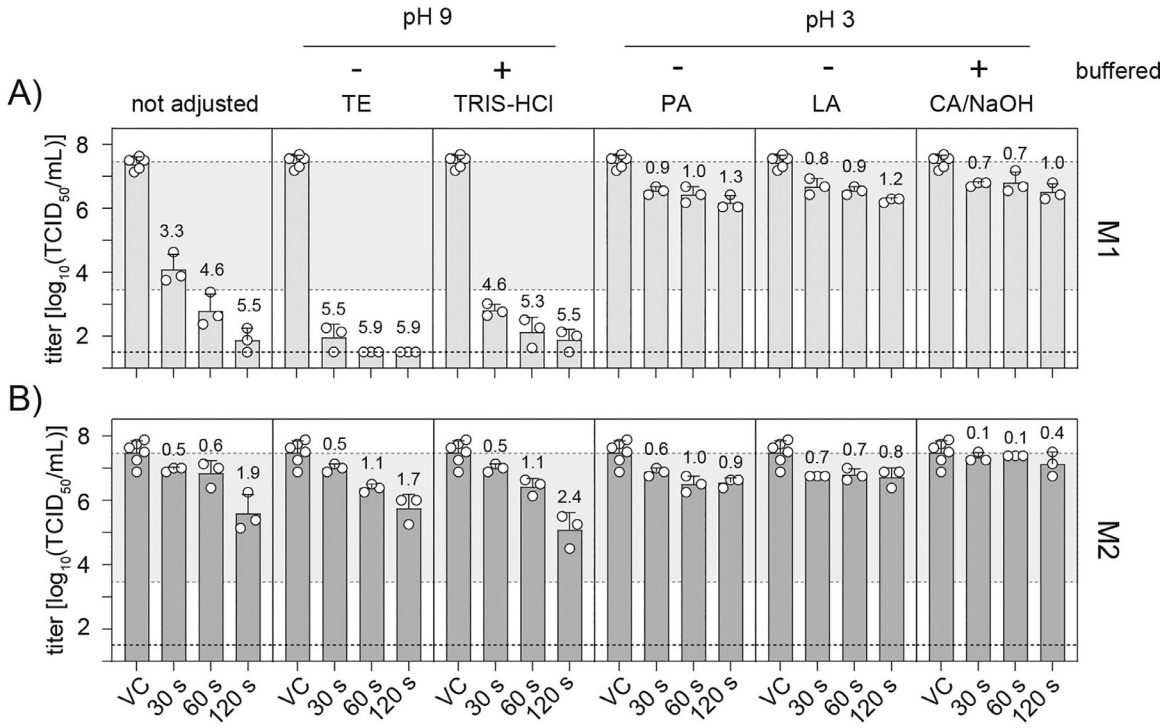

Fig 6. Virucidal activity of 2 commercially available hand disinfectants against AdV-5 at different pH values. AdV-5 inactivating activities of product M1 (52.3% ethanol and 24.6% 1-propanol (v/v)) (A) and M2 (52.0% 2-propanol and 34.4% 1-propanol (v/v)) (B) at different pH were measured in a suspension test under clean conditions. The pH value was either not adjusted, pH 9 adjusted with tetrahydroxypropyl ethylenediamine (TE), or pH 3 adjusted with phosphoric acid (PA) or LA (lactic acid). Titers are given at 30-, 60-, and 120-second exposure time. Bars represent mean values plus standard deviation of individual biological replicates (white dots), activity is indicated as reduction factor above bars compared to virus control (VC). Gray areas mark 4-$\log_{10}$ reduction.

exposure time (Fig 1A). For 1-propanol a 50% concentration with 60 seconds exposure time was sufficient to reach a reduction by 4 orders of magnitude (Fig 1B). In contrast, 2-propanol showed no activity even at the longest incubation time (Fig 1C). The ability to inactivate AdV-5 dropped at higher concentrations like 80% for ethanol and 1-propanol. A concentration of 97% ethanol and 1-propanol even failed to achieve any sufficient adenovirus inactivation (Fig 1A and B). The product M1, which is based on ethanol and 1-propanol reached a virus reduction by more than 4 $\log_{10}$ steps within 60 seconds (Fig 2A), whereas the second product M2 (mixture of 1- and 2-propanol) demonstrated nearly no reduction of AdV-5 titers after the same incubation time (Fig 2B).

Impact of increasing temperatures on the virucidal activity of alcohols against AdV-5

Next, ethanol (80%), 1-propanol (80%) and 2-propanol (80%) were tested in a temperature range from 20°C to 30°C with exposure times of 30 and 60 seconds (Fig 3). Increasing temperatures enhanced the virus-inactivating properties of ethanol from 2.1 RF at 21°C and 60 seconds to 4.6 RF at 25°C (Fig 3A). A regression analysis of these data sets showed that an increase of 1°C results in a 0.44 or 0.65 RF with a 30 second or 60 second exposure time, respectively (Fig 3B). Similar results were observed for 1-propanol in the suspension test (Fig 3C), which is also reflected in the regression analysis (Fig 3D). For both alcohols, the highest virucidal activity was found at a temperature of 30°C. The virucidal activity of 2-propanol could only be improved in a significant manner at 30°C and 60 seconds exposure time but did not reach a reduction by 4 $\log_{10}$ (Fig 3E and F). For the product M2, which was not active at room temperature after 60 seconds of exposure, 25°C and more were sufficient to reach a virucidal activity (Fig 4A) with an increase of 0.43 and 0.68 RF's per 1°C at the different exposure times (Fig 4B).

Influence of pH value on the virucidal activity of alcohols against AdV-5

To investigate, how the pH value of a formulation influences the virus-inactivating properties of alcohols against adenovirus at 20°C, samples with different pH values were tested in parallel to the unchanged alcohol concentrations of 80% (Fig 5). The adjustment of 80% ethanol (Fig 5A) and 80 % 1-propanol (Fig 5B) to a pH of 9 with TE achieved higher RF compared to the unchanged formulation, whereas an adjustment of 80% 2-propanol (Fig 5C) to a pH of 9 showed no effect. In contrast, the virucidal activity of ethanol and 1-propanol adjusted to pH 3 with phosphoric acid (PA) or lactic acid (LA) dropped independently of the acid used. These results could be confirmed for the different alcohols at a test concentration of 60% (Supplementary Fig 1). The pH adjustments were additionally tested for the products M1 and M2. As depicted in Figure 6A, the change of M1 to a buffered or unbuffered pH 9 could increase the reduction factors by 1-2 $\log_{10}$ at the 30-second exposure time, which could not be observed for pH 3 (Fig 6A). In case of the product M2, no improvement in the virucidal activity could be observed, neither at pH 9 nor at pH 3 (Fig 6B).

DISCUSSION

An outbreak of keratoconjunctivitis was caused by transmission of AdV-8 via the hand of healthcare workers.[1] Another outbreak in the hospital caused by AdV-4 was also considered by failure of contact infection control.[10] In addition, the use of 70% 2-propanol for surface disinfection in an ophthalmology clinic showed only limited activity against adenovirus.[4] Finally, the use of ethanol with a high

concentration as virucidal handrub was recommended.[11] Therefore, hand disinfectants mainly based on alcoholic solutions and alcoholic surface disinfectants in addition with a sufficient activity against human-pathogenic adenoviruses are critical in hospital hygiene.

In general, 1-propanol and ethanol or a mixture of both as in the test product M1 was able to reduce the test virus titer in a significant manner within 30 seconds, whereas a 2-propanol-based product failed. A review concerning the activity of ethanol against important viruses in hand disinfection confirmed our data showing an inactivation of AdV-5 by ethanol concentrations between 70% and 90% within 30 seconds.[11] Interestingly, in another study based on EN 14476 including ethanol and 2-propanol with different types of adenoviruses (Adv-8, -19, and -37) a RF only below 4 $\log_{10}$ steps was achieved after 120 seconds exposure time.[12] These differences in stability between different types of human pathogenic adenoviruses have to be clarified.

Of note, with increasing ethanol and/or 1-propanol concentrations a loss of activity against adenovirus was observed. This was already described in earlier investigations with adenovirus after introducing the 97% testing in EN 14476.[13] This phenomenon was not found with other nonenveloped test viruses such as polio- and norovirus.[13] The reason remains unclear and needs further investigations. It can be assumed that this effect might be based on denaturation of the special spike proteins of the adenovirus leading to loss of infectivity, which is hampered due to the loss of water. However, it must be an adenovirus specific effect due to the fact, that other nonenveloped viruses in the European norm missing this effect. Previous findings using atomic force microscopy could give new insights in the capsid damage of adenoviruses by ethanol alone and in combination with zinc salt[14] and it was shown that acidification of adenoviruses could influence the compaction of the nucleoproteic core.[15]

Additionally, we could show that increasing the test temperature demonstrated a strong effect on virus inactivation at concentration of 80% of the alcohols. These data might strengthen the discussion to increase the temperature in the suspension test when testing hand disinfectants in the laboratory because in reality higher temperature than 20°C can be expected on hand skin. Lower concentrations of the alcohols were not tested as 80% concentration in standard guidelines. The effect of changing pH values on the stability of AdV-5, which may be important for alcoholic surface disinfectants remains unclear. Irreversible aggregation of adenovirus particles and thus the protection of the virus from inactivation by chemical substances might be a possible explanation for the stability to alcohols at low pH values.

CONCLUSIONS

In summary, stability of AdV-5 as important prototype test virus in different European standards can be influenced by various factors. The kind of alcohol in a disinfectant and the test temperature in suspension test can influence the outcoming results with this test virus to a great extent. Also, a change in pH value might be important mainly for surface disinfectants based on alcohol. So, for the development and/or the improvement of a virucidal disinfectant for hands or surfaces these different parameters should be considered in general.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.ajic.2023.01.014.

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