Tropical Biomedicine 40(4): 478-485 (2023)
https://doi.org/10.47665/tb.40.4.014

RESEARCH ARTICLE

Stability of enteroviruses on toys commonly found in kindergarten

Baharin, S.N.A.N.[1], Tan, S.L.[2], Sam, I.C.[1], Chan, Y.F.[1*]

[1]Department of Medical Microbiology, Faculty of Medicine, University of Malaya
[2]Department of Orthopaedic Surgery, Faculty of Medicine, University of Malaya
*Corresponding author: chanyf@um.edu.my

ARTICLE HISTORY

Received: 14 April 2023
Revised: 4 September 2023
Accepted: 5 September 2023
Published: 15 January 2024

ABSTRACT

Hand, foot, and mouth disease (HFMD) is a contagious childhood disease caused by enteroviruses including enterovirus A71 (EV-A71), coxsackievirus A6 (CV-A6) and CV-A16 transmitted via direct and indirect contact. Different types of toy surfaces can affect the stability of viruses. Understanding the stability of enteroviruses on toys provides insightful data for effective disinfection in kindergartens or homes. Porous (ethylene-vinyl acetate mat foam, paper, pinewood, polyester fabric, and squishy polyurethane foam) and non-porous (acrylonitrile butadiene styrene plastic and stainless-steel coin) surfaces were inoculated with EV-A71 at 4, 24, and 35°C, and coxsackieviruses at 24°C. Infectious enteroviruses were recovered and titred in median tissue culture infectious dose assay ($TCID_{50}$). Atomic force microscopy (AFM) images were taken from surfaces to examine association of surface roughness with virus stability. Overall, infectious enteroviruses were persistent on all non-porous and porous surfaces. Virus persistence was longest at 4°C followed by 24°C and 35°C. EV-A71 half-lives ranged between 6.4-12.8 hours at 4°C, 2.4-6.7 hours at 24°C, and 0.13-2.7 hours at 35°C. At lower virus titres exposed to 24°C, half-lives of enteroviruses ranged from 0.1-1.4 hours. Surface roughness values from AFM suggested smooth surfaces of non-porous surfaces were associated with better virus stability. Temperature, enterovirus concentration, and type of surface affected persistence and stability of enteroviruses. Our findings suggest both porous and non-porous surfaces in kindergartens allow enterovirus persistence and should be frequently disinfected to curb HFMD outbreaks in kindergartens.

Keywords: enterovirus A71; hand-foot-and-mouth disease; stability; kindergarten; fomites.

INTRODUCTION

Enteroviruses are common causative agents of hand, foot, and mouth disease (HFMD). Although HFMD is self-limiting, severe cases have reported neurological complications including encephalitis, acute flaccid paralysis, and deaths. Signs and symptoms of HFMD include fever, sore throat, papulovesicular rashes on palms, foot, and buttocks in addition to ulcers in the mouth. (Chan *et al*., 2011) This contagious childhood disease transmits via the faecal-oral route either via direct or indirect contact involving the touching and passing of infectious materials from one surface to another. Apart from fomite-associated indirect transmission, HFMD-associated viruses can be detected in water, food, and sewage treatment plants, but infection by ingesting water contaminated by HFMD-associated viruses is not common (Okoh *et al*., 2010; Centres for Disease Control and Prevention, 2019).

HFMD is predominant in many parts of the Asia-Pacific region especially in China where 15 million probable HFMD cases were reported by the Chinese Centre for Disease Control and Prevention from 2013 to 2019 (Hong *et al*., 2022). In 2018, HFMD is the second most prevalent infectious disease in Malaysia with recurrent EV-A71 outbreaks reported every two to three years (Ministry of Health Malaysia, 2019; NikNadia *et al*., 2016). The national lockdown in 2020-2021 due to the COVID-19 pandemic resulted in the closure of childcare and kindergarten facilities, reduced human interaction, and stricter hygiene practices, which significantly reduced the number of HFMD cases (Bernama, 2021). After pandemic restrictions were lifted by 2022, HFMD cases rose 27-fold compared to the previous year resulting in the closure of 1,174 nurseries, kindergartens, and pre-schools due to HFMD outbreaks (Yusof, 2022).

There is no specific drug for HFMD or EV-A71 vaccine (outside China) available and treatment relies on relieving symptoms. Thus, prevention and control of HFMD are important measures particularly involving high-touch surfaces. Enteroviruses are non-enveloped RNA viruses resistant to commercial alcohol-based disinfectants and require at least 95% ethanol, or Virkon S or mixed solution of household bleach and water to be inactivated (Chan & Abu Bakar, 2005; Chang *et al*., 2013). Despite the anticipated risk of enterovirus transmission via fomites, availability of studies focusing on stability of non-polio enterovirus on fomites are limited (Mahl & Sadler, 1975; Sattar *et al*., 1988; Abad *et al*., 1994; Kadurugamuwa & Shaheen, 2011; Tuladhar *et al*., 2012; Thevenin *et al*., 2013a, 2013b; Firquet *et al*., 2015; Tamrakar *et al*., 2017).

This study aims to test stability of the common HFMD-associated enteroviruses EV-A71, CV-A16 and CV-A16 on different types of toy surfaces commonly found in kindergartens. We highlight the risk of fomite transmission of enteroviruses for porous and non-porous surface toys.

MATERIALS AND METHODS

Virus isolates and cell line

EV-A71 (strain 41 (5865/SIN/000009) subgenogroup B4, GenBank accession no. AF316321), CV-A6 (strain Gdula, GenBank accession no. AY421764), and CV-A16 (strain PM-22159-02, GenBank accession no. JQ746673) were cultured in human rhabdomyosarcoma (RD) cells (ATCC CCL-136) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% heat-inactivated foetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, and sodium pyruvate. At day 3 post-infection, 90% cytopathic effect (CPE) was achieved. The cells were freeze-thawed twice, and cell supernatant was centrifuged for ten minutes at 12 000 × g with an Eppendorf 5804R centrifuge (Eppendorf SE, Germany) at 4°C and kept at -80°C. Viral titres were determined via measurement of median tissue culture infectious dose ($TCID_{50}$) and calculated using the Reed-Muench method.

Toy materials

Toys commonly found in kindergartens were categorized into non-porous (a plastic brick made from thermoplastic polymer acrylonitrile butadiene styrene and a coin made from stainless steel), semi-porous (play mat made from ethylene-vinyl acetate), and porous (paper, wooden blocks made from pinewood, polyester fabric, and a squishy toy made from polyurethane foam) surfaces (Figure S1-supplementary data). Non-porous surfaces are rigid and smooth while semi-porous surfaces are rigid with the presence of pores. Porous surfaces were categorised by the presence of pores visible to the naked eye.

Each material was cut into pieces of 2×2 cm^2 surface area, disinfected by immersion in 70% ethanol, and allowed to air dry at room temperature in a class II biological safety cabinet (BSC) prior to the experiment. Coins were autoclaved prior to the experiment. For a surface control, a polystyrene well-plate was used due to its hydrophobic properties to allow comparison of virus recovery with toy surfaces made from a variety of materials that may affect absorption of virus suspension.

Stability of EV-A71 on toy surfaces

Thirty microliters of 10^8 $TCID_{50}$/mL EV-A71 suspension were pipetted on each toy surface and allowed to dry in the BSC for one hour. The titre was chosen to ensure persistence of EV-A71 was observable in the virus titration assay after drying of the virus inoculum. The toys were then placed in six-well plates and kept at one of three temperatures (4±1°C, 24±1°C, and 35±1°C). Infectious virus was recovered from toy surfaces by flushing 0.5 mL of DMEM supplemented with penicillin, streptomycin, L-glutamine, and sodium pyruvate. Triplicate samples were recovered from each toy surface at 0, 24, 48, 72, and 96 hours post-inoculation (hpi). Recovered samples were kept at -80°C prior to viral titration. Toy materials were kept in a fridge at 4±1°C, on the laboratory bench at 24±1°C, and in a glass chamber at 35±1 °C. The relative humidity was kept constant at 50±5% and measured using a digital hygrometer.

Comparison of stability between EV-A71, CV-A6, and CV-A16 on toys

Only the control surface, plastic brick, coin, paper, play mat, and squishy toy were used in this experiment. Thirty microliters of 3.2×10^5 $TCID_{50}$/mL virus suspension were pipetted on toy surfaces and allowed to dry for one hour in the BSC. This titre reflects clinically relevant viral titres cultured from the stool and bronchoalveolar lavage samples of a patient with EV-A71 infection (Cordey *et al*., 2012). After drying, toys were placed in six-well plates and kept at 24±1°C. The experiments were performed in triplicate for each toy and each virus. The relative humidity was kept constant at 50±5% and measured using a digital hygrometer.

Viral titre determination

RD cells were seeded into 96-well plates in DMEM supplemented with 10% FBS, penicillin, streptomycin, L-glutamine, and sodium pyruvate. Briefly, 45 µl of recovered samples were serially diluted in 405 µl of serum-free media (SFM) into 10^{-1} until 10^{-5} dilutions. Then, the media in the 96-well plates was replaced with 100 µl of diluted recovered samples. Each recovered sample was titred in quadruplicate. The inoculated well plates were incubated at 37°C for one hour. Then, 100 µl of DMEM supplemented with 2% FBS was added to each well resulting in a total volume of 200 µl for each well. Signs of CPE were recorded and calculated using the Reed-Muench method.

Log $TCID_{50}$ = log of the last dilution showing CPE above 50% –

$$\left[\frac{(x-50\%)}{(x-y)} \times \textit{log of dilution factor}\right]$$

$$TCID_{50}/mL = \frac{TCID_{50}}{\textit{volume of inoculum } (mL)}$$

Where

x (%) = % of CPE from wells at the last dilution showing above 50% CPE

y (%) = % of CPE from wells at the last dilution showing below 50% CPE

For half-life ($t\frac{1}{2}$):

$$\text{Viral titre after } \mathbf{t} = \text{initial viral titre} \times \left(\frac{1}{2}\right)^{t\frac{t}{\frac{1}{2}}}$$

Where **t** = time taken to reach undetectable infectious titre

Atomic force microscope (AFM) imaging of toy surfaces

AFM images of non-porous (coin and plastic brick), semi-porous (play mat), and porous (paper and squishy toy) surfaces were acquired using the BioScope Catalyst AFM (Bruker Corporation, USA) mounted onto a Leica DMI3000 B inverted fluorescence microscope body (Leica Microsystems Pte Ltd, Singapore) and controlled with a Nanoscope V controller (Bruker Corporation, USA). The standard ScanAsyst–Air AFM cantilever was used to scan the surfaces of the specimens with patented ScanAsyst mode parameters. All AFM images were acquired by scanning in the air using a standard air probe holder. The laser was aligned to focus on cantilever using the EasyAlign (Bruker Corporation, USA) by adjusting the photodetector signals.

Toy surfaces were prepared by cutting into 2×2 cm^2 pieces of less than 1 mm in height and disinfected using 70% ethanol before being air-dried. Specialized cleaning was not performed to preserve the state of surfaces. The coin was autoclaved prior to disinfection. Toys were glued to a standard 25×75 mm glass slide and further removal of fine dust was done using an air jet blower. The glass slides were kept in a sealed container to prevent contamination of the surfaces of the cleaned toys by dust and fine particles.

AFM images were acquired from a scan area of 100×100 µm, with a sampling resolution of 1024 samples/line. Root mean square roughness (Rq) of the surface was obtained using NanoScope Analysis v1.50r1sr3 software (Bruker) by drawing five identical-sized boxes from scanned 2D images (NanoScope Analysis 1.50). The boxes were drawn on flat surfaces indicated by a colour bar scale (relative to minimum data cursor) for the height in the AFM images. The higher the microscopic peaks of the material, the brighter the colour gradient presented in the image. Rq value indicates the root mean square average of heights of microscopic peaks on the toy surface over the selected area of scanned AFM image.

Statistical analysis

Data analysis and production of graphs were performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, USA). Half-life lines were plotted using simple linear regression. Kruskal-Wallis test was performed to compare the differences in recovery of infectious inoculum based on temperature and toy surface. If there was an overall significant difference (P<0.05), Dunn's test for multiple comparisons was performed to determine the groups showing significant differences.

RESULTS

Stability of EV-A71 on toy surfaces

We infer the virus stability using the concept of half-life. At 24°C, infectious EV-A71 was detectable beyond 96 hpi on plastic brick, coin, play mat, and paper with half-lives of 6.1-6.7 hours (Table 1). These surfaces showed reductions of infectious viral titre of 4.33-4.76 logs compared to other surfaces which had reductions ranging from 4.86-6.46 log reduction $TCID_{50}$/mL (Table S1-supplementary data). Infectious EV-A71 was recovered from the porous wooden block until 72 hpi with a half-life of 4.5 hours and was even lower on the polyester fabric and squishy toy at 48 hpi with a half-life of 2.9 hours and 2.4 hours, respectively (Figure 1). Shorter half-lives on most porous surfaces suggested the effect of adsorption affecting the stability of EV-A71 on porous surfaces compared to non-porous surfaces. The control surface of polystyrene plastic provided stability up to 72 hpi with a half-life of 3.4 hours.

At 4°C, infectious EV-A71 was recoverable beyond 96 hpi on all toy surfaces. Smaller reductions of infectious viral titre were obtained ranging from 2.25-4.53 log reduction $TCID_{50}$/mL (Table S1-supplementary data). Virus stability beyond the time interval of 96 hpi suggested cold temperatures could limit the inactivation of infectious EV-A71 on toy surfaces. The longest half-life was 12.8 hours on non-porous coin surface followed by 8.9 hours on plastic brick and porous paper. Half-lives between 6.4-8.3 hours were observed in other porous materials (Table 1). The control surface showed recoverable virus beyond 96 hpi with a half-life of 6.5 hours.

At 35°C, infectious EV-A71 was still recoverable from all toy surfaces but, over a shorter duration. The reduction of EV-A71 was drastic compared to 4°C and 24°C. The longest virus stability was on a non-porous plastic brick at 48 hpi with 5.38 log reduction $TCID_{50}$/mL of infectious viral titre (Table S1-supplementary data). The virus did not persist longer than 24 hpi on all porous surfaces and the non-porous coin (Figure 1). The virus half-life on stainless-steel coin was the shortest at only 0.1 hours suggesting exposure to hot temperatures plausibly affected the coin surface temperature that was in contact with EV-A71 suspension hence, leading to rapid inactivation of the virus (Table 1). The half-lives ranged from 1.3-2.7 hours on other porous surfaces.

Dunn's test for multiple comparisons showed EV-A71 titres were significantly higher at 4°C compared to 35°C for coin (P=0.031), paper (P=0.011), and plastic brick (P=0.048), suggesting temperature influences the stability of infectious EV-A71 on these surfaces.

Comparison of persistence between EV-A71, CV-A6, and CV-A16 on toys

Persistence of other HFMD causative agents on toy surfaces was compared to differentiate stability between enteroviruses. We tested enteroviruses EV-A71, CV-A6, and CV-A16 on non-porous plastic brick and coin, and porous paper and play mat. At 24°C and using a lower initial viral titre of 3.2×10^5 $TCID_{50}$/mL, enteroviruses showed reduced recovery from all surfaces compared to using a higher initial viral titre of 1.0×10^8 $TCID_{50}$/mL showing the effect of virus concentration on the recovery of infectious virus. Despite reduced recovery, infectious enteroviruses were still recoverable on non-porous and porous surfaces within the first 24 hpi (Figure 2). Log reduction of infectious titre ranged from 3.35-3.47 (Table S2-supplementary data). These values might be affected by the effect of drying of viral inoculum for one hour.

Recovery from the non-porous plastic brick was the longest for up to 16 hpi for EV-A71 and CV-A16, while CV-A6 showed persistence until 6 hpi. On the coin surface, virus stability was reduced until 4 hpi for EV-A71 and CV-A16 while CV-A6 showed persistence up to 6 hpi. Consequently, half-lives ranged from 0.5-1.4 hours on plastic brick and reduced to 0.3-0.5 hours on coin.

The semi-porous play mat showed similar persistence to non-porous surfaces in which EV-A71 (half-life 0.7 hours) persisted up to 8 hpi followed by CV-A6 (half-life 0.4 hours) and CV-A16 (half-life 0.3 hours) at 4 hpi. The poorest recovery was made from porous surfaces. On paper, EV-A71 was not detectable after drying, CV-A16 was recoverable at 2 hpi (half-life 0.2 hours) while CV-A6 was only infectious up to the end of the drying period of one hour (half-life 0.1). On the squishy toy, infectious EV-A71 survived the drying period of one hour (half-life 0.1) while CV-A16 and CV-A16 showed similar detection at 2 hpi (half-life 0.2). The control surface (polystyrene plastic) showed persistence of all enteroviruses at 4 hpi and half-lives ranged from 0.3-0.4 hours (Figure 2, Table 2). Overall, there were no significant differences observed between the persistence of enteroviruses on all surfaces at 24°C using a lower virus titre of 3.2×10^5 $TCID_{50}$/mL. This suggests the stability of HFMD-related enteroviruses when produced at a concentration similar to an HFMD patient, with infectious enteroviruses able to persist on non-porous and porous surfaces at room temperature.

Surface topography characteristics of porous and non-porous toy surfaces

We used AFM to explore toy surface properties to associate surface roughness with virus stability. Surfaces made from polystyrene plastic, plastic brick, paper, squishy toy, coin, and play mat were chosen based on the longest and shortest half-lives at 24°C (Table 1). Non-porous surfaces showed lower peak-to-valley height (seen in the height image bar) ranging from 3.9-8.8 µm compared to porous surfaces ranging from 19.6-33.4 µm (Figure 3). The reduced

Table 1. Half-lives of EV-A71 recovered from different toy surfaces

Materials	Half-life (hours)		
	4 °C	24 °C	35 °C
Coin	12.8	6.7	0.1
Plastic brick	8.9	6.1	2.7
Play mat	7.1	6.6	2.7
Paper	8.9	6.7	1.9
Polyester fabric	8.3	2.9	1.3
Squishy toy	7.2	2.4	1.7
Wooden block	6.4	4.5	1.3
Control (polystyrene)	6.5	3.4	1.6

Table 2. Half-lives of enteroviruses recovered at 24°C from different toy surfaces. Dashed line (–) indicates undetectable infectious viral titre after drying for one hour

Materials	Half-life (hours)		
	EV-A71	CV-A6	CV-A16
Coin	0.3	0.5	0.4
Plastic brick	1.4	0.5	1.4
Play mat	0.7	0.4	0.4
Paper	–	0.1	0.2
Squishy toy	0.1	0.2	0.2
Control	0.3	0.3	0.4

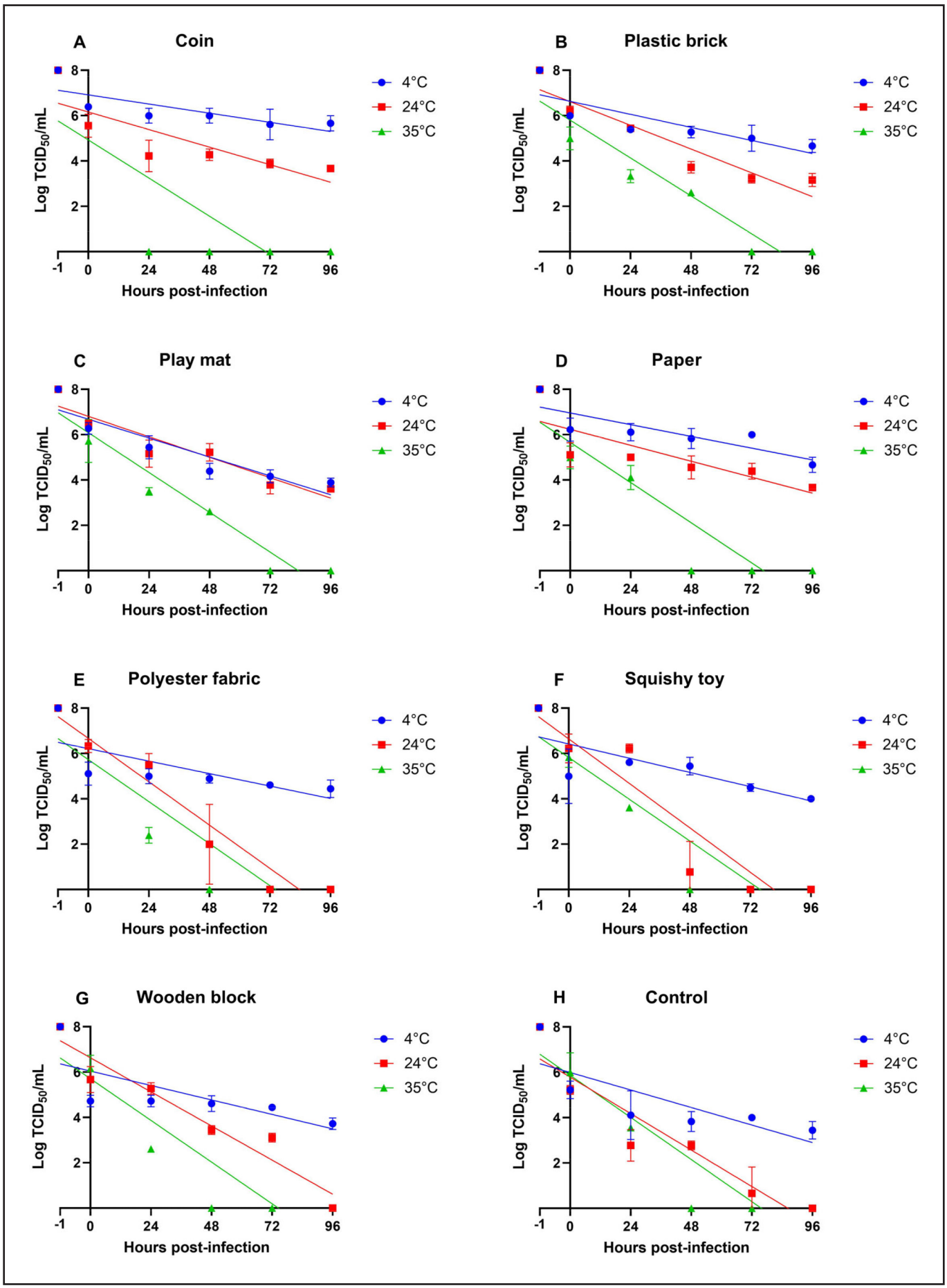

Figure 1. Infectious EV-A71 stability at 4°C, 24°C, and 35°C on **(a)** coin, **(b)** plastic brick, **(c)** play mat, **(d)** paper, **(e)** polyester fabric, **(f)** squishy toy, **(g)** wooden block, and **(h)** control. Data are expressed as log $TCID_{50}$/mL. The infectious titre shown at -1 on the x-axis indicates the titre prior to drying for one hour.

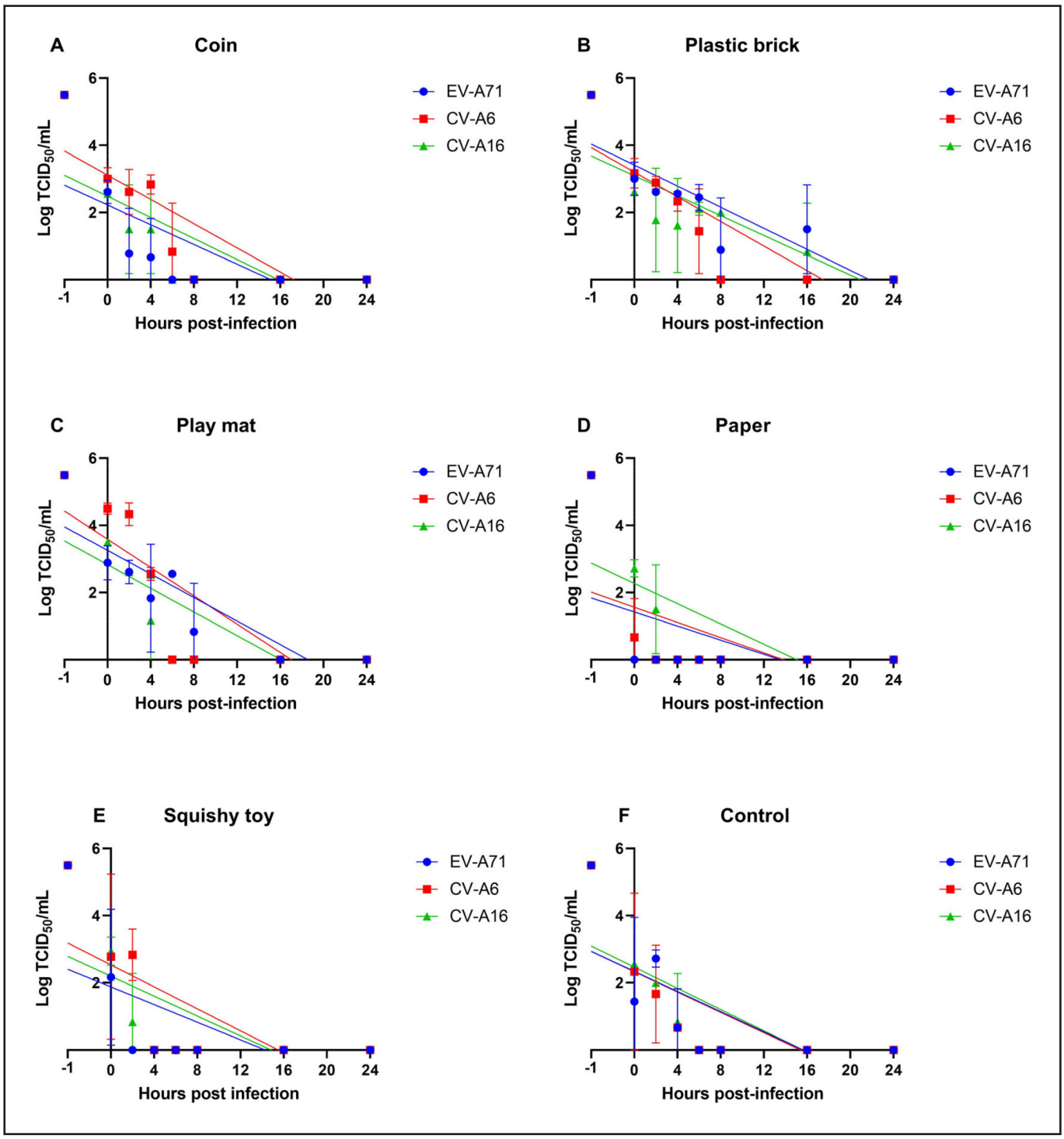

Figure 2. Viral titres of recovered EV-A71, CV-A6, and CV-A16 at 24°C from **(a)** coin **(b)** plastic brick, **(c)** play mat, **(d)** paper, **(e)** squishy toy, and **(f)** control. Data are expressed as log $TCID_{50}$/mL. The infectious titre shown at -1 on the x-axis indicates the titre prior to drying for one hour.

peak-to-valley height in non-porous surfaces resulted in lower surface root mean square roughness (Rq) ranging from 19.6-37.4 nm. The semi-porous play mat showed the lowest peak-to-valley height at 1.2 µm and Rq of 15.5 nm which indicated a smoother surface than non-porous surfaces. Both porous surfaces were significantly rougher (surfaces) compared to the semi-porous play mat (paper, P=0.0236; squishy toy, P=0.0007) with Rq from 269.6-792.6 nm (Table 3). Only the squishy toy was significantly rougher than the polystyrene plastic (P=0.0028) indicating its rougher surface would provide less stability towards the persistence of viral inoculum.

Table 3. Root mean square roughness (Rq) of toy surfaces

Materials	Rq average (nm)
Coin	37.3
Plastic brick	37.4
Play mat	15.5
Paper	269.6
Squishy toy	792.6
Control	19.6

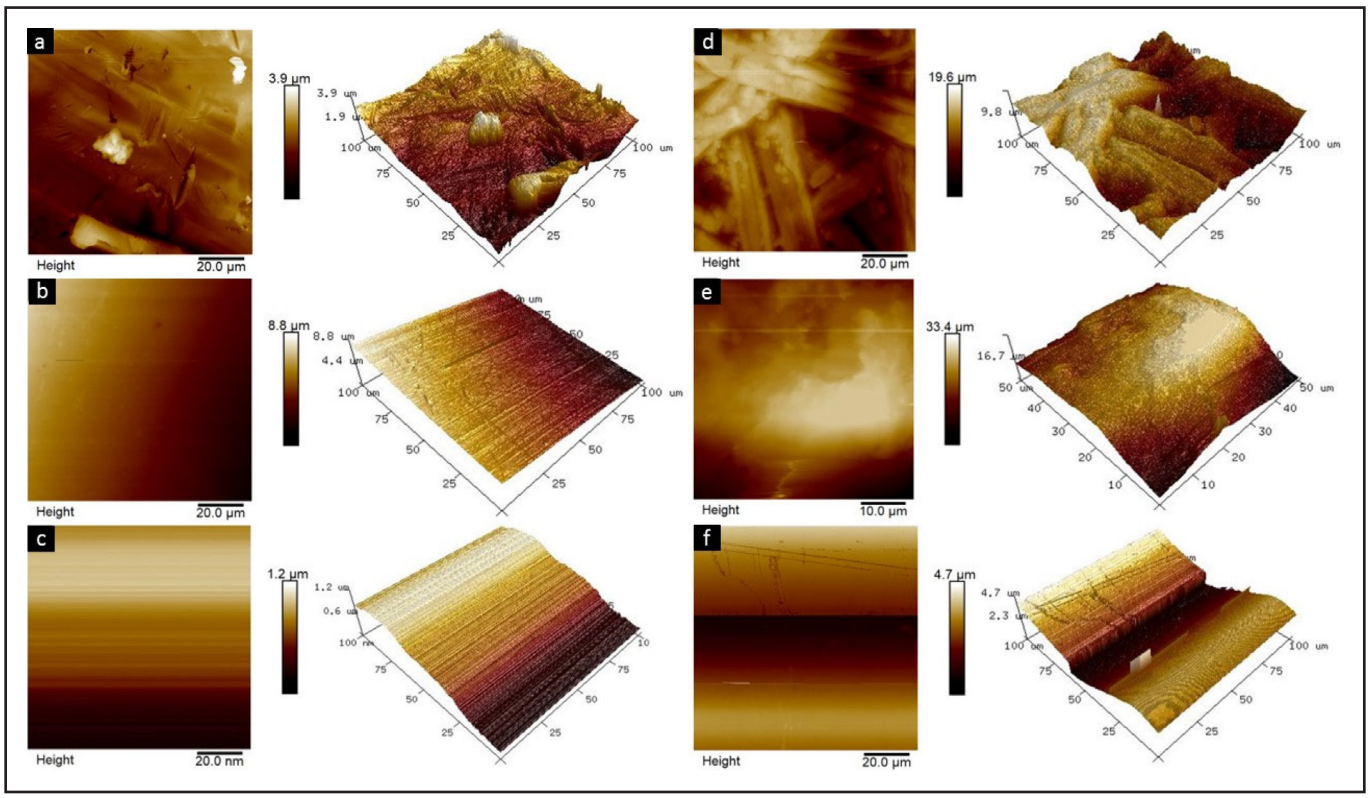

Figure 3. 2D and 3D AFM height images of **(a)** coin, **(b)** plastic brick, **(c)** play mat, **(d)** paper, **(e)** squishy toy, and **(f)** control surface (polystyrene plastic). The colour bar scale represents height gradients on the surface, with lighter colours indicating peaks and darker colours indicating valleys.

DISCUSSION

HFMD outbreaks are recurrent yearly in Malaysia and many parts of the world due to the many species of enteroviruses that do not provide cross-immunity, and the unavailability of EV-A71 vaccine globally except in China (Chan *et al*., 2012; Teo *et al*., 2018; Lin *et al*., 2019; Lee *et al*., 2021). As prevention and infection control are the main ways to control the spread of infection, we must focus on the basics of virus transmission and understand patterns of virus survival in the environment including any surface or fomite that has the potential to transmit enteroviruses (Tamrakar *et al*., 2017). This study compared the survival of infectious EV-A71 at three temperatures to determine the persistence and stability on different porous and non-porous toy surfaces. We showed here that the type of surface, temperature, and enterovirus concentration influences virus persistence and stability.

Our study did not reveal any significant differences between virus stability on porous and non-porous toys at the same temperature. While EV-A71 inoculated in this study was at a higher concentration than those produced by a typical HFMD patient, the persistence of EV-A71 on fomites was observed to be significantly longer at 4°C than 35°C. Similarly, Abad *et al*. observed inactivation of poliovirus, from the enterovirus genus, occurred at a slower rate when exposed to 4°C than 20°C on non-porous stainless steel, aluminium, china, and porous paper (Abad *et al*., 1994). The study highlighted that enteroviruses were stable at 4°C on both porous and non-porous toys, but interestingly HFMD occurs mainly in summer months and not during winter in temperate countries (Khetsuriani *et al*., 2006; Onozuka & Hashizume, 2011; Chang *et al*., 2012; Xu *et al*., 2020). This suggests temperature is not the sole factor influencing virus stability.

When different temperatures were used, we observed different endpoints of virus recovery for coin and plastic brick (non-porous), play mat (semi-porous), and paper (porous). However, these surfaces consistently had better virus stability than other porous surfaces used in this study at 4°C, 24°C, and 35°C. Abad *et al*. showed persistence of enteroviruses was shorter at high temperatures (Abad *et al*., 1994). This was seen with the coin showing the longest persistence and stability of EV-A71 at 4°C and 24°C with half-lives ranging from 6.7-12.8 hours but the shortest persistence at 35°C with 0.1 hours of half-life. At high temperatures, the heat-conductive stainless steel absorbs heat faster from the surroundings than other materials resulting in significant rapid inactivation of EV-A71. Placing toys outdoors where temperatures are high in tropical countries is a good option for inactivation of enterovirus which is cheaper and less hazardous compared to chemical disinfection. Exposure to sunlight further adds the virucidal benefit of ultraviolet radiation (Wommack *et al*., 1996; Espinosa-Garcia & Mazari-Hiriart, 2008).

When comparing different enteroviruses of the same species at titres mimicking those shed by EV-A71 patients, there was no significant trend towards greater stability on all the tested surfaces. Persistence of enteroviruses did not go beyond 24 hours after drying suggesting inactivation occurred earlier with lower virus titres. This suggests viral load plays an important role in effective fomite transmission. Furthermore, we observed no difference in the virus stability between enteroviruses on porous and non-porous toys. Modelling studies of EV-A71 and CV-A16 showed varied reproductive numbers (proxy for transmission) for EV-A71 was 5.06 (2.81-10.20), CV-A16 was 4.84 (3.00-9.00), and CV-A6 was 5.94 (3.27-10.00) (Zhang *et al*., 2021). Therefore, virus stability is not the only factor contributing to transmission. Other environmental effects like desiccation on different types of material also contribute towards

the persistence of infectious viruses based on differences in titre reduction found in this study. Resistance toward desiccation was described as a significant factor in the persistence of virus on fomites (Abad *et al*., 1994). Similarly, we found that EV-A71 viral titres recovered from all toys after one hour of drying at room temperature showed reduction of 1-2 logs compared to viral inoculum that was not dried.

Other enteroviruses like poliovirus type 1 can survive on paper at 20°C for more than 30 days and on cotton fabric for 143 hours (Abad *et al*., 1994; Tamrakar *et al*., 2017). Similarly, EV-A71 half-life was higher for paper than polyester fabric for all the tested temperatures. This again suggests the composition of the surface such as roughness could influence virus stability. Although information is limited regarding the persistence of non-enveloped enteroviruses associated with surface roughness, previous studies reported adhesion of viable particles tends to be weaker on smooth surfaces than on rough surfaces (Dika *et al*., 2013; Wu *et al*., 2018; Wang & Tarabara, 2022). Strong adherence on rougher surfaces may mean that infectious particles would less likely to be recovered resulting in inactivation over time by desiccation. In this study, infectious viruses were recovered for a shorter duration on the rougher squishy toy compared to the coin, indicating disruption to the virus inoculum which allows the virus to be less stable. Other fomite properties including surface texture, permeability, size of pores, and depth of pores may influence measures of the surface roughness. Under the influence of high temperatures, surface roughness may not be as impactful on the stability of EV-A71 as seen on the stainless-steel coin when other physical properties such as heat conductivity become influential.

Limitations in the study include the use of suspension media unrelated to the composition produced by humans which lacks the organic load that could influence virus stability. The presence of stool in suspension enhances enterovirus persistence on fomites which can be removed by disinfectants containing quaternary ammonium compounds and free chlorine (Abad *et al*., 1994; Tuladhar *et al*., 2012; Thevenin *et al*., 2013b). Another limitation includes the drying effect on virus inoculum which resulted in lower virus titres. We chose drying to mimic the presence of droplets on toys that might be dried by the time it is exposed to children when in kindergarten. It is also possible to explore other virus recovery methods such as soaking the contaminated toy surfaces and vortexing to obtain maximum recovery of virus from the surfaces.

Prevention of HFMD at kindergartens is done in several ways such as isolation of infected children, disinfection of premises, and closure of premises. HFMD guidelines from the World Health Organization and the Ministries of Health in Malaysia, Singapore, and Hong Kong recommend infected patients with suspected HFMD be isolated for the duration of fever (Rahmat *et al*., 2007; Centre for Health Protection, 2023; Ministry of Health Singapore, 2023). Disinfection and closure of premises are only necessary if there is a rise in cases reported within the same premise. A recent study in Taiwan reported the effectiveness of class suspension to reduce HFMD transmission (Lee *et al*., 2022). Our study provides implications for selecting materials for toys and furniture in kindergarten. Enteroviruses can survive on all tested surfaces, suggesting young children may be exposed to fomites by close contact in a congregated classroom or putting shared contaminated toys and utensils into their mouths leading to ingestion of infectious virus. An adequate time interval between period of disinfection and use of proper disinfectant might inactivate enteroviruses at a faster rate. Additionally, exposure to hot outdoor surroundings (in the tropics) may provide further inactivation of enteroviruses in a cost-effective and safe method for kindergartens.

CONCLUSION

Different types of toy surfaces affect the stability of viruses. Temperature, enterovirus concentration, and both porous and non-porous surfaces influence enterovirus persistence and stability in the environment.

ACKNOWLEDGEMENT

We are grateful to subproject leaders, Yin Kia Chiam and Umi Kalsum Mohd Salleh and other team members in the grant IIRG004-019HWB for discussions and advice. The research was funded by Universiti Malaya Impact Oriented Interdisciplinary Research IIRG004B-019HWB.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

Abad, F.X., Pintó, R.M. & Bosch, A. (1994). Survival of enteric viruses on environmental fomites. *Applied and Environmental Microbiology* **60**: 3704-3710. https://doi.org/10.1128/aem.60.10.3704-3710.1994

Bernama. (2021). Health DG: 106,477 HFMD cases as of June 18. *New Straits Times*. https://www.nst.com.my/news/nation/2022/06/807007/health-dg-106477-hfmd-cases-june-18. Accessed on 10 April 2023.

Centre for Health Protection. (2023). Hand, foot and mouth disease. Department of Health. The Government of the Hong Kong Special Administrative Region. https://www.chp.gov.hk/en/healthtopics/content/24/23.html. Accessed 10 April 2023.

Centres for Disease Control and Prevention. (2019). Hand, Foot, and Mouth Disease (HFMD). U.S. Department of Health & Human Services. https://www.cdc.gov/hand-foot-mouth/index.html. Accessed 10 April 2023.

Chan, Y.F. & Abu Bakar, S. (2005). Virucidal activity of Virkon S on human enterovirus. *The Medical Journal of Malaysia* **60**: 246-248.

Chan, Y.F., Sam, I.C, Wee, K.L. & AbuBakar S. (2011). Enterovirus 71 in Malaysia. A decade later. *Neurology Asia* **16**: 1-15.

Chan, Y.F., Wee, K.L., Chiam, C.W., Khor, C.S., Chan, S.Y., Amalina, W.M. & Sam, I.C. (2012). Comparative genetic analysis of VP4, VP1 and 3D gene regions of enterovirus 71 and coxsackievirus A16 circulating in Malaysia between 1997-2008. *Tropical Biomedicine* **29**: 451-466.

Chang, H.L., Chio, C.P., Su, H.J., Liao, C.M., Lin, C.Y., Shau, W.Y., Chi, Y.C., Cheng, Y.T., Chou, Y.L., Li, C.Y. *et al*. (2012). The association between enterovirus 71 infections and meteorological parameters in Taiwan. *PLOS ONE* **7**: e46845. https://doi.org/10.1371/journal.pone.0046845

Chang, S.C., Li, W.C., Huang, K.Y., Huang, Y.C., Chiu, C.H., Chen, C.J., Hsieh, Y.C., Kuo, C.Y., Shih, S.R. & Lin, T.Y. (2013). Efficacy of alcohols and alcohol-based hand disinfectants against human enterovirus 71. *Journal of Hospital Infection* **83**: 288-293. https://doi.org/10.1016/j.jhin.2012.12.010

Cordey, S., Petty, T.J., Schibler, M., Martinez, Y., Gerlach, D., van Belle, S., Turin, L., Zdobnov, E., Kaiser, L. & Tapparel, C. (2012). Identification of site-specific adaptations conferring increased neural cell tropism during human enterovirus 71 infection. *PLOS Pathogens* **8**: e1002826. https://doi.org/10.1371/journal.ppat.1002826

Dika, C., Ly-Chatain, M.H., Francius, G., Duval, J.F.L. & Gantzer, C. (2013). Non-DLVO adhesion of F-specific RNA bacteriophages to abiotic surfaces: Importance of surface roughness, hydrophobic and electrostatic interactions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **435**: 178-187. https://doi.org/10.1016/j.colsurfa.2013.02.045

Espinosa-Garcia, A.C. & Mazari-Hiriart, M. (2008). Solar radiation and enteric virus presence in irrigation water. *International Journal of Infectious Diseases* **12**: e443-e444. https://doi.org/10.1016/j.ijid.2008.05.1291

Firquet, S., Beaujard, S., Lobert, P.E., Sané, F., Caloone, D., Izard, D. & Hober, D. (2015). Survival of enveloped and non-enveloped viruses on inanimate surfaces. *Microbes and Environments* **30**: 140-144. https://doi.org/10.1264/jsme2.ME14145

Hong, J., Liu, F., Qi, H., Tu, W., Ward, M.P., Ren, M., Zhao, Z., Su, Q., Huang, J., Chen, X. *et al*. (2022). Changing epidemiology of hand, foot, and mouth disease in China, 2013-2019: a population-based study. *The Lancet Regional Health – Western Pacific* **20**: 100370. https://doi.org/10.1016/j.lanwpc.2021.100370

Kadurugamuwa, J.L. & Shaheen, E. (2011). Inactivation of human enterovirus 71 and coxsackie virus A16 and hand, foot, and mouth disease. *American Journal of Infection Control* **39**: 788-789. https://doi.org/10.1016/j.ajic.2011.01.015

Khetsuriani, N., LaMonte-Fowlkes, A., Oberst, S. & Pallansch, M.A. (2006). Enterovirus surveillance – United States, 1970-2005. *Morbidity and Mortality Weekly Report Surveillance Summary* **55**: 1-20. https://www.cdc.gov/mmwr/preview/mmwrhtml/ss5508a1.htm. Accessed 1 April 2023.

Lee, M.H.P., Chong, Y.M., Tay, C.G., Koh, M.T., Chem, Y.K., Noordin, N., Jahis, R., Sam, I.C. & Chan, Y.F. (2021). Detection of enteroviruses during a 2018 hand, foot and mouth disease outbreak in Malaysia. *Tropical Biomedicine* **38**: 150-153. https://doi.org/10.47665/tb.38.1.026

Lee, P.I., Tsai, T.C., Huang, Y.C., Wu, C.F., Hu, Y.L. & Lin, T.Y. (2022). Effectiveness of case isolation and class suspension in mitigation of enterovirus transmission in children. *Journal of Infection and Public Health* **15**: 594-598. https://doi.org/10.1016/j.jiph.2022.04.010

Lin, W.Y., Yu, Y.J. & Jinn, T.R. (2019). Evaluation of the virucidal effects of rosmarinic acid against enterovirus 71 infection via in vitro and in vivo study. *Virology Journal* **16**: 94. https://doi.org/10.1186/s12985-019-1203-z

Mahl, M.C. & Sadler, C. (1975). Virus survival on inanimate surfaces. *Canadian Journal of Microbiology* **21**: 819-823. https://doi.org/10.1139/m75-121

Ministry of Health Malaysia. (2019). Health Facts 2019: Reference Data for 2018. https://www.moh.gov.my/moh/resources/Penerbitan/Penerbitan%20Utama/HEALTH%20FACTS/Health%20Facts%202019_Booklet.pdf. Accessed 1 April 2023.

Ministry of Health Singapore. (2023). Hand, Foot and Mouth Disease: All You Need to Know. https://www.healthhub.sg/live-healthy/1932/Hand-Foot-and-Mouth-Disease-All-You-Need-to-Know. Accessed on 15 April 2023.

NikNadia, N.M.N., Sam, I.C., Rampal, S., WanNorAmalina, W.M.Z., NurAtifah, G., Verasahib, K., Ong, C.C., MohdAdib, M.A. & Chan, Y.F. (2016). Cyclical patterns of hand, foot and mouth disease caused by enterovirus A71 in Malaysia. *PLOS Neglected Tropical Diseases* **10**: e0004562. https://doi.org/10.1371/journal.pntd.0004562

Okoh, A.I., Sibanda, T. & Gusha, S.S. (2010). Inadequately treated wastewater as a source of human enteric viruses in the environment. *International Journal of Environmental Research and Public Health* **7**: 2620-2637. https://doi.org/10.3390/ijerph7062620

Onozuka, D. & Hashizume, M. (2011). The influence of temperature and humidity on the incidence of hand, foot, and mouth disease in Japan. *Science of The Total Environment* **410-411**: 119-125. https://doi.org/10.1016/j.scitotenv.2011.09.055

Rahmat, R., Abdul Rahman, H., Abd Wahab, Z., Kasri, A.R., Jahis, R. & Mohamed Ghazali, I.M. (2007). Hand Foot And Mouth Disease (HFMD) Guidelines. https://www.moh.gov.my/moh/resources/auto%20download%20images/589d71f714d23.pdf. Accessed on 1 April 2023.

Sattar, S.A., Dimock, K.D., Ansari, S.A. & Springthorpe, V.S. (1988). Spread of acute hemorrhagic conjunctivitis due to enterovirus-70: effect of air temperature and relative humidity on virus survival on fomites. *Journal of Medical Virology* **25**: 289-296. https://doi.org/10.1002/jmv.1890250306

Tamrakar, S.B., Henley, J., Gurian, P.L., Gerba, C.P., Mitchell, J., Enger, K. & Rose, J.B. (2017). Persistence analysis of poliovirus on three different types of fomites. *Journal of Applied Microbiology* **122**: 522-530. https://doi.org/10.1111/jam.13299

Teo, F.M.S., Nyo, M., Wong, A.A., Tan, N.W.H., Koh, M.T., Chan, Y.F., Chong, C.Y. & Chu, J.J.H. (2018). Cytokine and chemokine profiling in patients with hand, foot and mouth disease in Singapore and Malaysia. *Scientific Reports* **8**: 4087. https://doi.org/10.1038/s41598-018-22379-6

Thevenin, T., Lobert, P.E. & Hober, D. (2013a). Inactivation of an enterovirus by airborne disinfectants. *BMC Infectious Diseases* **13**: 177. https://doi.org/10.1186/1471-2334-13-177

Thevenin, T., Lobert, P.E. & Hober, D. (2013b). Inactivation of coxsackievirus B4, feline calicivirus and herpes simplex virus type 1: unexpected virucidal effect of a disinfectant on a non-enveloped virus applied onto a surface. *Intervirology* **56**: 224-230. https://doi.org/10.1159/000350556

Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Beumer, R.R. & Duizer, E. (2012). Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Applied and Environmental Microbiology* **78**: 7769-7775. https://doi.org/10.1128/AEM.02144-12

Wang, X. & Tarabara, V.V. (2022). Virus adhesion to archetypal fomites: a study with human adenovirus and human respiratory syncytial virus. *Chemical Engineering Journa* **429**: 132085. https://doi.org/10.1016/j.cej.2021.132085

Wommack, K.E., Hill, R.T., Muller, T.A. & Colwell, R.R. (1996). Effects of sunlight on bacteriophage viability and structure. *Applied and Environmental Microbiology* **62**: 1336-1341. https://doi.org/10.1128/aem.62.4.1336-1341.1996

Wu, S., Altenried, S., Zogg, A., Zuber, F., Maniura-Weber, K. & Ren, Q. (2018). Role of the surface nanoscale roughness of stainless steel on bacterial adhesion and microcolony formation. *ACS Omega* **3**: 6456-6464. https://doi.org/10.1021/acsomega.8b00769

Xu, J., Yang, M., Zhao, Z., Wang, M., Guo, Z., Zhu, Y., Rui, J., Wang, Y., Liu, X., Lin, S. *et al*. (2020). Meteorological factors and the transmissibility of hand, foot, and mouth disease in Xiamen City, China. *Frontiers in Medicine* **7**: 597375. https://doi.org/10.3389/fmed.2020.597375

Yusof, T.A. (2022). HFMD cases continue to rise, says Health DG. *New Straits Times*. https://www.nst.com.my/news/nation/2022/05/801028/hfmd-cases-continue-rise-says-health-dg. Accessed on 10 April 2023.

Zhang, Z., Liu, Y., Liu, F., Ren, M., Nie, T., Cui, J., Chang, Z. & Li, Z. (2021). Basic reproduction number of enterovirus 71 and coxsackievirus A16 and A6: evidence from outbreaks of hand, foot, and mouth disease in China between 2011 and 2018. *Clinical Infectious Diseases* **73**: e2552-e2559. https://doi.org/10.1093/cid/ciaa1853