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RESEARCH ARTICLE

Exploring the potential of enterovirus A71 fomite and respiratory transmission in a hamster model

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ABSTRACT

Enterovirus A71 (EV-A71) is a common pathogen of hand, foot, and mouth disease (HFMD) frequently contracted by young children. The virus commonly transmits by faecal contamination, and possibly through direct or indirect contact via fomite and respiratory routes. Transmission via fomites and the respiratory route via airborne or droplets is not clearly understood. Mouse-adapted EV-A71 (MP4 EV-A71) was used to study the effect of EV-A71 fomite-induced and respiratory transmission in one-week-old hamsters. For fomite transmission, the hamsters were exposed to coins contaminated with 10^4 50% tissue culture infectious dose ($TCID_{50}$) of EV-A71. All hamsters survived, showing self-limiting progression, and no significant loss of weight, but low viral RNA loads were detected in the oral washes and the mother of the exposed hamsters developed low neutralization titers. Despite the low fomite doses, transmission likely occurred in these hamsters. In respiratory transmission using an aerosol test chamber which was placed within the biological safety cabinet, self-limiting progression were seen in contact hamsters exposed to index hamsters orally infected with 10^4 $TCID_{50}$ of EV-A71. Index hamsters showed infection and died, but all contact hamsters survived. Computational fluid dynamics analysis showed that the transmission risk of the virus was heavily dependent on the cabinet airflow. Due to the strong convection flow, the exhaled air from the index-infected hamsters were defected, reducing the risk of infection to the contact hamsters. Taken together, our findings suggest that compared to control oral infections, fomites and respiratory transmission is less effective, but could still occur. This first animal model transmission study can be further refined with different virus dosages, exposure time and air flow to study fomite and respiratory transmission of EV-A71 in hamsters.

Keywords: Enterovirus A71; hand, foot, and mouth disease; hamster; fomite; respiratory.

INTRODUCTION

Classified in the *Picornaviridae* family of the *Enterovirus* genus, enterovirus A71 (EV-A71) belongs to a group of enteroviruses associated with faecal-oral transmission. Young children often develop hand, foot and mouth disease (HFMD), with papulovesicular rashes on the palms and feet. Direct contact via closed person-to-person interaction or indirect contact via contaminated surfaces or respiratory routes are also potential transmission routes. Secretions produced by an enterovirus-infected patient including saliva, blister fluid, and mucus are most infectious during the first week, with detection in the throat continuing for up to two weeks post-recovery. HFMD faecal specimens were reported to contain enterovirus up to 11 weeks post-recovery (Chung *et al.*, 2001). An incubation period of three to five days precedes symptoms including self-limiting fever and fatigue (Aw-Yong *et al.*, 2019). Treatment is provided to relieve symptoms, and there are no specific antiviral drugs and approved vaccines outside of China (Zhu *et al.*, 2014; Tong *et al.*, 2022).

HFMD is a contagious childhood disease, and young children possess higher risk of contracting HFMD due to behavioural and socioeconomic factors. Children from rural areas or less hygienic settings are often at higher risk (NikNadia *et al.*, 2016). In urban settings, children below the age of five years old are often enrolled in kindergartens or daycare centres where they interact closely with other children for prolonged periods. With a developing immune system, these children are prone to infectious diseases including HFMD which are transmitted via the faecal-oral route, close contact or contaminated surfaces. Lack of hand hygiene and personal hygiene awareness promotes spread of virus in these premises. However, there is a lack of information regarding EV-A71 transmission through contaminated surfaces and respiratory transmission. Respiratory droplets and aerosols are produced during coughing, sneezing, talking, and breathing. Droplets are particles of 30 to 50 µm in size that can be deposited within one meter, but smaller airborne particles of 5 µm can stay in the air and spread further distances (Ather *et al.*, 2023). Respiratory transmission could occur when pathogens are able to travel in aerosolized droplet nuclei and infect susceptible individuals.

Previous studies involving EV-A71 infection in animal models showed symptoms and pathology mimicking human infections (Chen *et al.*, 2004; Shih *et al.*, 2018). Syrian golden hamsters orally

fed with EV-A71 showed presence of lesions on the tongue, paws, and gastrointestinal tract (Phyu *et al.*, 2016; Phyu *et al.*, 2017). In the present study, one-week-old hamsters were orally infected with mouse-adapted EV-A71 (MP4 EV-A71) to study the dynamics of EV-A71 fomite and respiratory transmission.

MATERIALS AND METHODS

Cell cultures

Vero cells (ATCC catalogue no. CCL-81) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA), 1,000 U/mL penicillin-streptomycin (Thermo Fisher Scientific), and 2 mM L-glutamine (Thermo Fisher Scientific). For the virus neutralisation assay, DMEM was supplemented with 2% FBS, 1,000 U/mL penicillin-streptomycin, and 2 mM L-glutamine at 37°C with 5% CO_2.

Mouse-adapted EV-A71

The mouse-adapted EV-A71 virus, MP4, was obtained from BEI Resources (no. NR-472). Brains of mice infected by MP4 EV-A71 were harvested and propagated in Vero cells. By the fourth passage, virus stock was pooled and kept in -80°C. The virus was titered using Vero cells and determined via median tissue culture infectious dose ($TCID_{50}$). The virus stock was serially diluted 10-fold and inoculated into a monolayer of Vero cells that was grown overnight in a 96- well plate. The virus was incubated for seven days to observe for cytopathic effects (CPE). Wells containing more than 50% of CPE were marked and $TCID_{50}$ calculated using the Reed-Muench formula.

Virus neutralisation assay

Vero cells were seeded at 1×10^4 cells/well into 96-well plates one day prior to infection. The virus samples were 2-fold serially diluted in serum-free media at 1:8, 1:16, 1:32, and 1:128. Serum was collected from the dam and pups at 13 days post-infection (dpi). Two hundred microlitres of serum were incubated with 200 µl of MP4 EV-A71 for 2 hours at 37°C and transferred into cells for another hour of incubation. After washing, the cells were left in 37°C for 7 days to observe for CPE to determine the neutralising titer of serum against virus (NikNadia *et al.*, 2016).

Syrian golden hamster model

Experiment guidelines using Syrian golden hamsters (*Mesocricetus auratus*) were approved by the Institutional Animal Care and Use Committee from Monash University Malaysia Animal Ethics Committee (ethics project number: 30664). The experiments used one-week-old hamsters, which required presence of their dams in the same cage as a source of feeding and were not removed unless stated otherwise. At seven days of age, the hamsters were placed with their mother in an individually ventilated cage system in an animal biosafety level 2 laboratory at 20-24°C and with relative humidity between 45-65%. Sterile food pellets, distilled water, and corn cob bedding were provided. As hamsters are highly sensitive to smell, especially dams, hamsters were handled with nitrile gloves or sterile forceps (for hairless pups) to minimise transfer of scent from researcher to animal.

One-week-old hamsters (n=5) were orally inoculated with 100 µl of MP4 EV-A71 with the final dosage of 10^4 and 10^2 $TCID_{50}$ respectively to establish the oral infection. The hamsters were separated from their mother for 30 minutes to induce thirst before orally inoculating the virus. Hamsters were weighed every alternate day to determine the weight loss. Signs and symptoms were monitored, and we used a clinical score of 0-5; 0 – normal, 1 – ruffled fur, 2 – hind limb weakness, 3 – paralysis in one limb, 4 – paralysis in both limbs, 5 – moribund/death (Lin *et al.*, 2009; Lee *et al.*, 2021; Tan *et al.*, 2023).

Euthanization was performed when a hamster recorded a clinical score equal or higher than 4, or at the end of the experiment at 13 dpi. To perform euthanization, 500 µl of isoflurane was pipetted onto cotton balls placed in a beaker inside a sealed glass container chamber along with the hamsters. After 6-10 minutes, hamsters entered deep sedation and were taken out to perform cardiac puncture to obtain serum samples for neutralization assay.

Fomite-induced transmission

We selected stainless steel coins as a fomite to study fomite-induced transmission based on our previous study which reported that persistence and stability of EV-A71 was longest on this surface (Baharin *et al.*, 2023). Two groups of one-week-old hamsters (*n*=5 per group) labelled as R1 and R2 groups were placed in empty containers and exposed to five coins, each contaminated with 100 µl of MP4 EV-A71 at a final concentration of 10^4 $TCID_{50}$ per coin. Both groups of the one-week-old hamsters were left for one hour for three consecutive days before being placed in their original cage with the dam and observed for the next 13 days for signs and symptoms. For the control group, the experiment was repeated using PBS to coat the coins. For oral washing, 300 µL of PBS was used to flush the oral cavity at 0 (an hour after exposure), 1, 3, 5, 7, 10, and 13 dpi. The samples were kept in -80°C prior to RNA extraction and detection by PCR. Body weights of each hamster were recorded on the same day of oral wash collection.

Respiratory transmission

We simulated respiratory transmission between index and contact hamsters in the custom cage chamber placed within a biological safety cabinet. One-week-old hamsters (*n*=5) designated as index hamsters were orally inoculated with 100 µl of 10^5 $TCID_{50}$ MP4 EV-A71. Another group of one-week-old hamsters (*n*=5 per group) were assigned as contact hamsters. To test respiratory transmission, index-infected hamsters were put together with contact hamsters. The hamsters were placed in a custom cage chamber separated by wire mesh to prevent direct contact between groups (Turgeon *et al.*, 2019). The hamsters were exposed to each other for one hour for three consecutive days before returning to their original cage and observed for the next 13 days. The dams were not removed from their original cage during the experiment. Two separate groups of one-week-old hamsters served as controls for the index and contact groups respectively, remaining unexposed to the virus throughout the experiment in the custom cage chamber under same experiment setting. Oral wash samples from each hamster were collected and processed as previously described.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Viral RNA was extracted from the oral washes and serum-free media recovered from coins using NucleoSpin RNA Virus kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. To quantify viral RNA load, RT-qPCR was performed using primers (ENT-F 5'-CCCTGAATGCGGCTAAT-3' and ENT-R 5'-ATTGTCACCATAAGCAGCC-3' and TaqMan probe (5ATTO550N/ACCCAAAGTAGTCGGTTCCG/3IAbRQSp) targeting the 5'UTR of EV-A71. RT-qPCR was carried out with TaqMan Fast Virus 1-Step Master Mix (Thermo Scientific, USA) using the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) according to the following protocol: 50°C for 10 minutes, 95°C for 5 seconds, followed by 40 cycles of 95°C for 20 seconds and 60°C for 30 seconds. A standard curve was generated with standard RNA ranging from log 1 to log 8 RNA copies with 80% efficiency and correlation coefficient (R2) of 1 ± 0.02. The limit of detection and the limit of quantification for 5'UTR was determined as 100 RNA copies (data not shown).

Computational fluid dynamics modelling

Computational fluid dynamics (CFD) analysis was conducted to examine the flow field within the aerosol chamber. Carbon dioxide was used as a surrogate to represent the virus exhaled by the hamsters to predict the transmission risks of the virus from

the index group to the contact hamsters within the chamber. The CFD model of the aerosolization chamber was constructed using SOLIDWORKS 2017 (Dassault Systèmes, France). With the dimensions of the aerosol chamber of 1380 mm in length, 320 mm in width, and 360 mm in height, the chamber was divided into two compartments for the five infected index hamsters and five contact hamsters, each measuring 470 mm in length, 300 mm in width, and 340 mm in height.

The CFD model was input into Ansys-CFX software (Ansys, Inc, USA) to analyse the dispersion of the virus from the index group to the contact group. The inlet airflow rate of the aerosol chamber was assumed to be the same as that of the biosafety cabinet (0.5 m/s with a medium turbulence intensity of 5% and temperature of 24°C). Opening and pressure boundary conditions were assigned to the outlet of the aerosol chamber.

Statistical analysis

All graphs were plotted using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA). All quantitative data were presented in mean ± SD. A *p*-value < 0.05 was considered statistically significant. The survival rates of infected hamsters were determined with the Mantel-Cox log-rank test and Gehan-Breslow-Wilcoxon test.

RESULTS AND DISCUSSION

Enteroviruses transmit predominantly via the faecal-oral route since multiplication of the virus occurs in the gastrointestinal tract. Infective doses for enteroviruses were estimated between 30-1,000 viral particles (Racaniello, 2001). Nonetheless, indirect contact by fomites and respiratory transmission may occur. The golden Syrian hamster model for infection of enteroviruses was recently established, in which viruses could replicate and cause disease with lesions around paws and the oral cavity, and death at 4-8 dpi (Phyu *et al.*, 2016; Phyu *et al.*, 2017). Here, we housed the hamsters under different conditions to mimic fomite-induced and respiratory transmission of mouse-adapted EV-A71 in one-week old hamsters. We tested if MP4 EV-A71 could establish an oral infection in hamsters (Figure 1A). Oral infection of 104 TCID50 of EV-A71 in one-week-old hamsters initially caused gradual increase of body weight then decline starting day 5, coinciding with the onset of clinical symptoms (Figures 1B, 1C). Hamsters showed signs of hind leg weakness starting 4 dpi and all died by 7 dpi (Figures 1C, 1D). Viral load was inconsistently detected in the oral washes of all the five hamsters ranging from 0-9 $\log_{10}$ RNA copies/ml before the hamsters died (Figure 1E). After oral

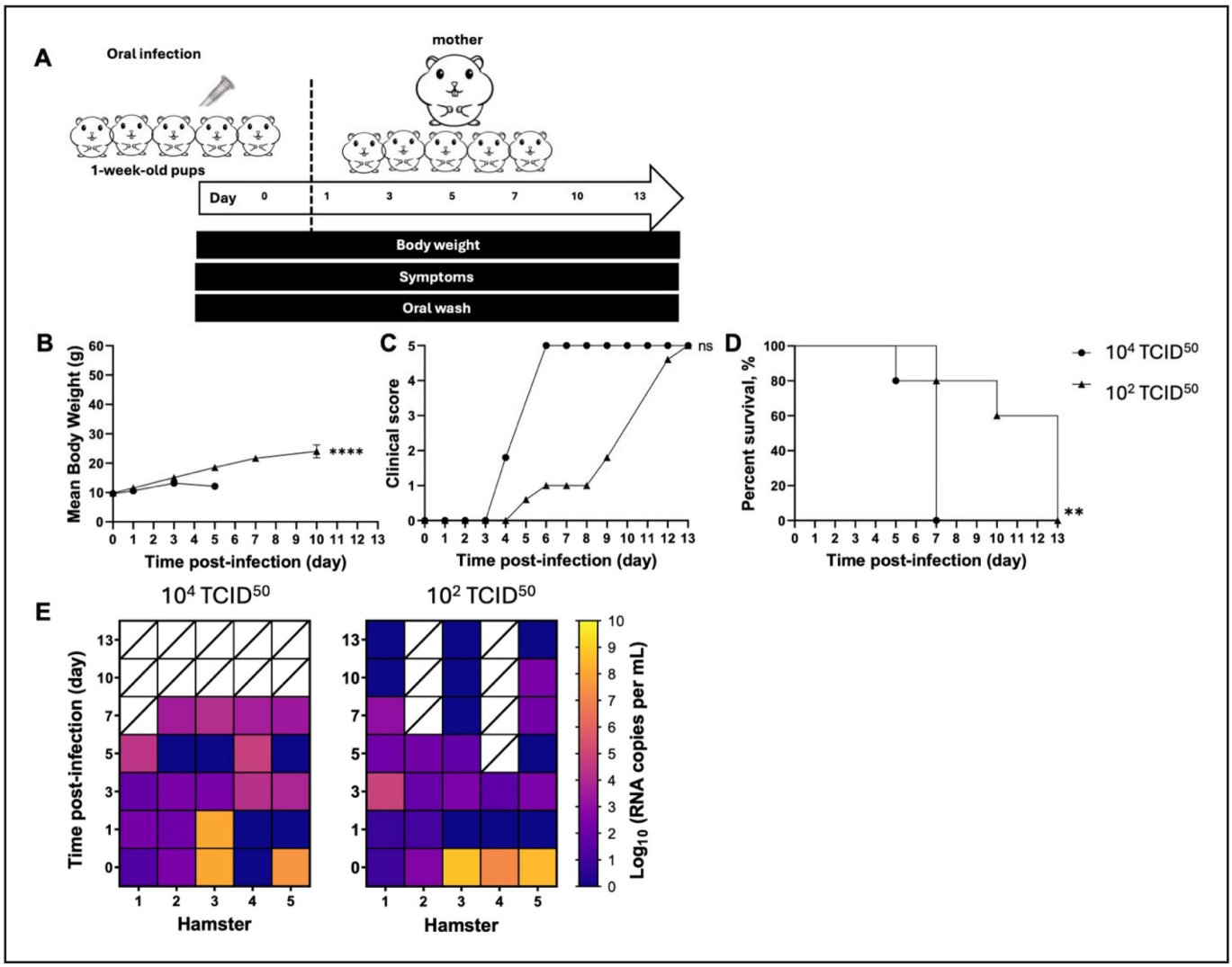

Figure 1. Susceptibility of one-week old hamsters to MP4 EV-A71 oral infection.

(A) Schematic of experiment to examine the susceptibility of hamsters to MP4 EV-A71 oral infection. One group of five hamsters was orally inoculated with MP4 EV-A71 to determine susceptibility against oral infection. (B-D) Mean body weights, mean clinical scores and survival curves of hamsters with infection of MP4 EV-A71. Disease symptoms were monitored and scored as: 0 – normal, 1 – ruffled fur, 2 – hind limb weakness, 3 – paralysis in one limb, 4 – paralysis in both limbs, 5 – moribund/death. (E) Viral loads measured from oral washes of infected hamsters at different time points are plotted in a heat map. Days that the oral washes were not taken are marked as (/). Hamsters orally inoculated with 10^4 $TCID_{50}$ were compared with those inoculated with 10^2 $TCID_{50}$ and statistically significant differences after Bonferroni correction are denoted with **** $p < 0.0001$, ns = non-significant.

infection of 10^2 $TCID_{50}$ of EV-A71, all hamsters increased body weight gradually till 10 dpi but ruffled fur was observed beginning 5 dpi, and all died by 13 dpi (Figures 1B, 1C, 1D). Detection of viral load with qPCR revealed initial high presence of virus (up to 9 log_{10} RNA copies/ml) in the oral cavity of three hamsters but declined to low or undetectable when the hamsters were dying (Figure 1D). These suggest successful recovery of initial input virus, susceptibility of hamsters to MP4 EV-A71 infection, and that MP4 EV-A71-infected hamster pups died in a dose-dependent manner. The higher viral dose killed the hamster pups as early as day 5, while the lower virus dose caused more gradual illness with limb weakness and deaths by day 13. Unlike previously reported, there were no obvious lesions around the paws and oral cavity, probably due to the use of a different EV-A71 strain (Phyu *et al.*, 2016; Phyu *et al.*, 2017).

Coins were used as the vehicle for evaluating fomite-induced transmission in hamsters. Body weight and clinical symptoms were recorded, and oral washes were taken (Figure 2A). We inoculated the highest virus dose on stainless steel coins to induce fomite transmission. We confirmed the successful contamination by recovery of MP4 EV-A71 from the surfaces of the coins (data not shown). The low MP4 EV-A71 virus titers readily recovered from the surfaces suggests that MP4 EV-A71 could be transferred from contaminated surface to one-week old pups' paws. The one-week-old hamsters were also not very active, limiting their exposure to the fomite. The mean body weight of the two fomite groups were similar to the control group and gradually increased up to 50 g at 13 dpi (Figure 2B). All hamsters did not show obvious clinical signs of disease and survived until 13 dpi (Figures 2C, 2D). In the fomite group R1, low presence of EV-A71 was detected ranging from 2-4 log_{10} RNA copies (except one hamster with 8 log_{10} at day 0) up to 13 dpi in their oral washes (Figure 2D). Serum taken from all pups and dams were used to perform viral neutralisation assay. As expected, the control hamsters showed no neutralization against EV-A71. All fomite groups also showed no neutralization against EV-A71 except one dam with 1:8 neutralizing antibody which suggests subclinical infection (data not shown). Taken together, low EV-A71 viral load was evident in the oral cavities of pups despite normal body weight increment and no signs of paralysis. However, the initiation of infection upon exposure to fomites in hamster pups does not necessarily mean that a transmission could occur. The self-limiting illness in all the infected hamsters suggest exposure of one hour for three consecutive days were not significant to induce any disease. Longer exposure time and continuous replenishing the virus inoculum may provide further opportunity for virus transfer.

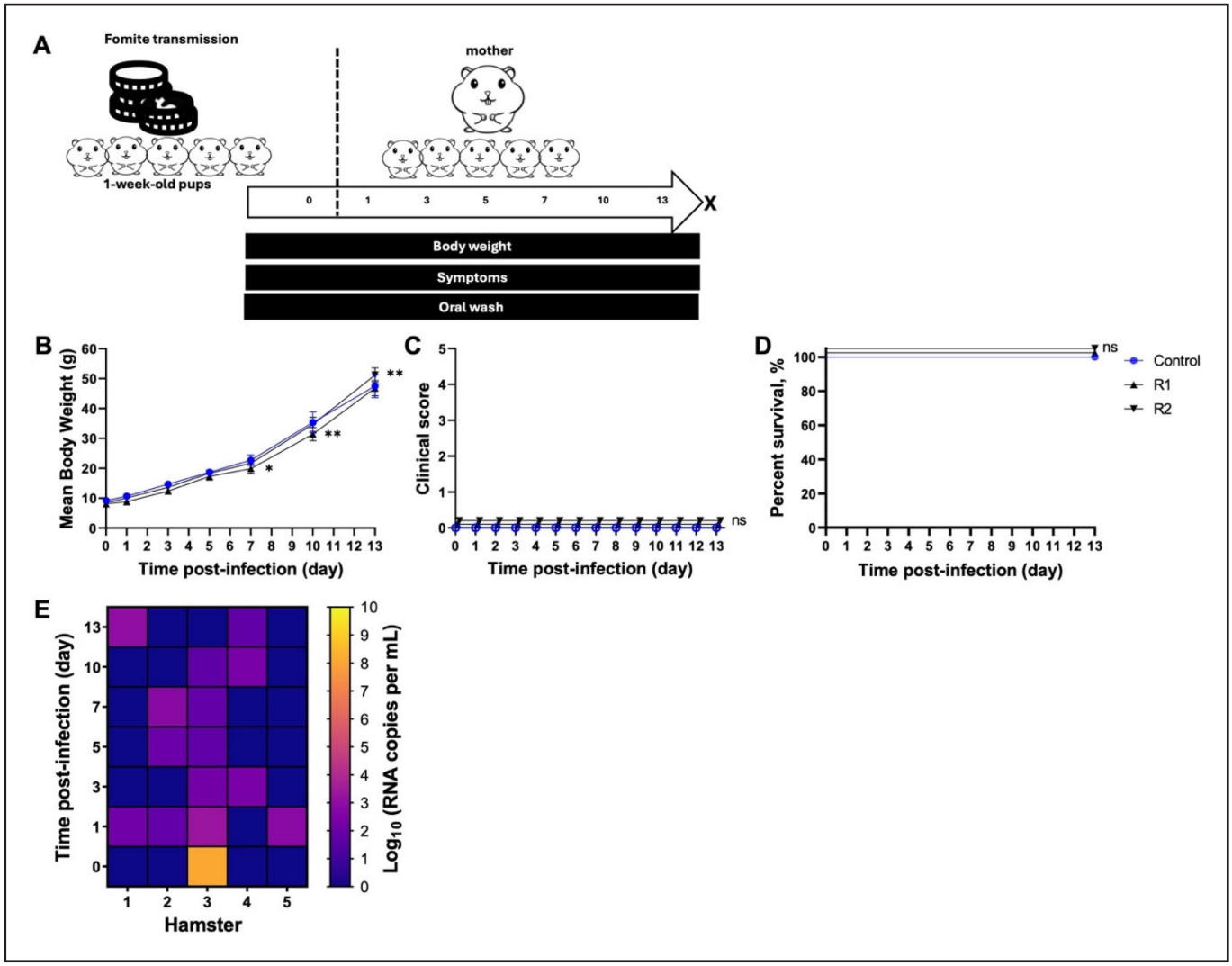

Figure 2. Fomite-induced transmission of MP4 EVA71 in one-week old hamsters.
(A) Schematic of experiment to examine fomite-induced transmission of MP4 EVA71. One group of 5 hamsters (R1 and R2) was exposed to contaminated coins as fomites (*n*=5). (B-D) Mean body weights, mean clinical scores and survival curves of hamsters with or without MP4 EV-A71 exposure. Disease symptoms were monitored and scored as: 0 – normal, 1 – ruffled fur, 2 – hind limb weakness, 3 – paralysis in one limb, 4 – paralysis in both limbs, 5 – moribund/death. Fomites groups were compared with control group and statistically significant differences after Bonferroni correction are denoted with * $p < 0.05$, ** $p < 0.01$, ns = non-significant. (E) Viral loads for one replicate experiment measured from oral washes of infected hamsters at different time points are plotted in a heat map.

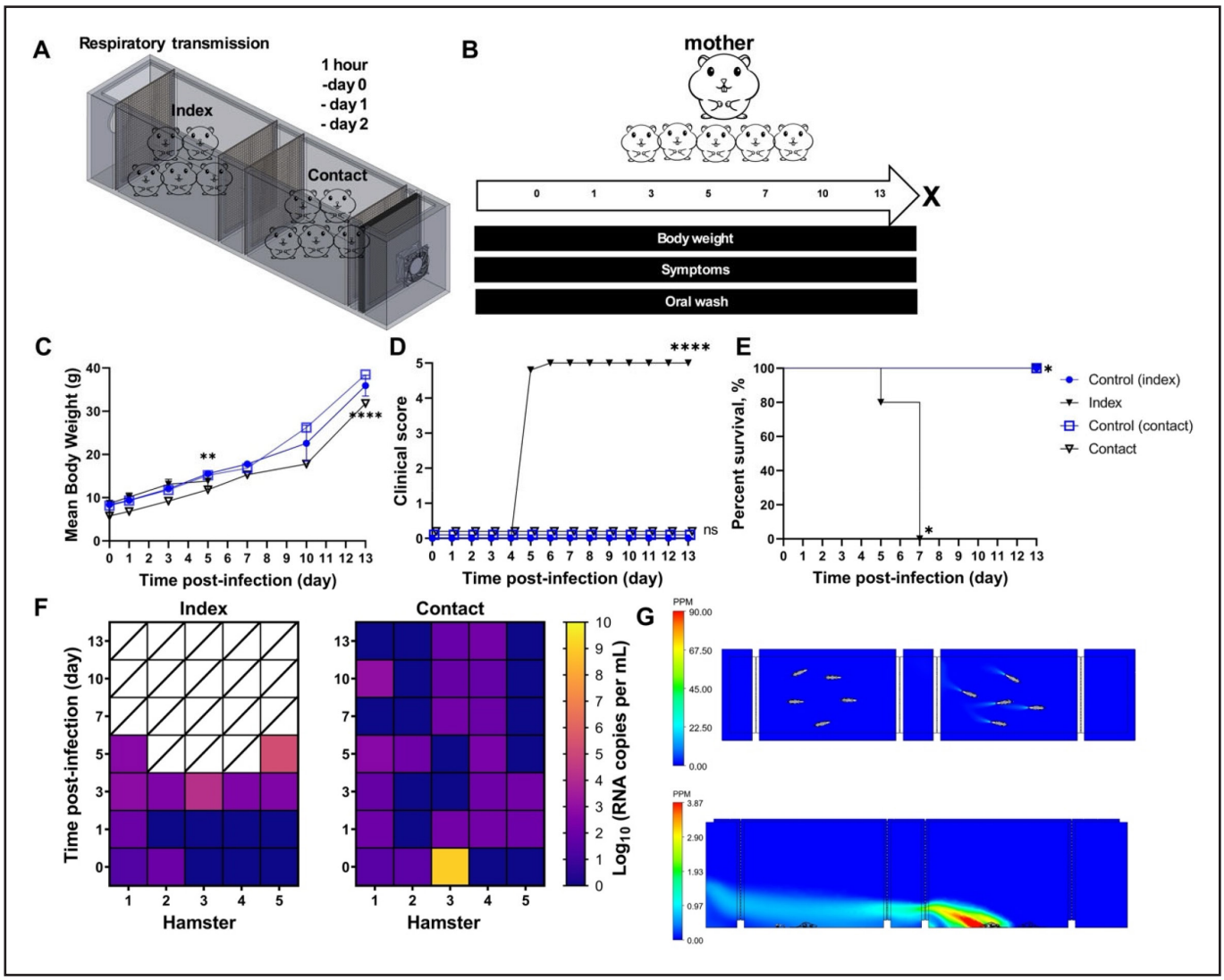

Figure 3. Respiratory transmission of MP4 EVA71 to one-week old hamsters.
(A) Schematic of experiment to determine respiratory transmission of MP4 EVA71. Five hamsters were exposed to infected index hamsters (*n*=5) via the oral route in the custom cage chamber separated by wire mesh to prevent direct contact between groups. The hamsters were exposed to each other for one hour for three consecutive days before returning to their original cage and observed for the next 13 days. (B) Recording of body weight and clinical symptoms as well as collection of oral washes from hamsters throughout the experiment. (C-E) Mean body weights, mean clinical scores and survival curves of hamsters with and without MP4 EV-A71 exposure. Disease symptoms were monitored and scored as: 0 – normal, 1 – ruffled fur, 2 – hind limb weakness, 3 – paralysis in one limb, 4 – paralysis in both limbs, 5 – moribund/death. (F) Index and contact groups were compared with corresponding control groups and statistically significant differences after Bonferroni correction are denoted with * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns = non-significant. Viral loads measured from oral washes of infected hamsters at different time points are plotted in a heat map. Days that oral washes were not taken are marked as (/). (G) Contour plot of the dispersion of exhaled CO_2 in the custom cage chamber. Plan view (top) and front view (bottom).

Next, to simulate respiratory transmission, an aerosol chamber was specifically designed to house the index and contact hamsters, keeping them separated by wire mesh (Figure 3A). In our physically separated aerosol chamber design, transmission between index and contact hamsters can be mediated by respiratory droplets and droplet nuclei. The index hamsters were orally fed with the virus prior to placement in the chamber. Due to biosafety concerns, the chamber was placed inside the biological safety cabinet during the contact time of one hour for three consecutive days. Body weight and clinical symptoms were recorded, and oral washes were collected for viral RNA analysis (Figure 3B). The control index and contact hamsters without infection showed gradual increase in mean body weight till 38 g at 13 dpi (Figure 3C). The index hamsters infected with EV-A71 had mean body weight increase similar to control groups but declining prior to death. As early as 5 dpi, the index group showed two-limb paralysis (Figure 3D). The contact hamsters appeared to have lower mean weight gain of 31 g compared to 38 g in the control group (Figure 3B). However, all the hamsters were healthy till 13 dpi and no death was observed (Figure 3E). In the index group, low viral load ranging from 1 to 5 $\log_{10}$ RNA copies/ml were detected in the oral washes (Figure 3F). In the contact hamsters, very low levels of 1 to 3 $\log_{10}$ RNA copies/ml were detected throughout the 13 dpi except for one hamster with a single high detection of 9 $\log_{10}$ RNA copies/ml (Figure 3F). Serum samples from all pups and dams were used to perform viral neutralisation assay, and only one pup had 1:8 neutralization antibody against EV-A71 suggesting subclinical infection. Hence the potential transmission of virus cannot be excluded.

An air sampler was placed for an hour at the end of the contact hamsters, but no presence of viral RNA was detected during the experiment (data not shown). As the respiratory transmission in hamsters was limited, we asked if the airflow in the biological safety cabinet could influence the transmission. To run the CFD model, the exhaled flow rate from a hamster was 0.073 L/min and the air density was 1.1839 kg/m^3. Based on the Office of Environmental Health Hazard Assessment, we assumed that the converted exhaled rate was about 1.44×10^{-6} kg/s and

the carbon dioxide exhaled was 100 ppm (Woods *et al.*, 2020). With the molecular weight of the carbon dioxide as 44.01 g/mol, the final converted concentration of carbon dioxide was 0.00018 kg/m^3 for the hamsters housed in the chamber (Figure 3G). As shown in Figure 3G, the contour plot with the highest concentration of carbon dioxide shaded in red were contributed by the exhaled carbon dioxide from the hamsters. The surrounding airflow effectively disperses the exhaled carbon dioxide, directing it swiftly towards the chamber outlet under the influence of continuous airflow from the cabinet. The dispersion pattern demonstrated that the carbon dioxide does not accumulate around the source but deflected from the hamsters and flowed towards the outlet of the chamber. The concentration of the carbon dioxide near to the compartment of the contact hamsters was very low and diluted by the incoming air (Figure 3G). As previously shown, the inflow and outflow air determined aerosol dispersion (Saw *et al.*, 2022). The transmission risk is heavily dependent on the airflow. Under strong convection flow, the exhaled particles were deflected, reducing the risk of infection in the contact group of hamsters. This observation highlights the role of ventilation design in maintaining good air quality which may explain the lack of respiratory transmission in these hamsters. Other possible explanations for the limited respiratory transmission could be due to limited coughing and sneezing from hamsters hampering adequate production of infectious aerosols and droplets in the air; varied viral shedding and clearance in different hamsters. Virus exposure could have varied significantly. To simulate person-to-person transmission, the index two-week-old hamsters orally infected with EV-A71 were exposed to contact hamsters for 2 to 12 hours (Phyu *et al*., 2017). In contrast, a study on coxsackievirus A16 kept the index and contact hamsters together throughout the 14 dpi period (Hooi *et al*., 2020). To improve this model in the future, longer exposure between index and hamsters are required to observe production of infectious aerosols and droplets in a room environment without air flow and ventilation.

Further experiments are required to clarify the significance of fomite and respiratory transmission in enteroviruses. In Syrian hamsters infected with SARS-CoV-2, disease severity and transmission efficiency were higher for airborne compared to fomite exposure (Port *et al*., 2021). Similarly, Nipah virus transmitted efficiently through direct contact but poorly via fomites, but not at all via aerosols (de Wit *et al*., 2011). This is the first animal study for fomite and respiratory transmission of enteroviruses. Further refinements such as age of the hamsters, virus dosages and exposure time will be required. Animals such as ferrets have been used as a model for influenza virus transmission and pandemic risk assessment (Belser *et al.*, 2018), so similarly, this hamster transmission model can serve as risk assessment model for more virulent emerging enteroviruses.

CONCLUSION

This study demonstrates that the role of fomite and respiratory transmission in EV-A71 infection cannot be excluded. Transmission of EV-A71 via fomite and respiratory exposure requires high concentrations of infectious virus and long exposure periods.

Conflict of Interest Statement

The authors declares that they have no conflict of interests.

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