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LETTER

# Reverse Genetic Analysis of Adaptive Mutations within the Capsid Proteins of Enterovirus 71 (EV-A71) Strains Necessary for Infection of CHO-K1 Cells

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Dear Editor,

Previous studies had described the adaptation of enterovirus 71 (EV-A71) strains that enabled entry and viral replication in Chinese Hamster Ovary (CHO) cell line (Zaini and McMinn 2012; Zaini *et al.* 2012). These adapted strains derived from serial passage of a clinical isolate in CHO cells exhibited an amino acid substitution at $VP2_{149}$, which enhanced viral replication by 100 ~ 1000-fold compared to the clinical isolate. The $VP2_{149}$ mutation was claimed responsible for adaptation to CHO-K1 cells without performing detailed molecular analyses to support these claims. In this study, we evaluate various VP1 and VP2 mutations in two CHO-adapted EV-A71 strains derived in our lab to assess their contribution to the phenotype of CHO cell adaptation.

Two EV-A71 strains derived in our laboratory and found to productively infect CHO cells, EV71:TLLm (Genbank accession no. KF514879) and EV71:TLLcho (Genbank accession no. KM508794.1), were evaluated in this study. The adaptation history of EV71:TLLm from the clinical isolate EV71:BS (Genbank accession no. KF514878.1) was described previously (Victorio *et al.* 2014). EV71:TLLcho was derived from serial adaptation of EV71:TLLm in Chinese Hamster Ovary, CHO-K1 cells (ATCC® CCL-61) for 20 cycles. To evaluate the infectivity of EV71:TLLm and EV71:TLLcho in CHO-K1 cells, we inoculated the viruses (10 MOI) onto a monkey kidney cell line (Vero; ATCC® CCL-81) and CHO-K1 cell line for 1 h at 37 °C, washed twice in PBS, and subsequently incubated in DMEM (1% FBS). At 48 h post-infection, live cell images were taken. Infected cells that have detached from the flask were coated onto Teflon slides (Erie, USA) and processed for immunofluorescent (I.F.) detection of viral antigens using pan-enterovirus monoclonal antibodies (Merck Millipore, USA) as previously described (Victorio *et al.* 2014). Viral titers in infected cell culture supernatants were also measured (Reed and Muench 1938; Victorio *et al.* 2014), and viral growth kinetics in CHO-K1 cells were compared by measuring viral titers at 6, 12, 24, 36, 48, and 54 h post-infection.

All three virus strains—EV71:BS, EV71:TLLm, and EV71:TLLcho—infected Vero cells, which exhibited lytic cell death and expressed viral antigens at 48 h post-infection (Fig. 1A). In contrast, only EV71:TLLcho productively infected CHO-K1 cells, as demonstrated by lytic cell death, viral antigen detection, and high viral titers from infected CHO-K1 cells (Fig. 1A, 1B). EV71:TLLm also infected CHO-K1 cells, although at a much slower replication kinetics and 100-fold lower titers (Fig. 1B) and despite the absence of lytic cell death (Fig. 1A). These findings confirm that EV71:TLLm and EV71:TLLcho strains, in contrast with the clinical isolate EV71:BS, were adapted to infect CHO-K1 cells.

The viral capsid mediates entry into host cells by interacting with specific cell-entry receptors. For EV-A71, the main cell-entry and uncoating receptor is the Scavenger Receptor Class B member 2 (SCARB2) protein (Yamayoshi *et al.* 2009). EV-A71 clinical strains are known to not infect rodent cells, primarily because of the low amino acid sequence similarity between primate and

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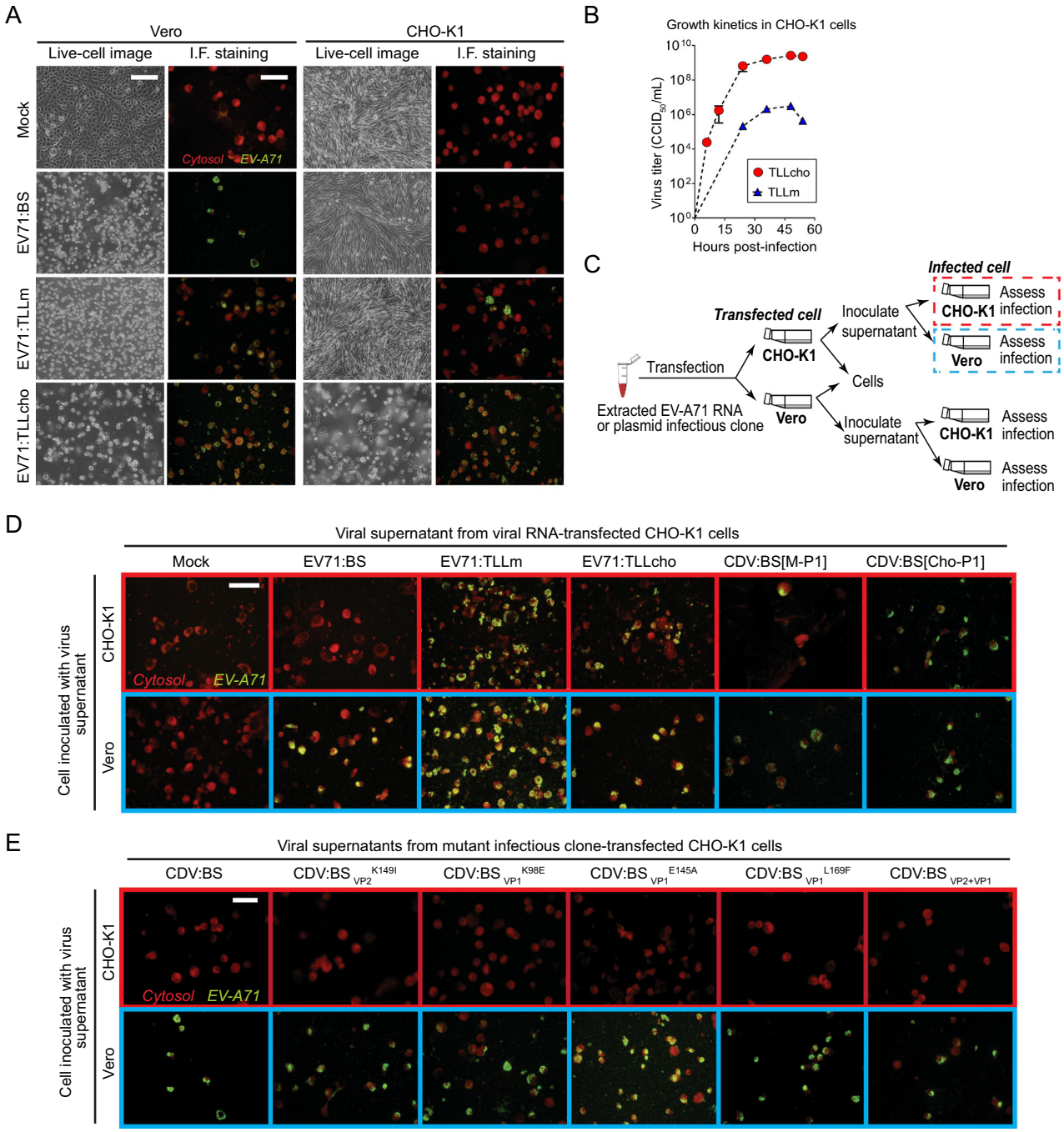


**Fig. 1** Evaluation of adaptive capsid mutations in CHO-adapted EV71:TLLm and EV71:TLLcho strains. (**A**) Representative live-cell and immunofluorescence (I.F.) staining images of virus-infected cells. Monkey kidney Vero cells and Chinese hamster ovary CHO-K1 cells inoculated with 10 MOI of either EV71:BS, EV71:TLLm or EV71:TLLcho were imaged at 48 h post-infection. The scale bars represent 50 μm. (**B**) Viral growth kinetics in CHO-K1 cells determined by measuring viral titers at various time-points post-infection. Titers are expressed as 50% cell-culture infectious dose ($CCID_{50}$) per ml. (**C**) Schematic of experimental approach for evaluating the infectivity of extracted viral RNA and plasmid infectious clones. (**D**) Representative images of I.F. staining of CHO-K1 and Vero cells inoculated with transfection supernatants from viral RNA-transfected CHO-K1 cells. (**E**) Representative images of I.F. staining of CHO-K1 and Vero cells inoculated with transfection supernatants from mutant infectious clone-transfected CHO-K1 cells. Cells were stained with pan-enterovirus antibody (green signals) and counter-stained with Evan's Blue (red background) Scale bars represent 50 μm.

human SCARB2 proteins (Victorio *et al.* 2016), which result in different conformations (Dang *et al.* 2014). Similarly, the SCARB2 protein expressed by CHO-K1 cells is more similar to mouse than human SCARB2 (Supplementary Figure S1), partly explaining why CHO-K1 cells are naturally resistant to infection with EV-A71 clinical strains.

We evaluated the contribution of the capsid in infecting CHO-K1 cells by transfecting genomic RNA extracted from EV71:TLLm and EV71:TLLcho into CHO-K1 cells. Transfection of viral RNA bypasses receptor-mediated cellular entry and introduces naked RNA directly into the cytoplasm. Subsequently, the resulting supernatant at 48 h post-transfection was re-inoculated onto both Vero and CHO-K1 cells to assess the production of viable infectious virus from transfection (Fig. 1C). Vero and CHO-K1 cells transfected with RNA extracted from either EV71:BS, EV71:TLLm or EV71:TLcho expressed viral antigens as detected by I.F. staining (Supplementary Figure S2A, S2B). Re-inoculation of transfection supernatants from CHO-K1 cells onto fresh Vero cells also resulted in positive I.F. detection of viral antigens (Fig. 1D). Re-inoculation of the same CHO-K1 transfection supernatants, with the exception of EV71:BS RNA, onto fresh CHO-K1 cells also resulted in cellular infection (Fig. 1D). We observed similar phenomena when supernatants from viral RNA-transfected Vero cells were passaged onto fresh Vero and CHO-K1 cells (Supplementary Figure S2C). These observations suggest that compared to the capsid protein of EV71:BS, EV71:TLLm and EV71:TLLcho capsids possess adaptive mutations that enable the virus to infect CHO-K1 cells.

To further confirm this finding, we synthesized chimeric EV71:BS infectious clones possessing the entire capsid protein derived from either EV71:TLLm or EV71:TLLcho as previously described (Victorio *et al.* 2016) (See Supplementary Materials for full details). These clones were transfected into Vero and CHO-K1 cells using Lipofectamine 2000 (Invitrogen, USA) to obtain clone-derived viruses (CDV:BS[M-P1] and CDV:BS[Cho-P1]). These chimeric viruses successfully infected CHO-K1 cells upon viral RNA transfection (data not shown). Re-inoculation of supernatants from CHO-K1 cells transfected with either CDV:BS[M-P1] or CDV:BS[Cho-P1] RNA onto fresh CHO-K1 cells also resulted in infection (Fig. 1D), suggesting that the adaptive mutations required for successful

**Table 1** Non-synonymous mutations in the capsid region (P1) EV71:TLLcho relative to EV71:BS.

| Genome region | Nucleotide changes | Amino acid changes | |
|---|---|---|---|
| | | Polyprotein numbering | Mature protein numbering |
| VP4 (747-953) | A809G | E21G | E21G |
| VP2 (954-1715) | G1359A | V204I | V135I |
| | G1385C | S213T | S144T |
| | A1400T | K218I | K149I |
| | G1429C | E228P | E159P |
| | A1430C | E228P | E159P |
| VP3 (1716-2441) | G1900C | A385P | A62P |
| | A2287G | T514A | T191A |
| | A2413T | S556C | S233C |
| | A2421G | I558M | I235M |
| VP1 (2442-3332) | T2462C | V572A | V7A |
| | C2530A | Q595K | Q30K |
| | A2719G | I658V | I93V |
| | T2724A | D659E | D94E |
| | A2734G | K663E | *K98E* |
| | A2753G | N669G | N104G |
| | A2876C | E710A | *E145A* |
| | A2943T | E732D | E167D |
| | C2947T | L734F | *L169F* |
| | C3165T | S806L | S241L |
| | T3175C | Y810H | Y245H |
| | A3286G | N847D | N282D |
| | G3319T | A858S | A293S |

Amino acid substitutions that fall within the VP1 canyon surrounding Gln-172 are given in italics.

infection of CHO-K1 cells reside in the capsid protein of these CHO-adapted virus strains.

To identify specific capsid mutations crucial to CHO cell adaptation, we performed Sanger sequencing of the EV71:TLLm and EV71:TLLcho genomes (Victorio *et al.* 2014). From the aligned capsid gene sequences, we identified 23 non-synonymous mutations that potentially contributed to the CHO-adapted phenotype (Table 1). These include $K_{149} \rightarrow I$ and $S_{144} \rightarrow T$ substitutions located in the EF loop ("puff region") of VP2, also previously identified as a virus neutralization epitope (Liu *et al.* 2011). This also corresponds to the single amino acid substitution reported between the CHO-adapted strains and parental clinical isolate (Zaini *et al.* 2012). We also observed six substitutions in the surface-exposed loops of VP1, including $K_{98} \rightarrow E$ in the BC loop, $E_{145} \rightarrow A$ in the DE loop, and $L_{169} \rightarrow F$ near the centre of the capsid canyon. We hypothesized that these surface-exposed residues, particularly those surrounding the capsid canyon (Rossmann 1989; Plevka *et al.* 2014) and interacting with virus receptors on host cells would have the greatest enhancement to cellular entry. To assess the contribution of these mutations in CHO cell adaptation, we constructed infectious clones where the VP2 ($K_{149} \rightarrow I$) and VP1 ($K_{98} \rightarrow E$, $E_{145} \rightarrow A$, and $L_{169} \rightarrow F$) mutations were individually introduced into the genome of EV71:BS. These amino acid substitutions appear to be stable in the genome of EV71:TLLcho from earlier passages (Supplementary Figure S3). However, with the exception of VP2 $K_{149}$, these positions did not show mutations between the CHO-adapted and clinical strains previously reported by the McMinn group (Supplementary Figure S4).

The mutant clone-derived viruses (CDVs) were inoculated onto CHO-K1 and Vero cells to assess cellular entry and infection (Fig. 1C). All evaluated CDVs productively infected Vero cells, but none was able to infect CHO-K1 cells (Fig. 1E). We therefore constructed an infectious clone where all the evaluated mutations in VP2 ($K_{149} \rightarrow I$) and VP1 ($K_{98} \rightarrow E$, $E_{145} \rightarrow A$, and $L_{169} \rightarrow F$) were simultaneously introduced into the EV71:BS genome. This CDV:$BS_{VP2+VP1}$ productively infected Vero cells but not CHO-K1 cells. These observations suggest that the VP2 and VP1 mutations evaluated are not sufficient to enable the EV-A71 clinical isolate (EV71:BS) to infect CHO-K1 cells.

In this study, we described two EV71 strains, EV71:TLLm and EV71:TLLcho, that productively infect CHO-K1 cells. EV71:TLLm was previously derived by adaptation in a mouse cell line (NIH/3T3) and infects a number of primate and rodent cell lines (Victorio *et al.* 2014). EV71:TLLcho is a new strain adapted to CHO-K1 cells and exhibits faster growth kinetics than EV71:TLLm. Compared to other EV-A71 strains derived from EV71:BS strain (Victorio *et al.* 2014), EV71:TLLcho is unique in its ability to induce lytic cell death in CHO-K1 cells. We determined that EV71:TLLcho successfully infects CHO-K1 cells owing to adaptive mutations in the capsid protein region. When we evaluated four non-synonymous mutations in VP1 and VP2 (out of the 23), which are predicted to be exposed on the virus surface, none of these mutations were sufficient to enable entry of EV71:BS into CHO-K1 cells. This was unexpected, considering our previous findings that three VP1 mutations ($K_{98} \rightarrow E$, $E_{145} \rightarrow A$, and $L_{169} \rightarrow F$) were necessary and sufficient to enable entry of EV71:BS into rodent cells (Victorio *et al.* 2016). Similarly, previous studies reported a single substitution in VP1-145 arising from mouse adaptation (Arita *et al.* 2008; Wang *et al.* 2011; Zaini and McMinn 2012) and monkey adaptation (Kataoka *et al.* 2015; Fujii *et al.* 2018). Our current findings do not fully support these previous observations and strongly suggest the need for cooperation of various adaptive mutations in the capsid to enable successful EV-A71 infection of a different host species. In the case of CHO cells or hamster infection, EV-A71 requires more than four capsid mutations for successful virus entry into hamster cells. Perhaps, it would be interesting to sequence the viruses re-isolated from EV-A71 infection models of hamster (Phyu *et al.* 2016) and gerbil (Xu *et al.* 2015) and compare with our findings.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and Human Rights Statement** This article does not contain any studies with human or animal subjects performed by any of the authors.

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