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ARTICLE in VIROLOGY JOURNAL · JUNE 2015

Impact Factor: 2.18 · DOI: 10.1186/s12985-015-0316-2

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# Validation and utilization of an internally controlled multiplex Real-time RT-PCR assay for simultaneous detection of enteroviruses and enterovirus A71 associated with hand foot and mouth disease

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## Abstract

**Background:** Hand foot and mouth disease (HFMD) is a disease of public health importance across the Asia-Pacific region. The disease is caused by enteroviruses (EVs), in particular enterovirus A71 (EV-A71). In EV-A71-associated HFMD, the infection is sometimes associated with severe manifestations including neurological involvement and fatal outcome. The availability of a robust diagnostic assay to distinguish EV-A71 from other EVs is important for patient management and outbreak response.

**Methods:** We developed and validated an internally controlled one-step single-tube real-time RT-PCR in terms of sensitivity, linearity, precision, and specificity for simultaneous detection of EVs and EV-A71. Subsequently, the assay was then applied on throat and rectal swabs sampled from 434 HFMD patients.

**Results:** The assay was evaluated using both plasmid DNA and viral RNA and has shown to be reproducible with a maximum assay variation of 4.41 % and sensitive with a limit of detection less than 10 copies of target template per reaction, while cross-reactivity with other EV serotypes was not observed. When compared against a published VP1 nested RT-PCR using 112 diagnostic throat and rectal swabs from 112 children with a clinical diagnosis of HFMD during 2014, the multiplex assay had a higher sensitivity and 100 % concordance with sequencing results which showed EVs in 77/112 (68.8 %) and EV-A71 in 7/112 (6.3 %). When applied to clinical diagnostics for 322 children, the assay detected EVs in throat swabs of 257/322 (79.8 %) of which EV-A71 was detected in 36/322 (11.2 %) children. The detection rate increased to 93.5 % (301/322) and 13.4 % (43/322) for EVs and EV-A71, respectively, when rectal swabs from 65 throat-negative children were further analyzed.

**Conclusion:** We have successfully developed and validated a sensitive internally controlled multiplex assay for rapid detection of EVs and EV-A71, which is useful for clinical management and outbreak control of HFMD.

**Keywords:** Hand foot and mouth disease, Enteroviruses, Enterovirus A71, Real-time RT-PCR, Diagnosis

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