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Universal PCR Primers Are Critical for Direct Sequencing-Based Enterovirus Genotyping

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I have read with great interest the recently published article by Van Leer-Buter et al., who employed PCR and direct sequencing to genotype enteroviruses (EVs) detected in a tertiary care hospital (1). Surprisingly, although some EVs of new species C were identified, a total of 8.5% (34/399) of the detected EVs failed to be genotyped (1), which was not addressed or discussed by the authors. The use of obsolete EV genotype classification and the adoption of non-universal PCR primers have gained my special attention, and the latter may be one explanation for the above-mentioned observation. Herein I would like to address these two important items which may affect the correct understanding and analysis of the results presented in this paper.

First, in the introduction section, based on two reports published in 1999 and 2009, Van Leer-Buter and colleagues stated that so far there are eight EV species (EV-A to EV-H) recognized (1). However, the EV genus has been classified into 12 species by the International Committee on Taxonomy of Viruses since 2013: i.e., EV-A to EV-H, EV-J, and *Rhinovirus* A (RV-A) to RV-C (2; http://www.picornaviridae.com). Of note, in order to avoid any confusion with the number 1 (one), EV-I was deliberately skipped from the new classification system (2; http://www.picornaviridae.com). Interestingly, as shown in Fig. 5 in their article, Van Leer-Buter and colleagues did include prototype strains of RV-A, -B, and -C in their phylogenetic analysis (1), which might confuse readers about the EV genotype classification adopted by the authors.

Second, in the Materials and Methods section, Van Leer-Buter et al. provided the information on the forward primer DK001 and reverse primer EV-AII (TTCTGIGTIGAIAC YTGWGCICCCAT), by which they amplified the 5′ untranslated region (5′-UTR) of the VP1 gene of EVs (1). To begin with, DK001 (5′-CAAGCACTTCTGTTTCCC-3′) was previously designed based on sequence alignment of RV 1b, 2, 14, 16, 39, and 89 (3). This suggests that this forward primer would not be a universal primer to amplify EV sequences of different genotypes, which has been confirmed by my bioinformatic analysis of divergent EV genotypes (data not shown). Besides, another bioinformatic analysis of the reverse primer EV-AII (data not shown) has also demonstrated that its 5′ end is also not common among different EV genotypes, although the 3′ end (CCCAT) of this primer appears to be universal to different strains. Merely matching with a portion of EV genotypes, the PCR primers would not bear sufficient sensitivity and capacity to amplify EV sequences of different known genotypes and would also lose the sensitivity to detect unknown EV genotypes from patient samples. This may explain the authors' finding that approximately 8.5% of the total EV detections could not be genotyped (1). Of course, this failure may additionally be due to other factors influencing PCR amplification or direct sequencing—e.g., EV viral load of the patients and sample quality. In brief, the proper design of universal primers for multiple EV genotypes has turned out to be a critical step for direct sequencing-based EV genotyping.

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