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Short communication

The high genetic similarity between rhinoviruses and enteroviruses remains as a pitfall for molecular diagnostic tools: A three-year overview

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ABSTRACT

Background: Enteroviruses (EVs) and rhinoviruses (RVs) belong to the *Enterovirus* genus within the *Picornaviridae* family, and show genetic similarities. These viruses are related to mild diseases, but EVs infections can sometimes lead to more severe complications. Current diagnostic molecular techniques should discriminate between the four EV and the three RV species that infect humans.

The aim was to revise the EV and RV PCR-confirmed specimens by sequencing for genetic characterisation.
Material and methods: Respiratory tract specimens were collected from patients with suspicion of respiratory infection. Respiratory viruses' laboratory-confirmation was performed by commercial multiplex real-time RT-PCR assays. Genetic characterisation of all EV and in a selection of RV was performed based on the phylogenetic analyses of partial VP1 and VP4/2 sequences, respectively.
Results: From 19,957 tested specimens, 309 (1.5%) were EV-positive, 2546 (12%) were RV-positive, and 233 (1%) were EV/RV co-detections. The phylogenetic analyses revealed that: among single EV detections, 177/309 (57%) were characterised as EV, 2/309 (1%) as RV, and 130/309 (42%) could not be typed; among single 1771 RV detections (Ct < 35), 1651/1771 (93%) were characterised as RV, 3/1771 (0.3%) as EV and 117/1771 (6.7%) could not be typed. Among EV/RV co-detections, 62/233 (27%) were characterised as EV, 130/233 (56%) as RV and 41/233 (18%) could not be typed.
Conclusions: A diagnostic method well considered for routine laboratory-confirmation of respiratory viruses should discriminate EV and RV targets. RVs are usually associated with mild respiratory disease, but the potential relatedness of EVs to neurological complications makes their monitoring mandatory. Therefore, an accurate detection and differentiation should be required in commercial diagnostic solutions.

1. Introduction

Human enteroviruses (EVs) and rhinoviruses (RVs) are small, non-segmented, positive-stranded RNA viruses belonging to the *Enterovirus* genus, within the *Picornaviridae* family (Jiang et al., 2014). Based on antigenic and genetic features, four EVs species (EV-A, -B, -C and -D) and three RVs species (RV-A, -B and -C) have been described, and a wide broad of types can be distinguished within them (Cabrerizo and G. P. E. E. Grupo Para El Estudio de Las Infecciones Por Enterovirus Y Parechovirus, 2017; McIntyre et al., 2013). More specifically, EV-A, -B, -C and -D have 25, 63, 23 and 5 different types, and RV-A, -B and -C, 80, 32 and 55, respectively (Oberste et al., 1999; Nikolaidis et al., 2019).

EVs and RVs are usually related to mild diseases, especially RVs, but these infections can lead to more severe complications in a few cases, such as lower respiratory tract infections (bronchiolitis or pneumonia) for RVs, in addition to myocarditis, encephalitis or acute flaccid paralysis (AFP) and poliomyelitis for EVs (Goffard et al., 2014; Rao et al., 2016). Infections caused by these two viruses can occur at any age, but the paediatric population is the most susceptible, particularly < 5 year-old children for EVs (Khetsuriani et al., 2006). The emergence, evolution and virulence of these viruses are through point amino acid changes and recombination events (Nikolaidis et al., 2019; Simmonds

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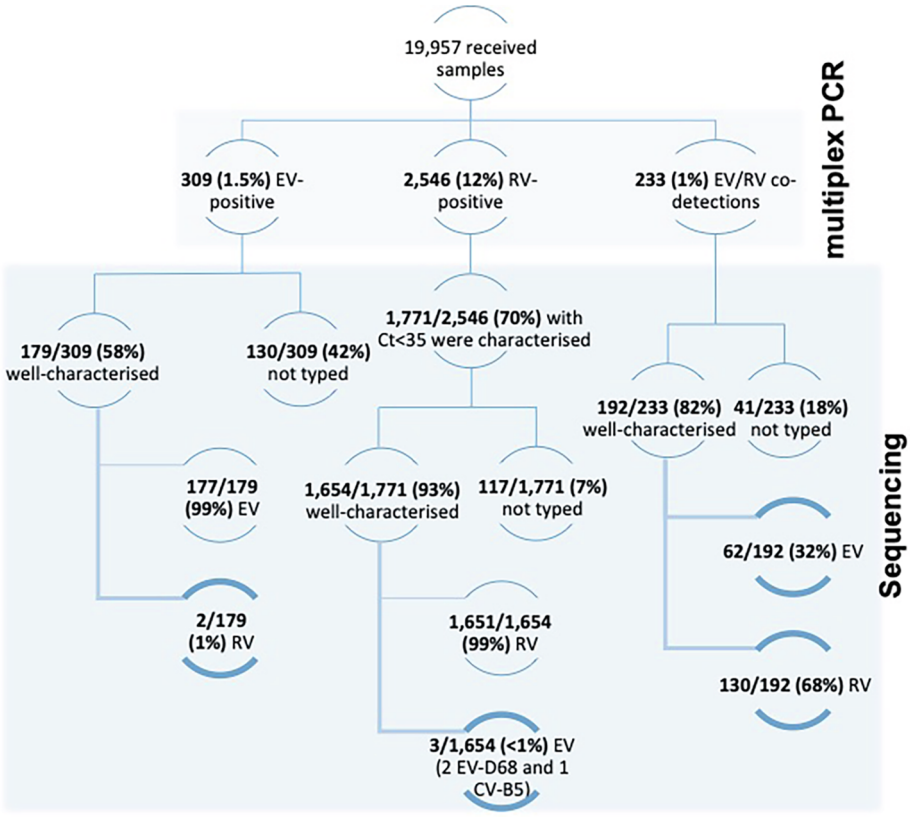

Fig. 1. Overview flowchart of the different steps followed to diagnose (multiplex PCR) and further characterisation (sequencing) of EV and RV-positive specimens for our laboratory surveillance. Those discordant results are highlighted in thick blue lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Welch, 2006).

The routine microbiological diagnosis of these viruses is currently based on molecular techniques in particular not only for their availability and their user-friendly applicability, but also for their high sensitivity values (To et al., 2017). The 5′UTR is usually the target of these diagnostic techniques, which is highly conserved among the *Enterovirus* genus because of its implication in viral replication and translation (Nikolaidis et al., 2019). However, since the genetic similarities between EVs and RVs, the molecular methods are still not able to correctly discriminate between these viruses or to detect the large number of different types for an accurate diagnosis.

The aim of the present study was to describe the discordances on the routine microbiological diagnosis for EV and RV based on real time PCR assays by using a sequencing method for their genetic characterisation.

2. Material and methods

2.1. Patients and samples

From October 2014 (40/2014) to May 2017 (20/2017), upper (nasopharyngeal aspirates or swabs) and lower (bronchoalveolar lavages, bronchoaspirates and tracheal aspirates) respiratory tract specimens were collected for respiratory viruses' laboratory-confirmation from patients with suspicion of acute respiratory tract infection who were attended at the emergency department or admitted to a tertiary hospital (Vall d'Hebron University Hospital) in Barcelona, Spain. Samples were processed within the first 24 h after collection, being kept at 2–4 °C in several aliquots until its use.

2.2. Detection of EVs and RVs from respiratory specimens

The detection of both viruses was performed by CE-marked commercial real-time multiplex RT-PCR-based assays (Anyplex II RV16 assay, from October 2014 to November 2016, or Allplex Respiratory Panel Assay, from December 2016 to May 2017, Seegene, Korea). Total nucleic acids were previously extracted using NucliSense easyMAG (bioMérieux, Marcy líEtoile, France) according to the manufacturer's instructions and kept frozen (−20 °C) until use. For samples received from October 2014 to November 2016, an additional real-time RT-PCR (Seegene, Korea) to Anyplex II RV16 assay was performed to improve the detection of all EVs due to the inaccurate detection of some of them, as previously described (Gimferrer et al., 2015).

2.3. Genetic characterisation by phylogenetic analyses

RVs were characterised based on the phylogenetic analysis of the partial coding sequence of the VP4/2 protein, with the primers and protocol from Savolainen et al. 2002 (Savolainen et al., 2002), as used in previous studies (Andrés et al., 2018a). Since the number of RV detections in respiratory tract specimens is high, a previous quantitative real-time RT-PCR (qRT-PCR) was carried out for RV-positive samples for the selection of those with cycle-threshold (Ct) cut-off values under 35 to be subsequently genetically characterised (Granados et al., 2012). All EVs were characterised by phylogenetic analyses of the partial coding sequence of the viral protein 1 (VP1) according to the protocol recommended by the World Health Organisation (WHO) (*Enterovirus Surveillance Guidelines - Guidelines for Enterovirus Surveillance in Support of the Polio Eradication Iniciative*, 2015) for the genetic characterisation of all Enterovirus genera (including RVs), as used in previous studies (Andrés et al., 2018b).

3. Results

During the study period, a total of 19,957 specimens were received for the laboratory-confirmation of respiratory viruses by real-time RT-PCR assays, of which 2546 (12%) were single RV laboratory-confirmed, 309 (1.5%) were single EV-laboratory-confirmed, and 233 (1%) were double EV and RV co-detections. Based on the sequencing of the partial VP1 and VP4/2 regions, these EV and RV confirmations were further confirmed or not. These results are summarised in Fig. 1.

4. Discussion

A good diagnostic method for routine laboratory-confirmation of respiratory viruses should be able to detect EV and RV targets. However, despite the latest advances in the technology applied on most of the current commercial and in-house methods, they are still not able to fully discriminate between them due to the genetic similarities of the targeted regions used for diagnosis or maybe due to recombination events in the 5′UTR among these viruses (Nikolaidis et al., 2019). This might have consequences on the patient management, or simply on the acquisition of a valuable epidemiological knowledge, as a result of an inaccurate diagnostic result. Nevertheless, recent evolutionary analyses only show recombination within each EV and RV specie, not between both species, so the EV and RV differentiation should be correctly done (Nikolaidis et al., 2019).

We revised the real power of discrimination of our routine molecular method for the laboratory-confirmation of respiratory viruses, including the full discrimination of EVs and RVs, by using the collected sequencing data through the virological surveillance. Our results confirmed that the percentages of discrepancies were particularly low when EV and RV were single-detected. In these cases, single RV and EV detections were successfully confirmed by sequencing of VP4/2 or VP1 coding-regions in the majority of the cases, respectively. However, although the number of double EV/RV co-detections was not high, sequencing confirmed that this commercial method is still unable to discriminate between both picornaviruses. As commented above, the close genetic similarity between some EVs and RVs remains as a pitfall for the specificity of this commercial method. Regarding the high number of samples that could not be successfully typed based on VP1 sequencing among single EV detections (42%) or double co-detections (18%) and in comparison to RV typing (7%), the main reason might be the lack of prior selection based on the viral load in the respiratory specimen.

According to our results, RVs are highly prevalent and usually associated to mild respiratory diseases in the population attended in our hospital (Andrés et al., 2018a), rather than EVs that are frequently related to lower respiratory tract infections and, in a very few cases, to myocarditis or even AFP (Andrés et al., 2018b). Although there are not vaccine or specific antivirals for EVs and RVs, and the clinical management is mainly supportive, patient isolation per etiological agent is an important measure to prevent nosocomial spread, because of the potential relatedness of EVs to these severe respiratory or even neurological complications. Therefore, the virological surveillance of RVs and, in particular EVs, is mandatory, especially for EV-A71 and EV-D68, which was also detected as a RV during the study period (Casas-Alba et al., 2017; Knoester et al., 2019). But this surveillance must be based on a good prompt detection as well as on a further genetic characterisation to better guarantee a correct attention and manage of the patient, especially children. Therefore, the suitability of routine commercial PCR-based assays for an accurate differentiation between these viruses should be assured.

In summary, based on the results of the EV and RV surveillance in our hospital, this three-year overview study highlights the fact that the full discrimination between RVs and EVs by commercial molecular methods is still compromising. Additional efforts in order to develop new diagnostic solutions for a better discrimination, as well as continuous revision of the diagnostic molecular methods currently in used according to updated surveillance data should be a priority in hospital laboratories.

Ethical approval

Institutional Review Board approvals (PR_AG_173/2017; PR_AG_201/2015) were obtained from the Clinical Research Ethics Committee of Hospital Universitari Vall d'Hebron. Participant informed consent was not necessary for this type of studies, because we only used the samples with the only fate to sequence the virus, as it is described.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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