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Nucleic acid amplification methods such as the PCR have had a major impact on the diagnosis of viral infections, often achieving greater sensitivities and shorter turnaround times than conventional assays and an ability to detect viruses refractory to conventional isolation methods. Their effectiveness is, however, significantly influenced by assay target sequence variability due to natural diversity and rapid sequence changes in viruses that prevent effective binding of primers and probes. This was investigated for a diverse range of enteroviruses (EVs; species A to D), human rhinoviruses (HRVs; species A to C), and human parechovirus (HPeV) in a multicenter assay evaluation using a series of full-length prequantified RNA transcripts. RNA concentrations were quantified by absorption (NanoDrop) and fluorescence methods (RiboGreen) prior to dilution in buffer supplemented with RNase inhibitors and carrier RNA. RNA transcripts were extremely stable, showing minimal degradation after prolonged storage at temperatures between ambient and -20°C and after multiple freeze-thaw cycles. Transcript dilutions distributed to six referral laboratories were screened by real-time reverse transcriptase PCR assays using different primers and probes. All of the laboratories reported high assay sensitivities for EV and HPeV transcripts approaching single copies and similar amplification kinetics for all four EV species. HRV detection sensitivities were more variable, often with substantially impaired detection of HRV species C. This could be accounted for in part by the placement of primers and probes to genetically variable target regions. Transcripts developed in this study provide reagents for the ongoing development of effective diagnostics that accommodate increasing knowledge of genetic heterogeneity of diagnostic targets.

Infections with picornaviruses, human rhinoviruses (HRVs), enteroviruses (EVs), and human parechoviruses (HPeVs) are frequent in human populations worldwide. Diseases associated with these viruses range from the common cold and exacerbation of asthma and bronchitis to severe infections of the central nervous system (CNS) and myocardium. EVs and HRVs are classified as members of the Enterovirus genus (27, 46), a diverse group of human, monkey, and other mammalian viruses, while HPeV is a member of the Parechovirus genus, along with the rodent Ljungan virus (13, 19, 49). The 93 human EVs fall into four genetically distinct species, human EV species A (EV-A) to EV-D (20, 46). Species B variants (echoviruses, Coxsackie B viruses, and Coxsackie A virus 9 [CAV-9]) are the mostly frequently identified viral causes of CNS-associated infections in Western countries and, along with species A serotype EV71, in Southeast Asia. Human rhinoviruses fall into three species, HRV-A to -C, containing 75, 25, and >60 types, respectively (45, 46). The disease associations of different HRV species and types are similar, although with increasing evidence of greater disease severity reported for species C (reviewed in reference 29). The common HPeV variants found in Europe are types 1, 3, 4, and 6, with a recently described association between type 3 infections and severe neonatal infections leading to sepsis (5, 6, 14).

Screening, surveillance, and investigation of the disease associations of different EV, HRV, and HPeV types require assays that are effective for the range of genetic variants found in diagnostic samples. Assays that target the highly conserved 5' untranslated regions (UTRs) of these viruses (typically by real-time PCR) (2, 9, 18, 37, 51) therefore need to accommodate naturally occurring sequence variability in this region to ensure equal sensitivity. Similarly, virus species and type identification through amplification and sequencing of coding regions such as VP1 in the case of EVs and HPeV (14, 35, 36) and VP4 for HRVs (31, 42) require often quite degenerate primer sequences to allow effective amplification of these more divergent regions of the genome.

In the current study, we have addressed one of the major problems with evaluating the performance of screening and virus typing assays for EVs and PeVs through the creation of a set of standardized RNA transcript controls. Each is quantified in absolute numbers of RNA copies, and together they represent the wide
range of naturally occurring EV, HRV, and HPeV variability. While RNA transcripts have been widely used as individual controls for the diagnostic screening of several human viruses, including noroviruses, flaviviruses, EVs, and rhinoviruses (11, 28, 44, 47, 48), this study extends their use to create larger panels representing the full genetic diversity of EV-A to -D, rhinoviruses A and B, recombinant (Ca) and nonrecombinant (Cc) variants of HRV-C (17), and HPeV type 1 (HPeV-1). Comparative evaluation of the sensitivity and amplification dynamics of a wide range of currently implemented real-time PCRs for these viruses, along with several different typing methods, provides considerable insights into the performance of these assays and how they may be potentially improved. For example, their use has revealed frequent potential sensitivity problems with HRV-C detection.

**MATERIALS AND METHODS**

**Human EV, rhinovirus, and HPeV-1 transcripts.** Full-length cDNA clones of CAV-16 (accession number U05876), echovirus 7 (E7) and E30 (AF465316 and GB-27 [unpublished]), CAV-21 (D00358), and EV70 (D00820) representing EV-A to -D, rhinoviruses A and B, recombinant (Ca) and nonrecombinant (Cc) variants of HRV-C (17), and HPeV type 1 (HPeV-1). Comparative evaluation of the sensitivity and amplification dynamics of a wide range of currently implemented real-time PCRs for these viruses, along with several different typing methods, provides considerable insights into the performance of these assays and how they may be potentially improved. For example, their use has revealed frequent potential sensitivity problems with HRV-C detection.
were as follows: PeV-CCF forward primer, CACTAGTTGTAAGGCCCA CGAA (300 nM); PeV-CCR reverse primer, GGCCCCAGATCAGATCCA (300 nM); PeV-CCP TaqMan probe, (Cy5/BHQ) CAGTGTCTCT TGTTACGCTGGGTACCTTCT (100 nM) (10).

Results from different referral laboratories were normalized to take into account differences in RNA (and, where relevant, cDNA) volumes in different assays.

Other PCR assays. EV, HRV, and HPeV sequences were amplified by a range of virus typing and reference tests used in the Specialist Virology Laboratory, University of Edinburgh. EV and HRV transcripts were assayed in six replicates by nested PCRs using the primer pairs from the 5' UTR (50) and the VP4/partial VP2 region (50). VP1 regions were amplified for EV-A, -B, and -D as previously described (16, 26). Species C sequences were amplified by newly designed primers from VP2 (outer sense, position 1172 [5' base numbered in the poliovirus Leon type 3 isolate, accession number K01392], TCN MRR GGR TGG TGG TGG AA; inner sense, position 1223, TTY GGN CAR AAY ATG TAY CAY TA; outer antisense, position 1731, CCR TTR AAY TCR CWR CAC ATN GG; inner antisense, position 1629, CCC CAR TTR TTR TGY TTN RCC AT). HPeV sequences were amplified in the 5' UTR (15) and the VP3-VP1 junction (14).

RNA stability. EV-B (E30) and -A (CAV-16) transcripts were investigated for stability at different temperatures. A 100-μl volume of a 10⁵-copy/μl dilution of each was incubated in storage solution for up to 30 days at ambient temperature, 4°C, and 37°C; an aliquot of each was also freeze-thawed three times. To determine the possible contribution of residual contaminating template DNA to RT-PCR results, control reaction mixtures without a reverse transcription step were prepared in parallel; all control reaction mixtures were negative at the highest transcript concentration tested (60,000 RNA copies/reaction mixture).

Nucleotide sequence accession numbers. Composite sequences of R4636/07 (HRV-Cpat19; Cc) and R3092/06 (HRV-C40; Ca) have been deposited in GenBank and assigned accession numbers JX276744 and JX276745, respectively.

RESULTS

Amplification of transcripts by real-time RT-PCR. Dilution series of the RNA transcripts ranging from 10⁵ to 10⁻² copies/μl were assayed by a previously described EV/HPeV multiplexed PCR method (3) (Fig. 1). The five EV transcripts showed highly reproducible amplification dynamics by real-time PCR with a linear relationship between the log₁₀-transformed RNA input copy number (x axis) and the cycle threshold (CT) value. Amplification efficiency was close to 100% (data not shown). For each transcript, the assay endpoint sensitivity lay between 0.9 and 9 RNA copies (Fig. 2A), apart from negative results for one of two of the replicate assays of E30 and EV70. Assay of the EV transcripts by other laboratories yielded similarly consistent results (Fig. 3), although there was marked variability in amplification rates between the laboratories (CT value of a nominal 1,000 RNA copies), ranging from around 32 (Edinburgh and Amsterdam) to 23 (Turku). There was a similarly wide range of endpoint sensitivities (0.4 to 20 RNA copies) between the laboratories (Fig. 2A), although sensitivity was generally consistent among the five EV transcripts tested by each laboratory. Two-log reduced sensitivity for the species C transcript was observed on testing by the Bristol and Turku laboratories and additionally for species D by the latter laboratory.

Much more variable amplification dynamics were observed with HRV transcripts, with assays from many laboratories showing reduced sensitivity for species B and C transcripts. For example, amplification of the HRV-A transcript in the multiplexed assay in Edinburgh showed amplification dynamics similar to those of EV-A to -D, while amplification of HRV-B and -C was much slower (Fig. 1B and 3A) and showed reduced assay sensitivity (Fig. 2A). Comparison of testing for HRV transcripts from each laboratory assay showed a consistent trend toward lower amplification rates and endpoint sensitivities for HRV than for EV and HPeV transcripts (Fig. 2A and 3), particularly for species C. In marked contrast, all of the assays detected HPeV RNA sequences with rates and sensitivities similar to those for EVs.

The observed variability of assay sensitivities and amplification efficiencies could be accounted for, at least in part, by the existence of sequence mismatches of primer and probe se-
quences and the target sequences (Fig. 4). The primers used for EV detection were targeted in conserved regions between positions 416 and 606 within the 5' UTR. With the exception of the sense primers used in Edinburgh and Glasgow (11), none of the primers or probes mismatched transcript target sequences. However, the one or two mismatches between the sense primer and EV evidently had little effect on assay performance; similar detection efficiencies were observed for transcripts from all four species (Fig. 2A and 3).

Primers and probes used for HRV detection were, in general, similarly conserved in the target regions among HRV variants (Fig. 4B). As for EV, few, if any, mismatches were observed with any of the real-time PCR methods, although many assays showed reduced sensitivity for species C rhinoviruses. In the Edinburgh real-time PCR, the poor sensitivity for species C might plausibly have arisen through the two mismatches toward the 3' end of the antisense primer. All of the HPeV primers and probes showed perfect matches to the HPeV1 transcript (data not shown).

Finally, although the transcripts used in the assay represent a substantial proportion of the naturally occurring sequence diversity of EVs, HRVs, and HPeV, additional variability is shown among the full currently described data set of known types within each species. To illustrate this, strict and 95% consensus sequences were constructed from alignments of each of the described types within EVs and HRVs (Fig. 4). Primers and probes showed many potential mismatches with one or more described variants within EVs and HRVs at numerous positions. For example, both sense and antisense primers for HRV used by Edinburgh and Glasgow targeted regions in the 5' UTR that were quite variable, with many positions showing less than 95% conservation among the broader set of HRV types (Fig. 4B). Better conservation was observed in EVs (Fig. 4A) and HPeV-1 to -6 (data not shown).
Sensitivity and specificity of primers used for EV and rhinovirus typing. RNA transcripts were amplified by nested PCR assays for capsid-coding regions (VP4/partial VP2) of EVs and HRVs, VP1 (EV-A, -B, and -D), partial VP2 (EV-C), and partial VP3/VP1 (HPeV), which are used for (sero)type identification (Fig. 2B; see Table S1 in the supplemental material). Dilutions ranging from $10^5$ to $10^{-12}$ copies/μl were assayed in 6-fold replicates. Frequencies of positives at each dilution were used to calculate detected RNA concentrations that were compared with input copy numbers to determine assay sensitivity. Amplification of EV, HRV, and HPeV RNA transcripts by nested PCR in the 5'UTR showed relatively high sensitivity and close concordance between the input RNA copy numbers and the RNA copy numbers detected (Fig. 2B). The sensitivities of the amplification methods for the coding regions VP4/VP2 (EVs and HRVs), VP1 (EV-A, -B, and -D), VP2 (EV-C), and VP3/VP1 (HPeV) ranged from equal sensitivity to an approximately 100-fold reduction. While these values may vary between serotypes within a species (and could not be assessed with the current panel of transcripts), these findings provide evidence that typing assay sensitivity is broadly comparable between types. 

RNA stability. Dilution of transcript RNA in a low-pH, RNase-free solution containing carrier RNA was designed to enhance their stability. 

![Primer matches to EV and HRV transcript sequences. Mismatches are highlighted with shaded boxes. Sequence alignments are numbered on the basis of the CAV-16 sequence (accession no. U05876).](Fig. 4)
longer-term stability and thus the reproducibility of assay evaluation in referral laboratories. To investigate how stable the RNA preparations actually were, two representative EV transcripts (E30, CAV-21) were subjected to a range of temperatures and freeze-thaw cycles and their RNA content was assessed by real-time PCR (Fig. 5). No or minimal changes in Ct values (reflecting residual RNA concentrations) were observed on freezing-thawing or incubation for up to 30 days at 4°C, while increases of approximately 2- and 8-fold were observed on incubation at room temperature and 37°C for 30 days (corresponding to approximately 4-fold and 250-fold reductions of amplifiable RNA sequences).

**DISCUSSION**

RNA transcripts of EV-A to -D, HPeV-1, and rhinoviruses A, B, Ca, and Cc were developed for use as molecularly calibrated RNA standards for validation and comparison of the assay sensitivities of a range of real-time PCRs and typing protocols. This Clinical Virology Network-initiated study is a response to the growing need for independent external validation of molecular-analysis-based diagnostic assays that have become the standard method of virus detection (33, 40). For all three target groups of viruses, substantial sequence diversity in most parts of the genome complicates the development of effective assays capable of detection and identification of all species and types. For each, the 5’ UTR is the most conserved region and is almost invariably targeted by existing real-time PCRs for diagnostic screening. In the case of EVs and HPeVs, screening of cerebrospinal fluid (CSF) in cases of suspected viral meningitis and neonatal sepsis requires high assay sensitivity and specificity due to the low viral loads in this compartment (8, 21, 32, 34). Recent developments of highly sensitive multiplexed PCR assays therefore represent a significant advantage in the screening of patients with these clinical presentations. Indeed, the similarity of the sensitivities of each of the real-time assays evaluated for different EV species provides reassurance that the rarity of species C and D detection in CSF samples cannot be directly attributed to assay insensitivity.

Rhinovirus screening presents a different set of difficulties. Despite its genetic diversity, PCR primers and probes for the 5’ UTR can also accommodate most of the sequence variability between species. However, these target the same conserved regions found in EVs and lead to substantial cross-amplification of EVs by HRV primers and vice versa (12). As demonstrated by the highly variable results from real-time screening of transcript dilutions, there were also substantial differences in assay sensitivity for different HRV species, most markedly for recombinant and nonrecombinant species C variants that were frequently undetected or showed ≥2-log reductions in sensitivity compared to species A and B (Fig. 2). These findings are consistent with previous studies documenting the difficulty in species C detection by PCR. Combined with its inability to be grown in cell culture, this may account for its relatively late discovery in 2006 to 2007 (1, 22–25, 30, 39). The demonstration of largely ineffective detection of species C will lead to improved assay design and modified primer/probe sequences. These can be reevaluated with the transcripts as part of its validation process; this process is under way in Edinburgh.

The same transcripts were used to evaluate the sensitivity of EV, HRV, and HPeV typing assays based on the amplification and sequencing of coding region sequences. This is necessary not only because sequence variability in the 5’ UTR is so restricted as to preclude (sero)type identification, but the occurrence of recombination between the 5’ UTR and the rest of the genome (41) and the consequent absence of species-specific 5’ UTR sequences prevent reliable species identification. A variety of typing assays have been developed, in the case of EV and PeVs, in the VP1 region (5, 14, 35, 36), where sequences have been shown to be highly predictive of EV and HPeV types. Similarly, VP4 sequences provide a reliable indication of HRV types, a more conserved region of the capsid gene that can be amplified with a common set of primers (31, 42, 50).

Variable amplification efficiencies with nested primers from the different capsid-encoding regions were observed (Fig. 2B), observations that derive from primer mismatches with target sequences in the transcripts that cannot be fully accommodated through the use of degenerate bases. However, sensitivity differences from screening (5’ UTR-based) assays and from calculated input RNA copy numbers were rarely greater than 100-fold (and usually much lower). In the specific cases of EV-B and HPeV, almost equivalent sensitivities compared to screening PCRs may underlie the previously described high frequency of successfully typed CSF samples despite their generally low viral loads (13, 14, 26).

The use of an RNA dilution buffer specifically designed to prevent RNA degradation through the incorporation of RNase inhibitors and a low concentration of carrier RNA proved highly effective at maintaining high stability despite a variety of mistreatments (Fig. 5). The minimal effect of freezing-thawing and its stability at both 4°C and ambient temperature suggest that dilution series of the RNA transcript standards can be relatively easily distributed without the need for frozen shipment. Combined with their lack of infectivity and extremely low cost of production and distribution, these transcripts can be readily supplied to diagnostic laboratories as a contribution to their quality assurance mechanisms and ongoing assay evaluations.

In summary, this study has successfully developed and evaluated a series of RNA transcripts that capture much of the diversity of EV and HRV and provide the means for laboratories to readily evaluate their screening and typing assays in absolute (RNA copy number) terms. These reagents could contribute substantially to
comparisons of assay sensitivities between laboratories, troubleshooting, and ongoing assay development as our understanding of picornavirus diversity increases.

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REFERENCES


