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Enterovirus subtyping in a routine UK laboratory setting between 2013 and 2017

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*Abstract

Abstract

Background

Human enteroviruses (hEV) are the leading cause of viral meningitis. hEV genotyping is predominantly performed through amplification and sequencing of viral capsid protein-1 (VP1), frequently by national reference laboratories (NRLs).

Objective

To determine the frequency of genotyping failure in our NRL-submitted samples and apply a superior alternative assay to resolve untyped specimens.

Study design

We initially audited genotyping data received for a cohort of patients in the East Midlands, UK by the NRL between 2013 and 2017, then identified an alternative RT-PCR typing method by literature review and evaluated primers from both assays *in silico* against comprehensive publicly available genomic data. The alternative assay was further optimised and applied to archived nucleic acids from previously untypable samples.

Results

Genotyping data showed a significant increase in untypable hEV strains through the study period ($p < 0.005$). Typing failure appeared unrelated to sample type or viral load. *In silico* analyses of 2,201 hEV genomes showed high levels of mismatch between reference assay primers and clinically significant hEV-species, in contrast to a selected alternative semi-nested RT-PCR VP1-typing assay. This alternative assay, with minor modifications, successfully genotyped 23 of 24 previously untypable yet viable archived specimens (EV-A, n=4; EV-B, n=19). Phylogenetic analyses identified no predominant strain within NRL untypable isolates, suggesting sub-optimal reference assay sensitivity across hEV species, in agreement with *in silico* analyses.

Conclusion

This modified highly sensitive RT-PCR assay presents a suitable alternative to the current English national reference VP1-typing assay and is recommended in other settings experiencing typing failure.

Highlights

- 32% of enteroviral samples referred to a national reference laboratory failed typing
- Regular re-evaluation of RT-PCR primers is required in enteroviral diagnostics
- Recommended WHO primers remain optimal for contemporary genomes, including EV-D68
- 23 of 24 (96%) viable but ‘untypable’ samples were typed by this alternative assay
- Simple and effective in-house enteroviral typing could support national surveillance

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1 # Introduction

2 Enteroviral infections in humans are caused by species A-D, commonly referred to as the
3 human enteroviruses (EV). Transmission of EVs occurs via the faecal-oral or respiratory route
4 through person-to-person contact (direct) or from contaminated environmental sources (indirect)
5 [1-3]. The majority of EV infections (50-80%) are asymptomatic or result in mild, self-limiting febrile
6 or respiratory illnesses that do not require treatment [1]. However, EVs are responsible for a diverse
7 range of clinical syndromes, from hand foot and mouth disease to acute haemorrhagic conjunctivitis,
8 and site of infection and severity varies by species, serotype and host physiology.

9 Due to their inherent neurotropism, EVs are the leading cause of viral meningitis and
10 meningoencephalitis in all age groups [1]. Young age, immunocompromise and diagnosis delay are
11 documented risk factors for developing severe or fatal EV infection [4-6]. Therapeutic options for
12 severe infections are limited, with sufficient evidence lacking to justify clinical use [7]. Symptom
13 management is the mainstay of patient management, with fast implementation of appropriate
14 infection prevention control (IPC) procedures the principle method of preventing and controlling
15 nosocomial outbreaks [8].

16 Fast, accurate diagnostic assays and international EV surveillance are essential for identifying
17 emerging virulent strains, monitoring shifts in serovar circulation and outbreak control [3, 8-10].
18 Reverse-transcription polymerase chain reaction (RT-PCR) assays, which target the highly conserved
19 5' untranslated region (UTR) genomic region, are the new gold standard for EV diagnosis due to their
20 superior sensitivity and turn-around time (TAT) compared to the previously favoured cell culture [3].
21 Routine RT-PCR targeting the 5' UTR has led to a rise in UK cases of EV being reported [11]. However,
22 high levels of genetic conservation make the 5' UTR an unsuitable target for EV typing assays. EV
23 type is ideally determined through amplification and alignment of variable regions within the VP1
24 gene [3]. It is well established that prevalent EV genotypes are constantly evolving both temporally
25 and spatially [12] and typing assays must be robust enough to detect and characterise all EV

26 subspecies to reflect this changing epidemiology. However, the effect of epidemiological changes on
27 current genotyping methodologies remains unknown.

28 In our regional diagnostic laboratory EV detection is determined by a commercial 5' UTR-
29 based assay, then positive predominantly neurological specimens are referred to the national
30 reference laboratory (NRL) for confirmation and genotyping [13, 14]. Due to an apparent increase in
31 diagnostically untypable specimens, we conducted a four-year audit of submissions, evaluated both
32 the current assay and an alternative recommended by the World Health Organisation (WHO, [8]), on
33 all available complete genome sequences *in silico*, then applied the optimised alternative assay on
34 available residual specimens previously determined as untypable.

35

36 # Materials and Methods

37 **Specimens and ethics**

38 All positive EV investigations performed within NUH Clinical Microbiology from October 1st
39 2013 to September 30th 2017 were included in this study. Samples were previously detected and
40 quantified predominantly by the Fast Track Diagnostics viral meningitis multiplex assay, generating
41 cycle threshold (cT) values and by the AusDiagnostics viral CSF multiplex generating copies per 10 µl
42 from September 2016 onward. Duplicate results were removed with neurological entries
43 preferentially retained if multiple EV positive specimens were investigated per patient. In the event
44 of duplicate specimens from the same anatomical site, the earliest total nucleic acid (TNA) extract
45 with an available typing result was retained. Ethical approval for the use of residual material was
46 provided under the Nottingham Health Science Biobank Research Tissue Bank, REC reference
47 15/NW/0685.

48 **cDNA Synthesis and Semi-Nested Polymerase Chain Reaction (snPCR)**

49 TNA extraction was performed on the bioMérieux™ NucliSENS® easyMAG® system [15]. and
50 resulting TNA eluates (50 µl) were stored at -80°C long term. cDNA was synthesized in 20 µl volumes
51 using RNA to cDNA EcoDry™ Premix with Random Hexamers (Clontech Laboratories) as per

52 manufacturer's instruction, except lyophilised RT mastermix was resuspended in 9 µl DEPC-treated

53 H_2O before addition of 10 µl TNA and 1 µl of 1pmol/µl enterovirus specific primers (AN32, AN33,

54 AN34 and AN35, Table 1) [16, 17]. 5µl cDNA was used in a modified version of the first round PCR

55 (PCR1) as described by Nix and colleagues [16, 17]. PCR1 comprised 1xHotStarTaq PCR buffer

56 (QIAGEN), primers p224/p222 (Table 1) at 50 pmol, 400 µM total dNTPs, 1.25 U HotStarTaq DNA

57 polymerase (QIAGEN) and molecular grade H_2O in a 50 µl volume, then thermocycled at 95°C (15

58 min), followed by 40 cycles at 95°C for 30 sec, 42°C for 30 sec, 72°C for 2 min, then 72°C for 2 min.

59 This PCR1 product (1µl) was used in a semi-nested (sn)PCR (25 µl volume), as per PCR1 except with

60 primers AN89/AN88 (10 pmol, Table 1), 0.625 U of HotStarTaq DNA polymerase and 40 cycles at

61 95°C for 30 sec, 60°C for 20 sec, 72°C for 30 sec.

62 snPCR products were separated and visualised on a 2% agarose gel and ranged from ~350-

63 400 bp due to variation in VP1 gene lengths across serotypes, as previously described [16, 18]. PCR

64 reactions were repeated once if no PCR product resulted. If unsuccessful after repeat, cDNA was

65 subjected to two quality control PCRs using primer pairs EQ1/EQ2 [19] and EQ2 with primer 91F

66 targeting the 5' UTR of the EV genome and generating ~150 bp and ~480 bp fragments respectively.

67 Reactions were performed in 15 µl HotStarTaq reactions under conditions above for snPCR with 55°C

68 annealing, 45 secs elongation time and 55 cycles total.

Primer Name	Sequence (5'- 3')	Gene	Location (nt)	Reference
cDNA Synthesis				
AN32	GTYTGCCA	VP1	3009-3002	[16]
AN33	GAYTGCCA	VP1	3009-3002	[16]
AN34	CCRTCRTA	VP1	3111-3104	[16]
AN35	RCTYTGCCA	VP1	3010-3002	[16]
First-step PCR (PCR1)				
p224	GCIATGYTIGGIACICAYRT	VP3	2207-2226	[16]
p222	CICCIGGIGGIAYRWACAT	VP1	2969-2951	[16]
Semi-nested PCR (snPCR)				
AN89	CCAGCACTGACAGCAGYNGARAYNGG	VP1	2603-2628	[16]
AN88	TACTGGACCACCTGGNGGNAYRWACAT	VP1	2977-2951	[16]
cDNA Quality Control PCR (cDNA-QC-PCR)				
EQ1	ACATGGTGTGAAGAGTCTATTGAGCT	5' NCR	408-433	[19]
EQ2	CCAAAGTAGTCGGTTCCGC	5' NCR	549-531	[19]

91F	ACCYTTGTRCGCCTGTTTT	5' NCR	69-87	N/A

69 **Table 1: Primers used in the study.** Sequence locations are relative to the PV1 Mahoney strain
70 (GenBank accession number V01149), with ascending and descending coordinates indicating forward
71 and reverse primers respectively.

72 **Sequencing and Sequence Analysis**

73 PCR products of expected size were diluted 1:10 in H_2O and subject to Sanger sequencing (Source
74 BioScience) with primer AN88 (3.2 pmol, [16]). Sequence identity was assessed using NCBI Standard
75 Nucleotide BLAST (BLASTn) [20] and the Genome Detective enterovirus typing tool:
76 https://www.genomedetective.com/app/typingtool/etv/ [21]. Resulting sequences and metadata
77 were submitted to Genbank under accession numbers MT614252-MT614274.

78 **Statistical Analyses**

79 Significance values were calculated in IBM SPSS Statistics for Mac (version 24.0) and GraphPad Prism
80 (version 8.3).

81

82 Results

83 **Audit of Local Enterovirus Typing Data**

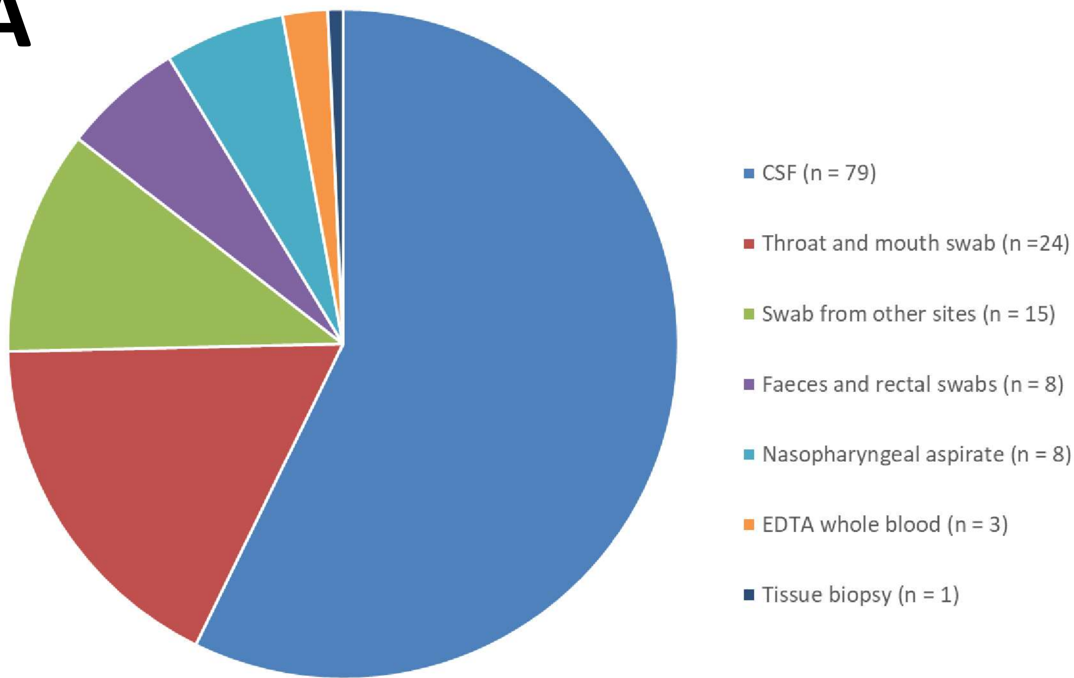
84 A retrospective audit of all EV investigations performed at NUH Clinical Microbiology from
85 October 1st 2013 to September 30th 2017 (inclusive, *n*=41,088) yielded 390 EV positive results. After
86 omission of duplicate patient results (n=69, see supplementary file) 321 unique infective episodes
87 were identified. These positive specimens principally included cerebrospinal fluid (CSF) (41.74%),
88 swabs (including skin) (24.92%), and samples from the upper respiratory tract (24.92%) and
89 gastrointestinal tract (4.98%).

90 EV typing at the NRL was requested for 138/321 positives (42.99%, **Figure 1, A**), 47 of which
91 (34.06%) were deemed 'untypable' or no typing result was received. For samples passing NRL quality
92 control (QC), no correlation between specimen type or EV viral load and an untypable result was
93 observed (see supplementary file). When analysed by date, a 5-fold increase in untypable results
94 (11.1% to 50.0%) was seen between 2013-14 and 2016-17 (**Figure 1, B**). This change in untypable

95 strain frequency was significant (p value = 0.0073) despite the declining number of specimen

96 referrals in 2016-17.

97



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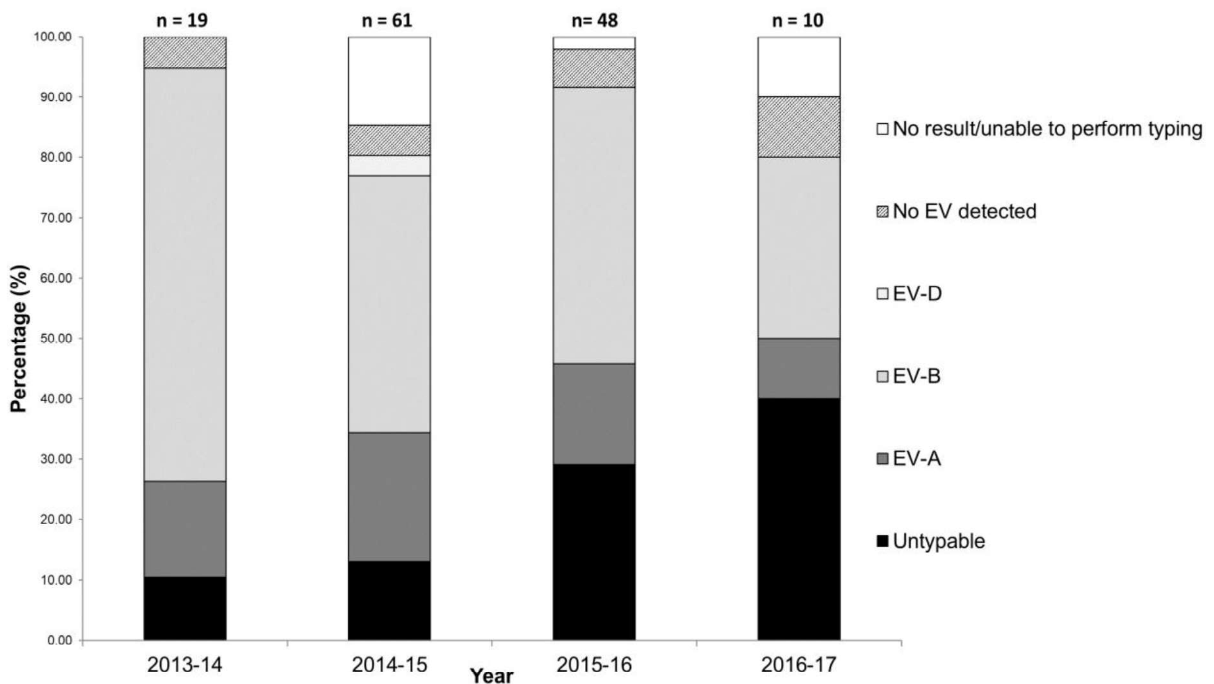

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101 **Figure 1: Breakdown of 138 EV positive eluates submitted for genotyping by specimen type,**
102 **detected in the four-year period between 2013-2017 (A) and EV typing data from the NRL, by**
103 **species identified or outcome, over successive 12-month periods (from September 30th**
104 **inclusive) (B).** Neurological specimens, cerebrospinal fluid; upper respiratory tract, nasopharyngeal
105 aspirate, throat swabs and mouth swab; lower respiratory tract, tracheal aspirate; blood, EDTA;
106 swabs from other sites include wound, skin and swabs from undefined sites. *n* = number of positive
107 EV specimens referred to the NRL for typing within each time period.

108

109 ***Re-screening of previously untypable enteroviruses***

110 Our literature review identified a RT-snPCR assay by Nix and colleagues [16] as the most
111 widely adopted worldwide for the typing of EVs (see supplementary file). Our *in silico* analysis of
112 primers from this publication [16] and the current NRL typing assay [13] was performed against
113 2,201 EV A-D complete genome sequences (see supplementary file). Primers designed by Nix and
114 colleagues gave the greatest coverage: only one potentially significant 3' terminal mismatch was
115 observed in a single primer / strain combination, in contrast to ≥4 mismatches in 50% of primer /
116 strain combinations for the NRL assay (see supplementary file). This provided strong evidence that
117 the pan-EV primers designed by Nix *et al* [16] still offer sufficient coverage of clinically relevant EV
118 species and serotypes since their design 14 years ago. A modified Nix VP1 RT-snPCR protocol was
119 therefore optimised on a panel of twelve EV positive TNA of known genotype (four EV-As, four EV-
120 Bs, one EV-C and three EV-D, see supplementary file) and 100% concordance was observed with
121 previous NRL typing.

122 The assay was then applied to the 28 available TNAs from the 47 samples that failed typing
123 by the NRL, with successful amplification achieved in 23 instances (82.1%), all of which were
124 successfully sequenced and typed (**Table 2**). Of the five extracts that failed RT-snPCR, two (Samples 4
125 and 10) failed cDNA quality control (QC) PCR (cDNA-QC-PCR) [19] indicating failure of the RT step
126 and likely degradation of extracted RNA. Two extracts (Samples 7 and 14) had <10 µl of original
127 extract available for analysis and cDNA was produced in half volume (5 µl) which was insufficient for
128 repeat after RT-snPCR failure. The final extract (Sample 11) failed RT-snPCR on repeat but passed
129 both 5' UTR cDNA-QC-PCRs with sequencing of the extended 5' UTR PCR suggestive of EV-A species
130 (**Table 2**).

131 Typing by phylogenetic analyses [21] showed broad distribution across EV-A and B species
132 (Table 2). The majority of the 23 newly genotyped samples were EV-B (n=19), with a predominance
133 of echoviruses (n=14, 73.7%): types E9 (n=3), E6 (n=3) and E5 (n=3) being the most common (Table

134 2). All CSF specimens were positive for EV-B, concurring with previous data showing predominance
135 of this species for causing viral meningitis [1].

136 In addition to multiple EV-B species, three A6 sequences (Table 2) were also observed and
137 collectively support reference laboratory typing failure by primer mismatch, rather than alternative
138 sporadic means. As samples were from deduplicated patient data, it is assumed they are separate
139 infective episodes and may be related to a local outbreak or cluster, however investigation of sample
140 clinical details was beyond the scope of this study.

Extract Reference	Specimen Extract Derived From	cT value (black) or EV copies / 10 µl (blue)	EV RNA Detected		Typing Result by PCR		Sequencing Result for 5' UTR	
			NRL	RT-snPCR	NRL	RT-snPCR	NRL	RT-snPCR
Sample 1	CSF	33.28	✓	✓	Untypable	E7	Suggestive of EV-B	n/a
Sample 2	CSF	39.92	✓	✓	Untypable (confirmed non D-68 strain)	E25	n/a	n/a
Sample 3	CSF	37.4	✓	✓	-	E6	n/a	
Sample 4	NPA	27.02	✓	x	Untypable	Untypable	Suggestive of EV-B	RNA QC Fail Unable to sequence
Sample 5	STHR	20.39	✓	✓	Untypable	E18	Suggestive of EV-B	n/a
Sample 6	CSF	30.2	✓	✓	Untypable	E9	Suggestive of EV-B	n/a
Sample 7	CSF	33.0	✓	✓	Untypable	Untypable	Suggestive of EV-B	RNA QC Pass Insufficient extract for 5' UTR sequencing
Sample 8	Whole Blood	36.19	✓	✓	Untypable	E9	n/a	n/a
Sample 9	STHR	30.3	✓	✓	Untypable	E9	Suggestive of EV-B	n/a
Sample 10	CSF	38.0	x	x	None	None	No RNA detected	RNA QC Fail Unable to sequence
Sample 11	STHR	31.6	✓	✓	Untypable	Untypable	n/a	RNA QC Pass Suggestive of E-71
Sample 12	CSF	29.85	✓	✓	Untypable	E6	n/a	n/a
Sample 13	NPA	17.7	✓	✓	Untypable (confirmed non D-68 strain)	CVB1	n/a	n/a
Sample 14	CSF	Unavailable	✓	✓	Untypable (confirmed non D-68 strain)	Untypable	n/a	RNA QC Pass Insufficient extract for 5' UTR sequencing
Sample 15	CSF	33	✓	✓	Untypable	E6	n/a	n/a
Sample 16	STHR	22.3	✓	✓	Untypable	CVA6	n/a	n/a
Sample 17	CSF	33.63	✓	✓	Untypable	CVB5	n/a	n/a
Sample 18	Swab	28.9	✓	✓	Untypable	CVA6	n/a	n/a
Sample 19	Skin Swab	28.6	✓	✓	Untypable	CVA6	n/a	n/a
Sample 20	STHR	30.75	✓	✓	Untypable	CVB1	n/a	n/a
Sample 21	CSF	38.27	✓	✓	Untypable	E5	n/a	n/a
Sample 22	CSF	33.81	✓	✓	Untypable	CVB3	n/a	n/a
Sample 23	CSF	33.78	✓	✓	Untypable	E25	n/a	n/a
Sample 24	CSF	32.78	✓	✓	Untypable	CVB3	n/a	n/a
Sample 25	STHR	13*	✓	✓	Untypable	EV-A71	n/a	n/a
Sample 26	CSF	44*	✓	✓	Untypable	E5	n/a	n/a
Sample 27	CSF	34*	✓	✓	Untypable	E5	n/a	n/a
Sample 28	CSF	584*	✓	✓	Untypable	E30	n/a	n/a

141

142 **Table 2: New typing results by sample for previously untypable Enteroviruses isolates.** Original specimens from which extracts were produced, viral loads
143 (copies/ µl) or Ct values (cycle threshold) and typing data received from the NRL for each specimen is shown for each sample. E, Echovirus; CVB, Coxsackievirus B; CVA,
144 Coxsackievirus A; EV-A71, Enterovirus 71. NPA, nasopharyngeal aspirate; SWAB, swab from undefined site; STHR, swab from throat; CSF, cerebrospinal fluid; NRL, national
145 reference laboratory; RT snPCR, reverse transcriptase semi-nested polymerase chain reaction. Enteroviral sequences located on Genbank under accession numbers
146 MT614252-MT614274.

147 # Discussion

148 We observed a significant (*p* 0.0028) rise in untypable EVs from clinical specimens processed by the
149 main EV typing and epidemiological data collection service in England so re-evaluated available
150 residual sample TNA extracts by an alternative methodology recommended by the WHO [8]. PCR-
151 based typing assays of RNA viruses requires regular primer review to monitor genome changes that
152 result from high rates of evolution and frequent recombination events, notably in some species of
153 EV between structural and non-structural genes [3, 22, 23]. EV typing is further challenged by the
154 extensive diversity of clinically relevant isolates, spanning 116 types across 4 species often sampled
155 in specimens with low viral load [24]. The current assay utilised by the English NRL employs species-
156 specific primers to amplify the *N*-terminus of the VP1 gene in EV species A-C only, overlooking EV-D
157 entirely [13]. Sequencing of this region is the gold standard for EV typing [3] and species-specific
158 primers can offer increased sensitivity compared to generic primers [3, 25, 26]. However, an *in silico*
159 review identified multiple mismatches between the primers, designed in 2006 [13] and publicly
160 available EV genomes in 2018. This concurs with an overall English NRL typing success rate of 69%
161 between 2015 and 2017 [10] and 68% in our cohort, highlighting not only the importance of
162 designing sufficiently degenerate primers to accommodate naturally occurring variation across EV
163 genomes, but also regular re-appraisal of primers for clinical diagnostics. If surveillance data are to
164 be consistent and reliable, this accommodation should be within and across EV species without
165 losing sensitivity and specificity [24].

166 Primer mismatch is one explanation for NRL typing failure. However, the sustained increase
167 in untypable isolates would not occur without also continued genetic drift or prevalence change in
168 circulating EV isolates [23], such as the recent emergence of EV-CVA6 [10] of which we identified 3
169 previously untypeable isolates. Previous analyses of NRL typing results demonstrated complex EV
170 epidemiology, with differing patterns of circulation and no evidence of any exclusively predominant
171 EV strains [10, 11]. Clinical audit and typing data presented here concurred with this finding, but EV-

172 B species were the most commonly isolated between 2013 and 2017 (**Figure 1b and Table 2**). The
173 diverse range of EVs typed in this study shows a cross-species reduction in reference laboratory
174 assay sensitivity.

175 Further coordination of surveillance data and genotyping methods from laboratories in the
176 UK, Europe and beyond would undoubtedly strengthen confidence in understanding of trends in
177 enteroviral epidemiology [3, 10]. Notably, surveillance centres identified a rise in cases of the highly
178 pathogenic strains EV-A71 and EV-D68 across Europe and North America in recent years [27, 28] but
179 EV-D68 has been missed by other molecular assays [29] and EV-D species are not targeted by the
180 NRL assay [13] necessitating the implementation of an additional EV-D68 specific assay in England
181 [30]. The current English NRL protocol therefore requires multiple PCR reactions to be performed
182 per specimen, increasing cost, and potentially also processing time and risk of contamination, and
183 may be unsuitable for low volume, low viral load, or non-repeatable samples, such as CSF, which is
184 critical for diagnosing viral meningitis/meningoencephalitis, neonatal sepsis and acute flaccid
185 paralysis/myelitis [3]. However, in principle, with suitably improved primers, this approach could
186 offer a more sensitive typing assay by reducing the diversity of coverage required from each primer.

187 The alternative RT-snPCR adopted in our study also targets conserved VP1 gene motifs, but
188 using universal primers designed by the CODEHOP strategy based on conserved amino acid motifs
189 within the target site, permitting broad target specificity and amplification of EV species A-D in one
190 workflow [16, 31]. By combining a conserved non-degenerate clamp at the primer 5' end and a
191 consensus degenerate core region at the 3' end, these snPCR primers continue to maintain broad
192 specificity and primer template stability.

193 Our *in silico* analyses showed a continued high level of EV genomic coverage and low
194 mismatch probabilities for all RT-snPCR primers designed by Nix *et al.* in 2006 [16]. Furthermore, our
195 retrospective *in vitro* evaluation showed the successful, specific VP1 amplification of 23 previously
196 untypable isolates from an array of clinical specimens confirming the method by Nix *et al* offers
197 current diagnostic and public health benefits (**Table 2**). Detection of all clinically relevant EV species,

198 including the highly pathogenic serotypes EV-C105 and EV-D68, can be achieved with this assay ([16]
199 and supplementary file).

200 Previous investigations of UK EV typing data attributed untypable results to low viral loads
201 and insufficient sample volume [11]. Samples of insufficient volume or failing initial NRL QC RT-PCR
202 were not classified as 'untypable' in the study presented here. Analysis of untypable isolate viral
203 loads showed EV was detected at Ct values 17.7 to 39.92 on the NUH diagnostic platforms between
204 2013-2017 (**Table 2**). No correlation was seen between these and cT values for isolates typed by the
205 NRL, suggesting typing assay success is not proportional to viral load. RNA quality and specimen
206 degradation post transit is controlled through additional 5' UTR PCR [13] so specimen transit is
207 unlikely to contribute to typing failure. The modified RT-snPCR assay successfully genotyped 82.1%
208 (23/28) of re-evaluated samples, failing to amplify VP1 in five samples. However, four had RNA of
209 poor quality, as determined by our QC PCR, or were of insufficient volume to repeat (**Table 2**),
210 leaving only one of the 24 viable samples (Sample 11) without a newly determined typing result.
211 Although sequencing of the 5' UTR suggested an EV-A (EV-A71), interspecies recombination within 5'
212 UTR is possible and thus this result is inconclusive [23].

213 In conclusion, this study highlighted declining reliability of a NRL EV typing assay over time
214 and a viable alternative assay, readily achievable by local hospital laboratories, was presented. Real-
215 time sharing of surveillance data to improve control of poliovirus and non-poliovirus EV strains, is an
216 important future direction of EV diagnostics [3, 10]. Therefore, regular evaluation and improvement
217 of regional and NRL EV molecular assays should be undertaken to ensure optimal coverage is
218 achieved, although service demand, focus and the available resources can vary considerably at local
219 and reference levels [10]. The ENPEN Network recently highlighted the variation in the quality of EV
220 diagnostics (detection and typing) across Europe [24], but also presents a roadmap for international
221 collaboration in to significantly progress EV diagnostics. Exponential increase in available reference
222 sequence data generated by contemporary advances in unbiased metagenomics sequencing makes
223 this target more achievable in the future.

224

225 **Declarations of interest:** none

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