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1 **The emergence of enterovirus D68 in England in autumn 2014 and the necessity**
2 **for reinforcing enterovirus respiratory screening.**

3 A.I. CARRION MARTIN*,^{1,2}, R.G. PEBODY², K. DANIS^{1,3}, J. ELLIS², S. NIAZI²,
4 S. DE LUSIGNAN^{4,5}, K.E. BROWN², M. ZAMBON², D.J. ALLEN^{2,6†}

5 ¹European Program for Intervention Epidemiology Training (EPIET), European
6 Centre for Disease Prevention and Control, (ECDC), Stockholm, Sweden

7 ²Public Health England (PHE), London, UK

8 ³ French Institute for Public Health Surveillance (Institut de Veille Sanitaire, InVS),
9 Paris, France

10 ⁴Royal College of General Practitioners (RCGP) Research and Surveillance Centre
11 (RSC), Euston, London, UK

12 ⁵ University of Surrey, Guildford, UK

13 ⁶NIHR Health Protection Research Unit in Gastrointestinal Infections, Colindale, UK

14 *Corresponding author.

15 †Present address: Pathogen Molecular Biology Department, Faculty of Infectious and
16 Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK

17 Corresponding (manuscript and reprints) author address:

18 Antonio Isidro Carrión Martín (AICM)

19 National Infection Service

20 Public Health England, 61 Colindale Avenue, London, UK, NW9 5EQ

21 Email: Isidro.carrion@phe.gov.uk

22 Tel: +44 (0)20 83276101

23 Fax: +44 (0) 2083276585

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SUMMARY

In autumn 2014, enterovirus D68 (EV-D68) cases presenting with severe respiratory or neurological disease were described in countries worldwide. To describe the epidemiology and virological characteristics of EV-D68 in England, we collected clinical information on laboratory-confirmed EV-D68 cases detected in secondary care (hospitals), between September 2014 and January 2015. In primary care (general practitioners), respiratory swabs collected (September 2013-January 2015) from patients presenting with influenza-like-illness were tested for EV-D68. In secondary care 55 EV-D68 cases were detected. Among those, 45 cases had clinical information available and 89% (40/45) presented with severe respiratory symptoms. Detection of EV-D68 among patients in primary care increased from 0.4% (4/1074; 95%CI: 0.1-1.0) (September 2013-January 2014) to 0.8% (11/1359; 95%CI: 0.4-1.5) (September-2014 to January-2015). Characterization of EV-D68 strains circulating in England since 2012 and up to winter 2014/2015 indicated that those strains were genetically similar to those detected in 2014 in USA. We recommend reinforcing enterovirus surveillance through screening respiratory samples of suspected cases.

BACKGROUND

The *Enterovirus* genus (family *Picornaviridae*) includes twelve species (A-J) of which four (A-D) are associated with disease in humans; each of these species encompasses multiple types. Enterovirus infection can be associated with a broad range of clinical presentations, including meningitis, encephalitis, hand, foot and mouth disease (HFMD), respiratory illness, and more rarely, neurological symptoms. Multiple enterovirus types co-circulate, and some types are known to emerge occasionally in a population to cause outbreaks or clusters of cases of acute neurological disease [1, 2]. Increasing recognition of the disease burden associated with enterovirus infections, and the description of ‘re-emerging’ enteroviruses such as EV-C99, EV-C104, EV-D70, EV-A71 [3, 4], provide evidence that enteroviruses have rapidly evolving genomes, are ubiquitous worldwide, spread rapidly via faecal-oral and environmental routes, but also via respiratory transmission, and have the ability to cause severe disease. Enterovirus D68 (EV-D68) is one of five enterovirus types assigned to *Enterovirus* species D. The virus was first described in 1962, isolated from children with pneumonia and bronchitis [5]; in 2002 a human rhinovirus 87 was reclassified as EV-D68, based on phylogenetic analysis [6]. In countries with enterovirus established surveillance EV-D68 has been detected sporadically in cases of acute respiratory illness, particularly in children, and occasionally was associated with acute neurological disease and other severe outcomes [7, 8].

In autumn 2014, the United States of America (USA) experienced a nationwide outbreak of EV-D68 associated with severe acute respiratory illness [9]. This outbreak coincided with an apparent increase in incidence of reported cases of neurological disease characterized as acute flaccid myelitis (AFM), suggesting that AFM is a rare yet severe clinical manifestation of EV-D68 infection [10-12].

75 In 2014, following the USA outbreak, EV-D68 was detected in European countries
76 [13]. In Norway, EV-D68 was detected in 11% of 303 paediatric nasopharyngeal
77 samples collected from children hospitalized with acute respiratory infection. EV-D68
78 was associated with acute flaccid paralysis in one child [14]. In the Netherlands, 1%
79 (18/1896) of respiratory samples taken from patients with respiratory symptoms tested
80 positive for EV-D68 [15]. Phylogenetic analysis showed that strains detected in the
81 Netherlands were genetically similar to those circulating in the USA in 2014 [15].

82 Public Health England (PHE) has an established national Enterovirus Surveillance
83 System (ESS), with emphasis on further investigation of enterovirus-positive cases
84 presenting with acute neurological symptoms as part of enhanced poliovirus
85 surveillance [16]. Following reports of the emergence of EV-D68 in USA, PHE issued
86 guidance on October 2014 that EV-D68 should be considered as a possible cause of
87 disease, particularly in children presenting with severe acute respiratory infections
88 and/or unexplained neurological symptoms, and where standard respiratory virus
89 screens were negative, or if a combined rhinovirus/enterovirus positive result was
90 initially detected.

91 This study aimed to quantify the circulation of EV-D68 in England in primary and
92 secondary care before and after the identification of EV-D68 in North America in
93 autumn 2014. Our objectives were i) to determine whether there was an increase in
94 number of cases between autumn/winter 2013-2014 and autumn/winter 2014-2015,
95 and ii) to describe the clinical presentation and phylogenetic characteristics of this
96 virus, including severity in England in the autumn/winter 2014-2015.

97 **METHODS**

98 A confirmed case was any patient presenting to primary or secondary care with a
99 specimen positive for EV-D68 detected initially by one-step real-time reverse
100 transcription PCR (rRT-PCR) [15] . Cases investigated in this study were identified
101 from two surveillance sources.

102 **1. Secondary care (clinical presentations)**

103 As part of the ESS, samples (including stool, cerebrospinal fluid (CSF) and
104 respiratory samples) from individuals who presented to hospitals with acute
105 neurological syndromes and in whom an enterovirus is detected are sent for
106 enterovirus typing. This typing is carried out at the Virus Reference Department, PHE,
107 Colindale. From November 2014, referrals to the ESS were enhanced, to include
108 enterovirus-positive paediatric cases with acute respiratory symptoms. The
109 Respiratory Diseases Department of the PHE Centre for Infectious Disease
110 Surveillance and Control (CIDSC) coordinated the epidemiological investigation of
111 each confirmed case.

112 Using a standardized questionnaire the treating clinicians collected information on all
113 confirmed EV-D68 cases from October 2014 to February 2015. The information
114 gathered included: patient characteristics, clinical presentation (including details on
115 the neurological and respiratory presentations), testing performed (including all
116 pathogens detected and type of samples) and disease outcome.

117 **2. Primary care (EV-D68 circulation)**

118 To estimate the rate of circulation of EV-D68 in the community, we tested respiratory
119 samples from the general practitioner (GP) influenza sentinel surveillance system.
120 Patients presenting with acute febrile respiratory illness considered by their GP to be
121 influenza-like-illness (ILI) had nasopharyngeal virology specimens sent to PHE, as

122 part of the Royal College of General Practitioners Research and Surveillance Centre
123 (RCGP RSC) influenza sentinel surveillance weekly returns service. These specimens
124 were tested for EV-D68. This service, run by RCGP RSC since 1957, provides
125 clinical surveillance data from a sentinel network of over 100 general practices (GP)
126 (primary health care centres) together with virological respiratory specimens, from a
127 subset of patients presenting with ILI across England [17]. Each GP sends around
128 five combined nose and throat swabs per week from patients consulting with ILI,
129 particularly during the winter season, from around week 40 of one year to around
130 week 20 of the next year. Among the samples received from the RCGP RSC from
131 week 39-2013 to week 15-2015 (September 2013 to April 2015) only those testing
132 negative for influenza virus were tested for EV-D68.

133 **Data analysis**

134 Changes in circulation of EV-D68 were estimated by calculating EV-D68 percent
135 positivity in two periods. Percent positivity was calculated as number of EV-D68
136 positive samples divided by total of samples tested for EV-D68 in each period.
137 Percent positivity in the period week 39-2013 to week 05-2014 (September 2013 to
138 January 2014) (in 2013 RCGP RSC influenza testing only started in week 39) was
139 compared to percent positivity in the period week 36-2014 to week 05-2015
140 (September 2014 to February 2015), using Chi-square test. For primary care samples,
141 we only looked at percent positivity and demographic characteristics (age and sex) of
142 cases.

143 **Laboratory detection and characterization**

144 Detection of EV-D68 was performed by rRT-PCR as previously described [15].
145 Further characterization of EV-D68 strains was performed by amplification and

146 partial sequencing of the VP1 coding region using a previously developed in-house
147 assay (M. Iturriza-Gómara, University of Liverpool, UK). Briefly, primers Ent68F
148 [5'-GAAGCCATACAAACTCGCAC-3'] and Ent68R [5'-
149 ATTWGCAATGCTCATGTATGG-3'] were used to amplify a ~670nt fragment of
150 the VP1 coding region. A high fidelity PCR system was used (Expand High Fidelity
151 PCR Kit, Roche, UK) according to manufacturer's instruction, with the following
152 specifications (per reaction): magnesium chloride final concentration 2.5mM, 40pmol
153 each primer, and 1.75U DNA polymerase. Amplification was performed under the
154 following conditions: initial denaturation: 95°C, 240 seconds; thermal cycling 95°C,
155 30 seconds – 42°C, 60 seconds – 72°C, 60 seconds for 40 cycles; final extension:
156 72°C, 240 seconds.

157 Sequence analysis was performed using Bionumerics v6.1 (Applied Maths,
158 Kortrijk,Belgium) and phylogenetic and molecular evolutionary analyses were
159 conducted using MEGA version 6 [18].

160 For phylogenetic analysis, we included representatives of EV-D68 strains detected in
161 the USA in 2014 [19] and 2015 [20], EV-D68 strains detected in England in 2010-
162 2013 as part of national enterovirus surveillance and enhanced poliovirus surveillance
163 [21] and representatives of other EV-D68 strains reported globally [22-24].

165 **RESULTS**

166 **Clinical presentation of EV-D68 in England**

167 From week 42-2014 to week 05-2015 (October 2014 to February 2015), 433
168 respiratory samples were collected from patients presenting to secondary care in

169 England. During this period, 13% (55/433) of these respiratory samples were positive
170 for EV-D68, compared with 11% (3/26) of EV-D68 positive samples detected in the
171 same period in the previous year (Figure 1). Sixty-nine percent (38/55) of the EV-D68
172 cases (October 2014 to February 2015) were in children under 12 years of age; the
173 median age of cases was 4 years (range 0-71), and there was no difference in the
174 gender distribution (46% male) (Figure 2). The majority of these EV-D68 positive
175 specimens were nasopharyngeal aspirates, 44% (24/55), and nose/throat swabs,
176 31%(17/55). The remaining 14 were sputum, mouth swabs and tracheal/broncho
177 alveolar aspirates. EV-D68 was not detected in any CSF specimen tested (n=562).

178 Detailed clinical information was available for 82% (45/ 55) EV-D68 cases (Table 1).
179 None of these cases reported having recently travelled outside of the United Kingdom
180 (UK). Ninety-one percent (41/45) of the cases presented with acute respiratory
181 symptoms, one case presented with HFMD, one case had leukaemia (respiratory
182 symptoms not reported) and two cases presented with acute neurological symptoms.
183 These cases showing neurological presentations were in children <5 years of age: one
184 case presented with meningoencephalitis and paucity of limb movements [22]; the
185 second presented with afebrile seizures and respiratory arrest. These cases were
186 previously healthy children who required intensive care unit (ICU) treatment and
187 subsequently recovered; in both cases, EV-D68 was detected in nasopharyngeal
188 aspirate specimens. Thirty-three percent (13/39) of the cases presented with asthma or
189 wheezing, 84% (11/13) of them were children under 15 years of age (median age: 2
190 years) (information for asthma was only collected for 39 patients). Forty-seven
191 percent (21/45) of the cases were immunocompromised or had an underlying chronic
192 condition. Eighty-two percent (37/45) of the cases were admitted to hospital, with
193 27% (12/45) requiring treatment in ICU, all of them children (median age: <1 year)

194 (Table 1). Two patients in whom EV-D68 was detected died: both were infants with
195 underlying neurological problems. Information on cause of death was only accessible
196 for one of them: it was likely due to complications in the underlying neurological
197 problems. Two other infant cases had never left the hospital since birth.

198 **Estimate of prevalence of EV-D68 in England**

199 A total of 3575 nose/throat swab samples were collected from week 39-2013 to week
200 15-2015 (September 2013 to April 2015) as part of the RCGP RSC influenza sentinel
201 surveillance weekly returns service. The median age of the patients providing these
202 samples was 33 years (range 0-99), 17% of them were children <12 years of age.
203 Sixty-one percent were female.

204 EV-D68 was detected in 0.4% (15/3575) of all the community respiratory samples
205 analysed. Among the cases, the median age was 40 years, (range 5-84 years); nine
206 (60%) of them were female (Figure 2). Among those 3575 samples, 2433
207 (1074+1359) had been collected during the two periods of comparison and were
208 included in the percent positivity analysis. Positivity in the autumn/winter 2013/14
209 (week 39-2013 to week 05-2014) was 0.4% (4/1074; 95%CI: 0.1-1.0). Positivity in
210 the autumn/winter 2014/15 (week 36-2014 to week 05-2015) was 0.8% (11/1359)
211 (95%CI: 0.4-1.5) (p value=0.17). All primary care (RCGP RSC) EV-D68 positive
212 samples in 2013 were collected between weeks 40 to 47, while all the community EV-
213 D68 positive samples from 2014 were collected between weeks 49 to 52 (Figure 1).

214 **Phylogenetics**

215 Partial VP1 sequence was obtained from 88% (57/70) of the EV-D68 cases

216 identified from primary and secondary care in this study (four from community cases
217 recruited via the RCGP network and 53 from cases referred to the ESS).

218 Of the 57 EV-D68 sequences detected in this study, 56 clustered with EV-D68 genetic
219 clades (A and B as described elsewhere [23]): 49 (85%) in clade B and 8 (14%) in
220 clade A. The remaining 2014 strain clustered with the prototype US/Fermon/1962
221 strain (Figure 3).

222 EV-D68 strains detected in England in 2014 and 2015 clustered both with strains
223 detected in the USA during the same period, and with strains circulating in England
224 prior to 2014. Among the clade B viruses there were the two sequences found in cases
225 with neurological presentation. Both of these sequences were highly similar to other
226 co-circulating viruses from the same period.

227 Amino acid sequences were highly similar within clades, with most amino acid
228 differences occurring in the BC and DE loops (data not shown). There were no
229 specific amino acid changes in either the BC or DE loop, or elsewhere in the regions
230 of VP1 sequenced that distinguished strains associated with neurovirulence from other
231 EV-D68 strains detected.

232 **DISCUSSION**

233 We used two established surveillance systems for this study. From the national
234 enterovirus surveillance system data we described the burden of illness due to EV-
235 D68 and the severity of clinical presentation in England. We used the influenza
236 sentinel GP surveillance system to look at the changes in EV-D68 circulation in
237 primary care. Clinical information from the cases identified in the ESS (secondary
238 care) found that cases with EV-D68 positive samples were mainly presenting with
239 severe respiratory symptoms in the autumn/early winter 2014-2015. In primary care,
240 retrospective testing of samples revealed a non-significant increase in circulation of

241 EV-D68 in autumn/early winter 2014-2015 compared to the same period the previous
242 year.

243 Our findings from secondary care suggest that, when detected, EV-D68 is most
244 frequently associated with respiratory disease, usually in children, and more rarely
245 with neurological disease. Previous studies presented comparable results [9, 24-27].
246 Further, in our study the majority of hospitalized cases presented with severe
247 respiratory disease and one third of those were admitted to ICU (all of them children)
248 and required respiratory support. Our results on cases with severe respiratory
249 presentations, mostly children, associated with EV-D68 infection were comparable to
250 similar findings in the Netherlands and the USA [15, 27]. We found that 33% of
251 cases presented with asthma/wheezing compared to the 70% of cases presenting with
252 these symptoms in the 2014 USA outbreak [27], this difference could be attributable
253 to the small number of cases for whom we had information on asthma/wheezing. In
254 our study, similarly to other studies, almost half of the patients admitted with
255 respiratory disease were immunosuppressed or had severe underlying conditions [15,
256 28]. Our findings reinforce the importance of clinicians considering EV-D68 (and
257 other enteroviruses) as one of the differential diagnoses of severe acute respiratory
258 illness, particularly in children.

259 In the only two acute neurological presentations in our study, EV-D68 was not
260 detected in the CSF of these children and the detection of EV-D68 in the respiratory
261 samples may have been coincidental. However, both cases were previously healthy
262 and no other infectious cause was identified for the disease. Therefore we cannot
263 exclude the possibility of an association between EV-D68 and their severe acute
264 presentations. Both children made a complete recovery. Severe neurological
265 presentations associated with EV-D68 in previously healthy children with a preceding

266 respiratory infection have been reported in different European countries: in Norway,
267 two cases (Autumn 2014) [29]; a case in France (September 2014) [30] and two cases
268 in Wales (UK) (January 2016) [31], EV-D68 was not identified in the CSF in any of
269 these cases, but rather from upper respiratory tract samples. Similarly, we found that
270 EV-D68 RNA was detectable in respiratory specimens, but that other sample types
271 (e.g. CSF or stool) were not sensitive for detection of EV-D68. We were able to
272 recover infectious EV-D68 virus by inoculation of permissive cell lines with RT-PCR-
273 positive respiratory specimens, but not other specimen types. This highlights the
274 importance of collecting all clinically relevant samples for differential diagnosis in
275 such acute presentations and indicates that in EV-D68 suspected cases respiratory
276 specimens are the most appropriate for diagnostic testing and virological
277 characterization.

278 The rate of positivity in cases of acute respiratory disease presenting to primary care
279 in England doubled from autumn/winter 2013-2014 to autumn/winter 2014-2015.
280 Nonetheless this increase was not statistically significant and the number of cases and
281 positivity rates were low. This suggests that the high number of severe cases observed
282 in secondary care in the period post autumn 2014 is presumably explained by the
283 change in case definition and enhanced case ascertainment. Similarly, in the
284 Netherlands, a non-significant increase in the percentage of EV-D68 positives was
285 observed from 2013 (0.3%) to 2014 (0.6%) among patients presenting to the GP [32].
286 In another similar study in Germany from 2014, a higher percentage (7.7%) of EV-
287 D68 positive samples from outpatients presenting with ILI, and/or acute respiratory
288 infection was reported [28]. Our primary care cases had similar distribution across all
289 age groups, but in those in secondary care, age was skewed toward infants and young
290 children (Figure 2). This could be related to the PHE guidance, suggesting considering

291 EV-D68 as a possible cause of disease in children with severe respiratory symptoms.
292 Or, taking into account similar results from other studies, it may demonstrate a
293 different age profile of the EV-D68 cases presenting with severe symptoms in
294 secondary care compared to the profile of the mild presentations in primary care.

295 We characterized EV-D68 strains circulating in England since 2012 and up to winter
296 2014-2015, among which we identified strains genetically similar to those detected in
297 2014 in USA. These findings are consistent with similar results from Netherlands and
298 Germany [15, 28]. These studies describe emergence of distinct genetic clusters,
299 which reflects the continuous evolution of EV-D68 strains in circulation.

300 With extensive genetic variation in the EV-D68 genome, continuous collection of
301 genomic data by representative surveillance systems is required to ensure that PCR-
302 based detection methods are capable of detecting contemporary circulating viruses.
303 Genotyping assays are generally based upon sequence analysis of the VP1 coding
304 region, whilst primary detection assays usually target the 5'-untranslated region of the
305 genome. With recognized sequence variation in both regions, complete genome
306 sequences representing contemporary circulating viruses is essential in assuring
307 performance of molecular diagnostic and characterization assays.

308 However, to fully understand the clinical significance of detection of EV-D68 in
309 patients, alongside genomic characterization, isolation of the virus, characterization by
310 neutralization and measurement of virus-specific antibody titres is required.

311 Integrating 'classical' virology approaches and molecular techniques will better
312 establish links between infection and disease in patients, as well as provide valuable
313 technical information for improving laboratory diagnostics, monitoring genetic drift in
314 the virus genome which may result in degradation of PCR primer/probe-binding sites.

315 Some molecular assays have been found to exhibit cross-reactivity between EV-D68
316 and rhinovirus which can lead to false-positive results. Furthermore, with reports of
317 other emerging and novel enterovirus types [1, 2, 33], it is important to continually
318 enhance the virological, genomic and epidemiological understanding of these viruses
319 by maintaining up-to-date diagnostic and reference methods, and monitoring the
320 factors determining local and temporal differences in atypical disease outbreaks due to
321 enteroviruses.

322 The detection of EV-D68 worldwide in 2014-2015 may be the ‘tip-of-the-iceberg’ of
323 circulation of EVD-68. EV-D68 is rarely associated with severe disease, which is
324 probably why fewer cases have been detected by current surveillance systems that are
325 designed to capture more severe (neurological) infections. In our study, detection of
326 the virus in community and in hospitalized patients with respiratory disease suggest
327 EV-D68, and perhaps other enteroviruses, are a more important cause of infection and
328 potentially disease than captured by current surveillance systems.

329 **STRENGTHS AND LIMITATIONS**

330 Our study had some strengths and limitations. In terms of strengths, we were able to
331 look at the EV-D68 epidemiology and virology in primary care before and after the
332 signal in the US, using an identical surveillance system, which allowed the
333 comparison between the two periods with no ascertainment bias. In terms of
334 weaknesses, first, we could not compare the EV-D68 positivity rate in ESS with
335 previous years given the change in case definitions in autumn 2014 following the PHE
336 guidance mentioned before. Second, the RCGP RSC influenza sentinel surveillance
337 samples are predominantly restricted to the winter months when influenza is
338 circulating, considering the previously described EV-D68 seasonal pattern, majority

339 of cases occurring in late summer and autumn [34, 35], we may have missed cases if
340 they had occurred in summer or late spring. Furthermore, the representativeness of the
341 RCGP RSC influenza sentinel surveillance is not ideal for an estimate of the real
342 circulation of this virus in patients presenting to primary care, as the patients tested
343 present with acute febrile respiratory illness, and during the 2014 outbreak in the US
344 only 48% of the hospitalized cases presented with fever; additionally EV-D68 may be
345 a more common cause of mild respiratory illness both in primary care and in the
346 community among cases who self-manage without seeking healthcare intervention.
347 Nonetheless, the RCGP RSC is a representative network, with only small differences
348 with the national population and therefore the results of this study provide a useful
349 baseline for future monitoring of the circulation of EV-D68 and its severity in
350 England[17]. Finally, we did not sequence the whole genome which might contain
351 virulence markers elsewhere. For example, changes in the 5'UTR and IRES have been
352 proposed to associate with changes in translation initiation [36] and virulence [37, 38].

353 **CONCLUSIONS**

354 In this study we detected EV-D68 in cases of severe acute respiratory illness and, less
355 commonly, in neurological illness, particularly in children and those with underlying
356 disease from autumn 2014 when enterovirus surveillance system was enhanced and
357 respiratory sampling was included. We also provided evidence of EV-D68 in cases of
358 ILI presenting in primary care before and after autumn 2014, with strains genetically
359 similar to those detected in 2014 in the USA.

360 EV-D68 should be considered as a possible cause of disease in patients presenting
361 with severe acute respiratory infections and/or with unexplained neurological
362 symptoms. Our findings emphasize the necessity of reinforcing enterovirus

363 surveillance in England. We recommend the screening of respiratory samples for
364 enterovirus, particularly EV-D68, from patients presenting with severe acute
365 respiratory infections and/or with unexplained neurological symptoms, when all other
366 respiratory virus screen are negative or if an indeterminate rhinovirus/enterovirus
367 positive result was initially detected. The surveillance of enterovirus, specifically EV-
368 D68, in respiratory samples will help us to better understand the epidemiology of EV-
369 D68 and to inform surveillance and laboratory-testing guidance.

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384 **Conflict of interest**

385 None

386 **Ethical approval**

387 No ethical approval was required for this study.

- 389 (1) **Horner LM, et al.** Acute Flaccid Paralysis Associated with Novel Enterovirus C105.
390 *Emerging infectious diseases* 2015; **21**(10): 1858-1860.
- 391 (2) **Osterback R, et al.** Echovirus 30 meningitis epidemic followed by an outbreak-specific RT-
392 qPCR. *Journal of clinical virology : the official publication of the Pan American Society for Clinical*
393 *Virology* 2015; **69**: 7-11.
- 394 (3) **Qiu J.** Enterovirus 71 infection: a new threat to global public health? *The Lancet Neurology*
395 2008; **7**(10): 868-869.
- 396 (4) **Fischer TK, et al.** Emergence of enterovirus 71 C4a in Denmark, 2009 to 2013. *Euro*
397 *surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease*
398 *bulletin* 2014; **19**(38).
- 399 (5) **Schieble JH, Fox VL, Lennette EH.** A probable new human picornavirus associated with
400 respiratory diseases. *American journal of epidemiology* 1967; **85**(2): 297-310.
- 401 (6) **Ishiko H, et al.** Human rhinovirus 87 identified as human enterovirus 68 by VP4-based
402 molecular diagnosis. *Intervirology* 2002; **45**(3): 136-141.
- 403 (7) **Imamura T, et al.** Enterovirus 68 among children with severe acute respiratory infection, the
404 Philippines. *Emerging infectious diseases* 2011; **17**(8): 1430-1435.
- 405 (8) **Kreuter JD, et al.** A fatal central nervous system enterovirus 68 infection. *Archives of*
406 *pathology & laboratory medicine* 2011; **135**(6): 793-796.
- 407 (9) **Midgley CM, et al.** Severe respiratory illness associated with enterovirus D68 - Missouri and
408 Illinois, 2014. *MMWR Morbidity and mortality weekly report* 2014; **63**(36): 798-799.
- 409 (10) **Greninger AL, et al.** A novel outbreak enterovirus D68 strain associated with acute flaccid
410 myelitis cases in the USA (2012-14): a retrospective cohort study. *The Lancet Infectious diseases*
411 2015.
- 412 (11) **Messacar K, et al.** A cluster of acute flaccid paralysis and cranial nerve dysfunction
413 temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. *Lancet*
414 2015.
- 415 (12) **Pastula DM, et al.** Acute neurologic illness of unknown etiology in children - Colorado,
416 August-September 2014. *MMWR Morbidity and mortality weekly report* 2014; **63**(40): 901-902.
- 417 (13) Control ECfDPA. Enterovirus 68 detected in the USA, Canada and Europe Second update, 25
418 November 2014. In. [http://ecdc.europa.eu/en/publications/Publications/Enterovirus-68-detected-in-](http://ecdc.europa.eu/en/publications/Publications/Enterovirus-68-detected-in-the-USA-Canada-Europe-second-update-25-November-2014.pdf)
419 [the-USA-Canada-Europe-second-update-25-November-2014.pdf](http://ecdc.europa.eu/en/publications/Publications/Enterovirus-68-detected-in-the-USA-Canada-Europe-second-update-25-November-2014.pdf), 2014.
- 420 (14) **Bragstad K, et al.** High frequency of enterovirus D68 in children hospitalised with
421 respiratory illness in Norway, autumn 2014. *Influenza and other respiratory viruses* 2015; **9**(2): 59-
422 63.
- 423 (15) **Poelman R, et al.** The emergence of enterovirus D68 in a Dutch University Medical Center
424 and the necessity for routinely screening for respiratory viruses. *Journal of clinical virology : the*
425 *official publication of the Pan American Society for Clinical Virology* 2015; **62**: 1-5.
- 426 (16) England PH. PHE national polio guidelines. Local and regional services. In.
427 [https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/497601/PHE_Polio_g](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/497601/PHE_Polio_guidelines.pdf)
428 [uidelines.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/497601/PHE_Polio_guidelines.pdf). Public Health England, 2016.
- 429 (17) **Correa A, et al.** Royal College of General Practitioners Research and Surveillance Centre
430 (RCGP RSC) sentinel network: a cohort profile. *BMJ open* 2016; **6**(4): e011092.
- 431 (18) **Tamura K, et al.** MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.
432 *Molecular biology and evolution* 2013; **30**(12): 2725-2729.
- 433 (19) **Brown BA, et al.** Seven Strains of Enterovirus D68 Detected in the United States during the
434 2014 Severe Respiratory Disease Outbreak. *Genome announcements* 2014; **2**(6).

- 435 (20) **Wylie TN, et al.** Development and Evaluation of an Enterovirus D68 Real-Time Reverse
436 Transcriptase PCR Assay. *Journal of clinical microbiology* 2015; **53**(8): 2641-2647.
- 437 (21) **Kadambari S, et al.** Enterovirus infections in England and Wales, 2000–2011: the impact of
438 increased molecular diagnostics. *Clinical Microbiology and Infection* 2014; **20**(12): 1289-1296.
- 439 (22) **Varghese R, et al.** Sampling the upper respiratory tract for enteroviral infection is important
440 in the investigation of an acute neurological illness in children. *European journal of paediatric*
441 *neurology : EJPN : official journal of the European Paediatric Neurology Society* 2015; **19**(4): 494-
442 495.
- 443 (23) **Tokarz R, et al.** Worldwide emergence of multiple clades of enterovirus 68. *The Journal of*
444 *general virology* 2012; **93**(Pt 9): 1952-1958.
- 445 (24) **Farrell JJ, et al.** Enterovirus d68-associated acute respiratory distress syndrome in adult,
446 United States, 2014. *Emerging infectious diseases* 2015; **21**(5): 914-916.
- 447 (25) **Oberste MS, et al.** Enterovirus 68 is associated with respiratory illness and shares biological
448 features with both the enteroviruses and the rhinoviruses. *The Journal of general virology* 2004;
449 **85**(Pt 9): 2577-2584.
- 450 (26) **Zhang T, et al.** Enterovirus d68-associated severe pneumonia, china, 2014. *Emerging*
451 *infectious diseases* 2015; **21**(5): 916-918.
- 452 (27) **Midgley CM, et al.** Severe respiratory illness associated with a nationwide outbreak of
453 enterovirus D68 in the USA (2014): a descriptive epidemiological investigation. *The Lancet*
454 *Respiratory medicine* 2015; **3**(11): 879-887.
- 455 (28) **Reiche J, et al.** Low-level Circulation of Enterovirus D68-Associated Acute Respiratory
456 Infections, Germany, 2014. *Emerging infectious diseases* 2015; **21**(5): 837-841.
- 457 (29) **Pfeiffer HC, et al.** Two cases of acute severe flaccid myelitis associated with enterovirus
458 D68 infection in children, Norway, autumn 2014. *Euro surveillance : bulletin Europeen sur les*
459 *maladies transmissibles = European communicable disease bulletin* 2015; **20**(10): 21062.
- 460 (30) **Lang M, et al.** Acute flaccid paralysis following enterovirus D68 associated pneumonia,
461 France, 2014. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European*
462 *communicable disease bulletin* 2014; **19**(44).
- 463 (31) **A.; Mason B. WCTRPTMLGSRMCJRHRBHA.** Cluster of atypical adult Guillain-Barré
464 syndrome temporally associated with neurological illness due to EV-D68 in children, South Wales,
465 United Kingdom, October 2015 to January 2016. *Eurosurveillance* 2016; **21**(4).
- 466 (32) **Meijer A, et al.** Continued seasonal circulation of enterovirus D68 in the Netherlands, 2011-
467 2014. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European*
468 *communicable disease bulletin* 2014; **19**(42).
- 469 (33) **Oberste MS, et al.** Species-specific RT-PCR amplification of human enteroviruses: a tool for
470 rapid species identification of uncharacterized enteroviruses. *The Journal of general virology* 2006;
471 **87**(Pt 1): 119-128.
- 472 (34) **Prevention CfDca.** Clusters of acute respiratory illness associated with human enterovirus
473 68--Asia, Europe, and United States, 2008-2010. *MMWR Morbidity and mortality weekly report*
474 2011; **60**(38): 1301-1304.
- 475 (35) **Poelman R, et al.** European surveillance for enterovirus D68 during the emerging North-
476 American outbreak in 2014. *Journal of clinical virology : the official publication of the Pan*
477 *American Society for Clinical Virology* 2015; **71**: 1-9.
- 478 (36) **Kaida A, et al.** Enterovirus 68 in children with acute respiratory tract infections, Osaka,
479 Japan. *Emerging infectious diseases* 2011; **17**(8): 1494-1497.
- 480 (37) **Li R, et al.** Molecular analysis of virulent determinants of enterovirus 71. *PloS one* 2011;
481 **6**(10): e26237.
- 482 (38) **Gromeier M, Alexander L, Wimmer E.** Internal ribosomal entry site substitution eliminates
483 neurovirulence in intergeneric poliovirus recombinants. *Proceedings of the National Academy of*
484 *Sciences of the United States of America* 1996; **93**(6): 2370-2375.

TABLES AND FIGURES

Table 1. Clinical presentation and severity of EV-D68 positive cases in secondary care in England, September 2014 to January 2015.

Clinical Presentations-Severity (N=45)	n	%
Respiratory symptoms (including one case with neurological symptoms)	41	89
Asthma or wheezing	13	33 (N=39)
Immunocompromised or severe underlying diseases (all)	21	46
Admitted to hospital	37	82
Respiratory presentations	34	75
Intensive Care	12	27
Neurological presentations	2	4
Deaths	2	4

Figure 1. Distribution of EV-D68 cases detected in the Royal College of General Practitioners (RCGP) influenza sentinel surveillance system and in the Enterovirus Surveillance System (ESS) (including positives from before September 2014 and other later cases for whom clinical information is not available) by week of sample, England, September 2013 to March 2015.

Figure 2. Number of EV-D68 cases by sex, age and surveillance system where detected, England September 2013- January 2015. ((RCGP: Royal College of General Practitioners influenza sentinel surveillance system, (primary care.) EES: Enterovirus Surveillance System (secondary care))

Figure 3. Phylogenetic analysis of partial VP1 sequences of EV-D68 covering the BC and DE loops of the VP1 region. The evolutionary history was inferred using the Neighbour-Joining method and the optimal tree with the sum of branch length = 0.95104738 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (where >90%). The tree is drawn to scale. Analyses were conducted in MEGA6 [18]. ■(green)=ESS specimens; ■(blue)=RCGP specimens; ●=Cases associated with neurological illness; ▲=Reference sequences





