Emergence and epidemic occurrence of enterovirus 68 respiratory infections in The Netherlands in 2010

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ABSTRACT

Following an increase in detection of enterovirus 68 (EV68) in community surveillance of respiratory infections in The Netherlands in 2010, epidemiological and virological analyses were performed to investigate the possible public health impact of EV68 infections. We retrospectively tested specimens collected from acute respiratory infections surveillance and through three children cohort studies conducted in The Netherlands from 1994 through 2010. A total of 71 of 13,310 (0.5%) specimens were positive for EV68, of which 67 (94%) were from symptomatic persons. Twenty-four (34%) of the EV68 positive specimens were collected during 2010. EV68-positive patients with respiratory symptoms showed significantly more dyspnea, cough and bronchitis than EV68-negative patients with respiratory symptoms. Phylogenetic analysis showed an increased VP1 gene diversity in 2010, suggesting that the increased number of EV68 detections in 2010 reflects a real epidemic. Clinical laboratories should consider enterovirus diagnostics in the differential diagnosis of patients presenting with respiratory symptoms.

INTRODUCTION

Viruses of the family *Picornaviridae* genus *Enterovirus* cause a wide spectrum of disease in humans including upper and lower respiratory tract disease, pleurodynia, gastro-enteritis, hand-foot-and-mouth disease, herpangina, exanthema, myocarditis, pericarditis, paralysis, aseptic meningitis and generalized disease of the newborn ([Khetsuriani et al., 2006] , [Rotbart, 1995] and [Zaoutis and Klein, 1998]). Human

enterovirus species D type 68 (EV68) has been detected almost exclusively in respiratory tract specimens and has been associated with respiratory disease since its discovery in 1962 ([Khetsuriani et al., 2006] , [Oberste et al., 2004] and [Schieble et al., 1967]). When first described, EV68 was found to be relatively acid resistant and was distinguished from the acid sensitive human rhinovirus type 87 (HRV87) on this basis ([Kapikian et al., 1971] and [Schieble et al., 1967]). Recently, HRV87 was reclassified as EV68 based on phylogenetic analysis and cross-neutralization, and several laboratories have confirmed acid sensitivity of EV68 ([Blomqvist et al., 2002] , [Ishiko et al., 2002] and [Savolainen et al., 2002]). Distinguishing between rhinovirus and enterovirus by the acid sensitivity of isolates is therefore not appropriate for EV68.

In the USA, EV68 was detected only sporadically from 1970 through 2005 (Khetsuriani et al., 2006). EV68 infections have since been reported more frequently, but almost always associated with respiratory disease ([de Almeida et al., 2010] , [Imamura et al., 2011] , [Kaida et al., 2011] , [Petitjean-Lecherbonnier et al., 2011] , [Piralla et al., 2011] , [Rahamat-Langendoen et al., 2011] , [Renwick et al., 2007] , [Savolainen-Kopra et al., 2009] , [She et al., 2010] , [Smura et al., 2010] , [Tokarz et al., 2011] and [Wang et al., 2010]). Information on clinical severity of EV68 infections is limited, but recent studies show the association of EV68 infection with severe respiratory disease requiring hospitalization, disease of the central nervous system, and death following pneumonia in two patients and following disease of the central nervous system in one patient ([Imamura et al., 2011] , [Kreuter et al., 2011] , [Petitjean-Lecherbonnier et al., 2011] , [Piralla et al., 2011] and [Rahamat-Langendoen et al., 2011]).

In the Dutch enterovirus surveillance to document the absence of poliovirus circulation, EV68 has not been detected since 1963 (van der Sanden et al., manuscript in preparation). This is likely because of the surveillance design, which focuses on stool testing and therefore would not detect most respiratory pathogens. Furthermore, patients with respiratory disease are not routinely tested for enteroviruses, and EV68 might be misidentified as rhinovirus because of its acid sensitivity and reactivity in certain presumed rhinovirus specific PCR assays ([de Almeida et al., 2010] and [Centers for Disease Control and Prevention (CDC), 2011] ; Jaramillo-Gutierrez et al., manuscript in preparation). For these reasons, cases of EV68 infection may have gone unnoticed in The Netherlands and elsewhere. Furthermore, the 100% seroprevalence recently described in pregnant women in Finland (Smura et al., 2010) suggests that EV68 infections may be common.

In The Netherlands, we noticed an increased detection of enteroviruses and rhinoviruses from the sentinel general practice (GP) network surveillance for respiratory illness from early September 2010, which coincided with an increased incidence of influenza-like illness (ILI) (Fig. 1). As the summer wave of rhinovirus infections was not accompanied with increased incidence of ILI and the enteroviruses were almost exclusively EV68, we started to investigate the characteristics of EV68 viruses and associated respiratory illness. To this end, we retrospectively screened our biobank of historical upper respiratory tract specimens, collected through the acute respiratory infections surveillance and through three children cohort studies, for the presence of EV68 and we analyzed associated epidemiological and clinical data. In addition, we studied the genetic diversity and population dynamics of EV68 using partial VP1 gene sequences to assess whether the increase in number of EV68 detections reflected a real epidemic or was the result of a surveillance artifact.

[FIGURE 1]

RESULTS

Rates of enterovirus 68 detection

The rate of EV68 detections in biobanked specimens from patients with respiratory symptoms collected as part of the GP surveillance from 1994 through 2010, varied between seasons (mean 18%, median 14%, range 0–69%; Table 1). In total, 240 (2.4%) of the 9979 specimens analyzed were enterovirus positive, and of these 57 (24%) were characterized as EV68. Between 2000 and 2004 respiratory specimens were also collected from 567 control persons without respiratory symptoms as part of a case–control study as described previously (van Gageldonk-Lafeber et al., 2005). Only one of these control persons was positive

for EV68. In the three children cohort studies from 2004 through 2009, 76 of 2764 (2.7%) specimens analyzed contained enterovirus of which 13 (17%) from a total of 12 patients were identified as EV68 (Table 2).

[TABLE 1, TABLE 2]

Except for 2010 in which EV68 detections peaked during a 6-week period, only sporadic cases were identified in the other years of the surveillance (Fig. 2). However, almost all EV68 were detected in September through November throughout the study period and the distribution of EV68 detections across The Netherlands, including during the peak in 2010, was not confined to any particular region (data not shown).

[FIGURE 2]

Clinical manifestations from GP surveillance

EV68-positive patients with acute respiratory symptoms were compared with patients diagnosed at corresponding times with acute respiratory symptoms due to other causes. Demographic patient characteristics, clinical symptoms and diagnosis at initial consultation in the period 1996–2010 are summarized (Table 3). The highest prevalence of EV68-positive patients was among persons aged 50–59 years, while the highest number of EV68-negative patients was in the < 10 years age group (Table 3). The male/female ratio among EV68-positive and EV68-negative patients was 1.5 and 0.8 ($\chi 2$; $p = 0.001$), respectively. EV68-positive patients had significantly more dyspnea, cough, and bronchitis when compared to EV68-negative patients (Table 3). Patients with dyspnea had significantly higher incidence of bronchitis than those without dyspnea, 22.9% versus 4.5% (Fisher's exact; $p < 0.0007$). Similarly, patients with cough had significantly more bronchitis than patients without cough, 8.7% versus 0.5% (Fisher's exact; $p < 0.00001$). EV68-positive patients had significantly less fever, malaise, myalgia and diarrhea than EV68-negative patients (Table 3). Considering the epidemic year 2010 only, similar results were obtained (data not shown).

[TABLE 3.]

After stratification for the diagnosis “ILI” and “another acute respiratory infection (ARI)”, in both groups EV68-positive patients had significantly less fever and more dyspnea than EV68-negative patients ($\chi 2$; $p < 0.02$ in all comparisons). In addition, EV68-positive ARI patients had significantly more bronchitis than EV68-negative ARI patients ($\chi 2$; $p = 0.0002$). After stratification for EV68 and rhinovirus infection, EV68-infected patients also had significantly more dyspnea and bronchitis than rhinovirus-infected patients ($\chi 2$; $p \leq 0.002$ in all comparisons).

Follow-up questionnaires from 14 EV68-positive and 76 EV68-negative patients with an acute respiratory infection from the 2010 epidemic period were available for analyses (Table 4). There were no significant differences in follow-up between EV68-positive and EV68-negative patients for any of the conditions analyzed.

[TABLE 4.]

Clinical manifestations from children cohort studies

In the three children cohort studies 9 of 12 EV68-positive persons showed mild respiratory symptoms, with cough being the most common symptom in 7 out of 9 cases (Table 2). None of the ill persons developed complications and respiratory symptoms resolved within 4 weeks following detection of EV68. The remaining 3 EV68-positive persons were sampled at the time when they did not show respiratory symptoms (Table 2).

Phylogenetic analysis and evolutionary dynamics of enterovirus 68

To gain insight in the molecular epidemiology of EV68 and to assess whether the increase in the number of EV68 detections in 2010 reflected a real epidemic, partial VP1 sequences from The Netherlands were analyzed along with available EV68 VP1 sequences from GenBank using Bayesian relaxed molecular clock methods that allow the evolutionary rate to vary among branches on the tree. Phylogenetic analysis revealed that EV68 strains from several parts of the world (US, Finland, France, The Netherlands, and Japan) formed three major evolutionary lineages, each supported by 100% posterior probabilities (Fig. 3).

[FIGURE 3]

Viruses belonging to these clusters were distinguishable by a cluster specific signature of amino acid substitutions relative to the Fermon strain (Fig. 4). Dutch strains from 2010 primarily belonged to cluster 3 together with Japanese strains (5 of 39 available sequences) from 2010. The main Dutch variant circulating in the community in 2009, which was only detected in hospitalized patients in 2010 in The Netherlands, formed cluster 1 with Japanese strains (7/39) from 2010. Only 1 Dutch strain from 2009 was in cluster 2 with the majority of Japanese strains (27/39) from 2010.

[FIGURE 4]

A Bayesian skyline plot (BSP) (Drummond and Rambaut, 2007) of the partial VP1 sequence data was calculated to visualize temporal changes in relative viral genetic diversity of the Dutch EV68 strains using Bayesian coalescent approach (Drummond et al., 2006). The BSP revealed relatively constant viral diversity from the first EV68 detection in 1996 through approximately 2000 after which there is a slight increase in relative genetic diversity that likely reflects increased circulation (Fig. 2). From 2003 onwards viral diversity decreased slowly, until a sharp rise is observed in 2010, reflecting the increased detection of EV68 in that period (Fig. 2). The observed dynamics could not be explained by bias due to model selection or fluctuations in sampling density through time, as trends were similar when different model parameters were used in the BSP reconstruction. Down sampling of VP1 sequences from 2010, to correct for potential bias from increased sampling density during the EV68 epidemic, also revealed similar patterns of viral diversity (data not shown).

Amino acid sequence analysis

To identify potential viral factors in the increased incidence of EV68, we characterized all available partial VP1 gene sequences for amino acid changes relative to the EV68 Fermon reference strain from 1962, following phylogenetic analysis of all unique amino acid signature sequences in the dataset (Fig. 4). The majority of amino acid substitutions were found at residues 90–99, 140–145, 148, and 152, relative to the EV68 Fermon reference strain from 1962 (Fig. 4). In addition, an amino acid deletion occurred at position 140 in all viruses belonging to cluster 3 (Fig. 4). On the basis of the previously published crystal structure and alignment of VP1 sequences with those of other enterovirus serotypes, these regions are predicted to be located in the putative immunogenic BC loop and DE loop, both reported to be associated with infectious properties of enterovirus particles ([Chambon et al., 2004] , [Nix et al., 2006] , [Norder et al., 2003] and [Oberste et al., 1999]). These loops are exposed on the outside of the virus in the folded VP1 capsid protein. Four of the 5 remaining fixated substitutions were located in the vicinity of the BC and DE loops (residue positions 76, 84, 110, and 131).

DISCUSSION

We have demonstrated the presence of EV68 in The Netherlands since 1996 among patients with acute upper respiratory tract disease, and provided evidence for annual circulation during September through November. As we tested the specimens using consistent diagnostic methodologies with the capability to detect EV68 (Table 5), these results likely give a true relative estimate of the extent of EV68 circulation in

The Netherlands. Although the overall prevalence of enteroviruses appears low – 0.3% to 5.0% of patients with ILI or ARI per year from the community surveillance – compared to other respiratory cohorts (e.g. reviewed in Andréoletti et al., 2009), the data confirm that there was a large community-wide outbreak in 2010. A similar observation was made through community surveillance of patients presenting with ILI in the New York City area, with a 3% annual prevalence of enteroviruses, where EV68 peaked in September 2009 (Tokarz et al., 2011). The age distribution of EV68-infected persons in the current study was different from the typical age distribution for enteroviruses that affect younger age groups ([Khetsuriani et al., 2006] , [Rotbart, 1995] and [Zaoutis and Klein, 1998]). In the USA, EV68 infections most commonly occurred in children aged 1–4 years (n = 26), although 25% of EV68-infected persons were aged ≥ 20 years (Khetsuriani et al., 2006). Male predominance among EV68-infected persons in our study was similar to that for other enterovirus infections ([Khetsuriani et al., 2006] and [Rotbart, 1995]).

[TABLE 5]

Recent reports of EV68 detection are mainly from studies targeting patients with severe respiratory disease requiring hospitalization ([Imamura et al., 2011] , [Petitjean-Lecherbonnier et al., 2011] , [Piralla et al., 2011] and [Rahamat-Langendoen et al., 2011]). Combined, our data show that EV68 infections in the community are more often associated with relatively severe acute respiratory disease, i.e. dyspnea and bronchitis, but do not more often lead to complications compared with patients diagnosed with ILI or another ARI because of other causes. As EV68 was also sporadically detected in respiratory specimens of asymptomatic persons, we conclude that the spectrum of clinical disease associated with EV68 infection ranges from symptomless to severe respiratory disease requiring hospitalization, with some cases proving fatal. Next steps in the investigation of the clinical presentation of EV68 infection should include studies on the pathogenesis and host factors related to a severe outcome of EV68 infection.

The public health importance of EV68 infections as measured by ILI consultation rate was low, with about 30 ILI cases/100,000 population/week at the peak of EV68 detections in 2010. This is well below the epidemic threshold of 51 ILI cases/100,000 population/week for influenza in The Netherlands (Donker, 2011). However, of all EV68 patients only 44% were clinically diagnosed with ILI (Table 3) and therefore GP ILI consultation rates likely underestimate the public health importance of EV68 infections. Furthermore, our study was limited to patients with acute upper respiratory infections. Therefore, further investigation into the true public health impact of EV68 infections is needed.

The observed seasonality of EV68 detections to the autumn in our study was similar to that of EV68 described for Caen in France, Italy and the Philippines in 2008 ([Imamura et al., 2011] , [Petitjean-Lecherbonnier et al., 2011] and [Piralla et al., 2011]), New York City in 2009 (Tokarz et al., 2011) and Japan in 2010 (Kaida et al., 2011). It is also similar to detection of EV68 infections in camps of military recruits in the USA and Finland during 2004–2005 ([Savolainen-Kopra et al., 2009] and [Wang et al., 2010]), and in USA enterovirus surveillance (Khetsuriani et al., 2006). The confinement of circulation of EV68 to autumn is different from the typical period of circulation of other enteroviruses in the summer and early autumn ([Rotbart, 1995] and [Zaoutis and Klein, 1998]).

Analysis of virus population dynamics shows a clear expansion of the population diversity in 2010. This is not likely due to sampling bias because the GP surveillance in The Netherlands has been stable and consistently included the capability to detect EV68 over the period of the study. In addition, the coalescence rate for the specimens obtained in 2010 was extremely rapid, with most lineages quickly coalescing to a single recent common ancestor, indicating true exponential growth of the virus population in that period. Reducing the number of VP1 sequences from 2010 in the analysis did not alter the dynamics observed, consistent with extensive simulations of human influenza virus A(H3N2) demographics where demographic dynamics were reconstructed independently of sampling heterogeneity (Rambaut et al., 2008).

We found that, in the late 1980s, the 1990s, and in the early 2000s, major bifurcations occurred in the evolution of the EV68 VP1 that corresponded to amino acid substitutions in the putative BC and DE loops relative to the EV68 Fermon strain. This is in line with the finding that the geometric mean titers of neutralizing antibodies against the prototype Fermon strain (isolated in 1962) declined from 178.8 in 1983 to 88.1 in 1993 and 44.5 in 2002, as determined by a study in Finland in pregnant women (Smura et al., 2010). In addition, a single amino acid loss at position 84 in the BC loop of the VP1 protein of coxsackie

B4 virus was shown to reduce virus neutralization and one amino acid exchange at position 86 in the middle of the BC loop led to the complete loss of reactivity with specific antibodies (McPhee et al., 1994).

Although we found some interesting patterns in amino acid mutations in the VP1 protein that may help to explain the biological basis for the increase in incidence of EV68, deletions and mutations in other parts of the genome may also change virus behavior, for instance mutations in the 5′-UTR that might increase translational efficiency or virulence or both (Kaida et al., 2011). Full genome analysis of a systematic sample of EV68 is therefore needed to address these issues.

CONCLUSIONS

Our findings demonstrate circulation of EV68 during the autumn in The Netherlands. We found that upper respiratory tract disease was more severe in EV68-positive patients than in patients with other respiratory tract infections sampled at corresponding times. Phylodynamic analysis of partial VP1 sequences showed increased genetic diversity in 2010, indicating true epidemic spread of EV68 in 2010. Amino acid variation in the putative BC and DE loops of the immunogenic VP1 protein suggests that antigenic drift might explain the increase in incidence of EV68 in 2010 in The Netherlands. We recommend that clinical laboratories consider enterovirus in the differential laboratory diagnosis of patients presenting with respiratory symptoms.

MATERIAL AND METHODS

Sentinel surveillance

ILI incidence data and nose and throat swabs with associated demographic and clinical data of patients with ILI or another ARI were collected through the Dutch continuous morbidity registration sentinel GP network as described ([Donker, 2011] and [Pel, 1965]). Respiratory specimens, collected since 1994, are shipped to the National Institute for Public Health and the Environment, The Netherlands and PCR-based diagnostic testing and virus isolation are conducted as described (Andeweg et al., 1999). Patient data and enterovirus-positive specimens from week 43/1994 through week 49/2010 were included in the study.

Children cohorts

Enterovirus-positive respiratory specimens and clinical data of EV68-positive persons of 3 young children cohort studies in The Netherlands were included in the study (Table 2). These studies were the Wheezing Illnesses Study Leidsche Rijn (WHISTLER) in which infants were followed from birth through their first year of life in the period 2005–2008 and swabbed during episodes of respiratory tract infection ([Katier et al., 2004] and [van der Zalm et al., 2011]); a cystic fibrosis (CF) case–control study in which, during the 2004–2005 winter season, children were swabbed when having respiratory illness, and also swabbed bi-weekly independent of respiratory symptoms (van Ewijk et al., 2008); and The Netherlands amniotic fluid (NAF) study in which children born in a secondary or a tertiary hospital from 2006 through 2009 were swabbed in their first year of life during each episode of respiratory disease (Houben et al., 2009). Clinical data were collected with the specimens and on frequent intervals by clinical interview. The local medical ethics committee approved the studies and the parents gave written informed consent.

Enterovirus detection and EV68 identification

In the primary diagnostic process, freshly extracted nucleic acid was subjected to reverse transcriptase (RT)-PCR for several respiratory pathogens using primers and probes targeting the 5′ noncoding region of the genome of rhinoviruses and enteroviruses (Table 5). Block-based nested 1-step RT-PCR with probe hybridization to distinguish enteroviruses from rhinoviruses was performed as described ([Andeweg et al., 1999] and [Kämmerer et al., 1994]) (Table 5). Similar primers were subsequently used in a 2-step real-time RT-PCR assay with probes to distinguish enteroviruses from rhinoviruses (Table 5). A 2-step real-time RT-PCR for enteroviruses (Noordhoek et al., 2008) was used for further confirmation of enterovirus detection (Table 5). Combined historical results of RT-PCR and virus isolation (Andeweg et al., 1999) were used to

identify enterovirus-positive patients for this study. For EV68 identification and genetic and amino acid sequence analyses, freshly purified RNA from stored (− 80 °C) enterovirus-positive clinical specimens was used for partial sequencing of the VP1 gene as described (Nix et al., 2006).

Epidemiological analyses GP surveillance data

Patient demographic data, respiratory symptoms and clinical diagnosis at the time of GP consultation were used to compare ill persons infected with EV68 with all other ill persons of whom respiratory specimens were taken in the same year and week. Data were analyzed for the period 1996–2010 and separately for the year 2010 because of the epidemic nature of EV68 detections in 2010.

To compare the course of respiratory disease of EV68-positive and EV68-negative patients, sentinel GPs were asked to complete a follow-up questionnaire about the course of disease for 4-weeks following the onset of disease for patients who were swabbed during weeks 35–40 in 2010, the period of the EV68 epidemic. The questionnaire addressed the time to recovery of symptoms, repeat consultation and reasons for repeat consultation, complications, hospitalization, death, and underlying diseases.

Descriptive analyses were performed to compare demographic patient characteristics and acute phase and follow-up clinical data. Differences in proportions were analyzed using Pearson's chi-square test or Fisher's exact test if ≥ 50% of the cells have a count less than 5, with p-values ≤ 0.05 considered significant. All analyses were performed in SAS software version 9.2 (http://www.sciencedirect.com.proxy.library.uu.nl/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_issn=00426822&_origin=article&_zone=art_page&_plusSign=%2B&_targetURL=http%253A%252F%252Fwww.sas.com%252Fsoftware%252Fsas9).

EV68 VP1 sequences

Partial VP1 sequences (nucleotides 132 through 471 relative to the VP1 gene of the Fermon strain of EV68 [GenBank ID: AY426531], comprising the putative BC and DE loops of the folded VP1 protein) from this study are available from GenBank IDs: JF830017–JF830085. For the evolutionary analyses all EV68 VP1 sequences available from GenBank by March 31, 2011, with GenBank IDs: AF081348, AY062278–AY062280, AY42653, AY208116, EF107098, AY355268, AY426486–AY426500, GQ466485–GQ466487, and AB614406–AB614444, were included. Data on geographic origin and date of sampling were retrieved from the GenBank entry or available literature ([Kapikian et al., 1971] , [Oberste et al., 2004] , [Savolainen-Kopra et al., 2009] and [Schieble et al., 1967]). Partial VP1 sequences from 6 patients hospitalized at the University Medical Center Groningen (UMCG), Groningen, The Netherlands, of which clinical details have been published elsewhere (Rahamat-Langendoen et al., 2011), were kindly provided at the initiation of this study by J.C. Rahamat-Langendoen and H.G.M. Niesters.

Phylogenetic analysis and evolutionary dynamics

The genetic relationships of EV68 strains was studied using a Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in BEAST version 1.5.4 (http://www.sciencedirect.com.proxy.library.uu.nl/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_issn=00426822&_origin=article&_zone=art_page&_plusSign=%2B&_targetURL=http%253A%252F%252Fbeast.bio.ed.ac.uk) that incorporates the date of virus sampling (Drummond and Rambaut, 2007). A maximum clade credibility (MCC) tree was inferred using partial VP1 sequences of Dutch and international EV68 strains, taking into account the year of specimen collection. The Hasegawa–Kishino–Yano nucleotide substitution model with a discrete gamma distribution to accommodate rate variation among sites in the alignment and the uncorrelated exponential relaxed molecular clock model (Drummond et al., 2006), selected on the basis of Bayes factor testing (Suchard et al., 2003), were used for the analysis. Alignment sites were partitioned into first plus second codon positions and third codon position to allow different rates of substitution for these codon positions. The MCMC analysis was run for 100,000,000 generations and stationarity and mixing efficiency were examined using Tracer software (http://www.sciencedirect.com.proxy.library.uu.nl/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_issn=00426822&_origin=article&_zone=art_page&_plusSign=%2B&_targetURL=http%253

A%252F%252Ftree.bio.ed.ac.uk%252Fsoftware%252Ftracer). To test the reliability of the tree, posterior probabilities for the branching events were calculated following removal of the appropriate burn-in.

To infer the population dynamics of EV68 through time, we employed a Bayesian skyline plot model implemented in BEAST (Drummond et al., 2005) using the partial VP1 sequences of the Dutch EV68 strains and the full date of specimen collection. We specified 20 groups of coalescent intervals to capture the past population dynamics in the piecewise constant demographic function. The posterior distribution for the Bayesian skyline plot parameters yields the most plausible piecewise constant expectations for the coalescence rates through time in the genealogies, which in turn, represent the most plausible evolutionary histories for the sequence data. The same settings and run conditions as described above for the temporal phylogenetic analysis were used. The Bayesian skyline plot was visualized using Tracer software.

To test the robustness of the model selection on reconstruction of the phylogenetic tree and the population dynamics, the analyses were also performed using multiple combinations of nucleotide substitution and molecular clock models in addition to the optimal models selected on the basis of Bayes factor testing as described above.

Amino acid sequence analysis

A phylogenetic tree was inferred from all unique partial EV68 VP1 amino acid signature sequences, determined among all Dutch VP1 sequences and international VP1 sequences available in GenBank by March 31, 2011, by the maximum-likelihood statistical method using the Nearest-Neighbor-Interchange heuristic method to infer the tree, substitution model Jones–Taylor–Thornton with gamma distribution (chosen by best model test), gap treatment by partial deletion with a cutoff of 95% and 1000 bootstraps to test the phylogeny, using MEGA 5.03 software (http://www.sciencedirect.com.proxy.library.uu.nl/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_issn=00426822&_origin=article&_zone=art_page&_plusSign=%2B&_targetURL=http%253A%252F%252Fwww.megasoftware.net). Based on the consensus bootstrap tree, the amino acid sequence alignment was ordered for analysis of amino acid variation.

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TABLES AND FIGURES

Fig. 1. Weekly ILI consultation rates and absolute number of total enterovirus (including EV68) (in blue) and EV68 detections (in red) for the GP sentinel surveillance year 2010, plotted by week of specimen collection.

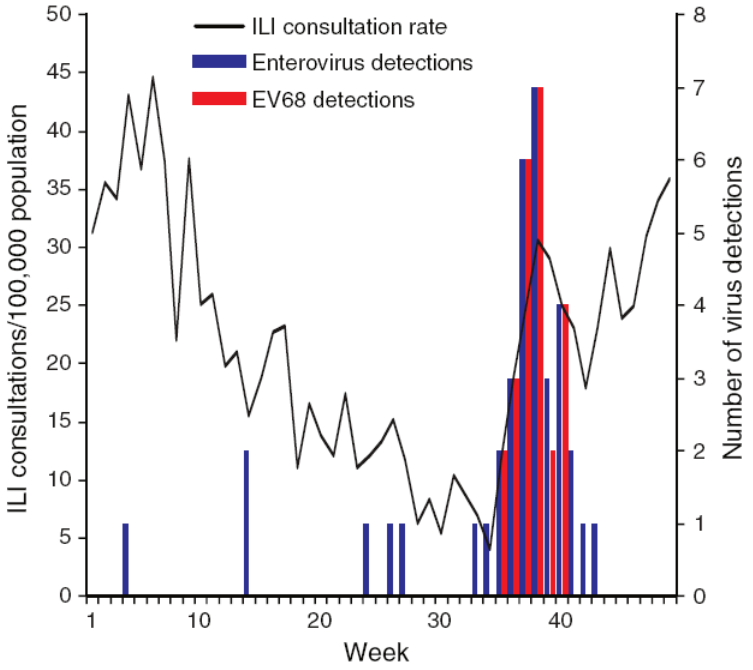

Table 1
Enterovirus and EV68 detections and success rate of VP1 sequencing of sentinel GP surveillance specimens from clinically ill patients, by year of specimen collection.

Year	Specimens tested No.	Enterovirus-positive No. (% of specimens tested)	VP1 sequence obtained[a] No.	Sequencing success %	EV68 No.	Proportion EV68 among enteroviruses % (95% CI)[b]
1994[c]	99	5 (5.0)	5	100	0	0 (0–43)
1995	847	6 (0.7)	4	67	0	0 (0–39)
1996	354	7 (2.0)	4	57	1	14 (3–51)
1997	490	18 (3.7)	7	39	1	6 (1–26)
1998	465	14 (3.0)	7	50	1	7 (1–31)
1999	365	6 (1.6)	3	50	1	17 (3–56)
2000	278	7 (2.5)	5	71	1	14 (3–51)
2001	210	5 (2.4)	4	80	2	40 (12–77)
2002	207	5 (2.4)	2	40	0	0 (0–43)
2003	295	10 (3.4)	2	20	2	20 (6–51)
2004[d]	201	5 (2.5)	0	0	0	0 (0–43)
2005[d]	308	1 (0.3)	1	100	0	0 (0–79)
2006	610	28 (4.6)	18	64	5	18 (8–36)
2007	812	18 (2.2)	16	89	4	22 (9–45)
2008	1054	15 (1.4)	15	100	9	60 (36–89)
2009	2340	55 (2.4)	48	87	6	11 (5–22)
2010[e]	1044	35 (3.4)	34	97	24[f]	69 (52–81)
Total	9979	240 (2.4)	175	73	57	24 (19–30)

[a] Partial VP1 sequences, nucleotides 132 through 471 relative to the VP1 gene of the Fermon strain of EV68 (GenBank ID: AY426531) were directly obtained from the clinical specimen to avoid sequencing of nucleic acid and amino acid substitutions possibly induced by virus isolation in tissue culture.
[b] Determined using Willson's estimate of the 95% confidence interval (CI) of proportions in the statistical software package WINPEPI version 10.9 (Abramson, 2011).
[c] Start at week 43.
[d] No PCR used during the season 2004–2005, only virus isolation.
[e] Through week 49.
[f] In 1 specimen EV68 was identified by sequencing of the 5′ noncoding region diagnostic RT-PCR product only.

Table 2
Enterovirus and EV68 detections with patient characteristics in 3 children cohorts in the period 2004–2009.

Study[a]	Patients No.	Specimens No.	Specimens/patient Median; range	Enterovirus No. (no. patients)	EV68		
					No. (no. patients, gender)	Age patient at sampling (range)[b]	Symptoms
WHISTLER	170	1456	11; 1–19	16 (14)	1 (1, F)[c]	0.8 mo	None
CF (patients)	21	356	18; 1–23	13 (7)	5 (4, 3 M, 1 F)[d]	7.7 mo–3 y plus 7.8 mo	3/4 Cough 1/4 Rhinorrhea 1/4 None
CF (controls)	19	303	17; 6–20	12 (9)	1 (1, F)[e]	7.6 mo	None
NAF	255	649	2; 1–9	35 (30)	6 (6, 6 M)	1.8 mo–9.7 mo	6/6 Common cold symptoms: acute onset, fever and rhinorrhea 4/6 Cough 1/6 Otalgia and ororrhea 1/6 Wheezing (happy wheezer)
All combined		2764		76 (60)	13 (12)[d]		

[a] WHISTLER = Wheezing Illnesses Study Leidsche Rijn study, CF = cystic fibrosis case–control study, NAF = Netherlands amniotic fluid study.
[b] Age in months (mo) or years (y) plus months (mo).
[c] EV68 identification by sequencing of the 5′ noncoding region diagnostic RT-PCR product only.
[d] One patient was sampled on 2 subsequent days and both specimens contained identical EV68 VP1 sequences.
[e] Twin sister without symptoms who was found positive for EV68 one week before the twin brother with cystic fibrosis became positive for EV68 with respiratory symptoms.

Fig. 2. Number of EV68 detections from the GP sentinel surveillance (in red) and children cohorts (in green) plotted by week and year of specimen collection. Superimposed on this graph is the Bayesian skyline plot (uninterrupted line) with 95% highest posterior density (HPD) intervals (dashed lines; a Bayesian analog to a confidence interval), determined using Dutch EV68 partial VP1 sequences with the full date of specimen collection included. It shows the relative measure for genetic diversity through time with the values plotted on the secondary y-axis.

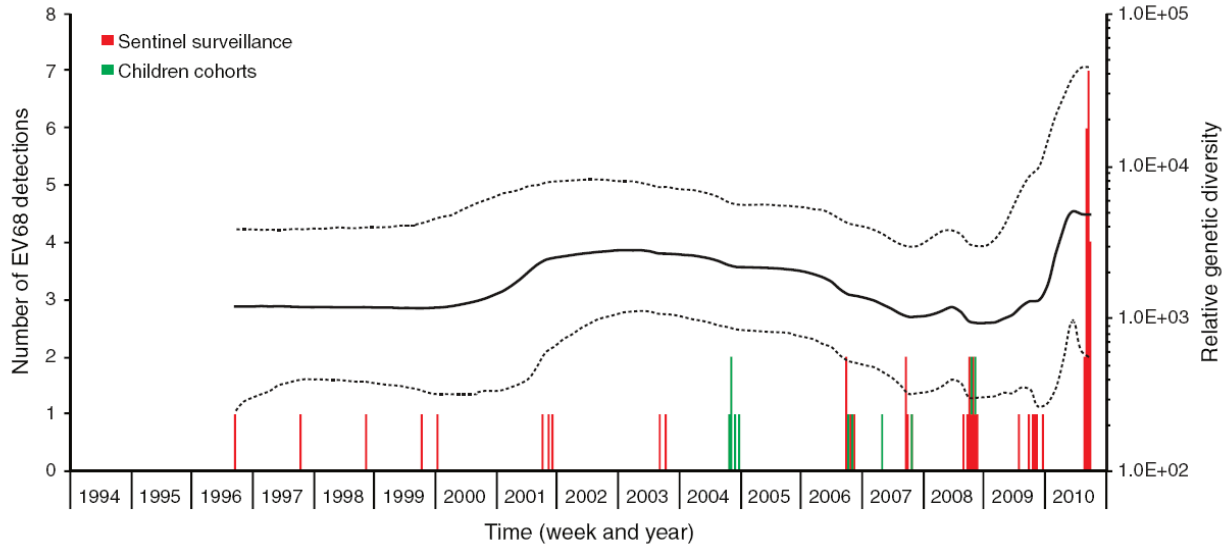

Table 3
Age distribution and clinical symptoms and diagnosis of sentinel GP surveillance patients with influenza-like illness or another acute respiratory infection; EV68-negative versus EV68-positive patients 1996–2010 collected in the same week.

Characteristic	EV68-negative patients No. = 833		EV68-positive patients No. = 57		χ^2 p-value[b]
	No.[a]	%	No.[a]	%	
Age group					
<10	190	22.81	8	14.04	0.1233
10–19	125	15.01	9	15.79	0.8729
20–29	108	12.97	2	3.51	**0.0358**
30–39	121	14.53	6	10.53	0.4036
40–49	116	13.93	10	17.54	0.4484
50–59	80	9.60	15	26.32	<**0.0001**
60–69	54	6.48	4	7.02	0.8742
70–79	28	3.36	2	3.51	0.9524
80–89	9	1.08	1	1.75	0.6405
>89	1	0.12	0	0	0.7935
Clinical symptoms					
Acute	630	82.46	44	84.62	0.6917
Fever	564	68.61	34	42.11	<**0.0001**
Malaise	498	60.58	34	42.11	**0.006**
Headache	312	38.28	14	25.45	0.0571
Myalgia	381	46.35	17	29.82	**0.0154**
Sore throat	441	53.65	27	47.37	0.358
Cough	622	75.67	50	87.72	**0.0381**
Dyspnea[c]	22	10.95	11	36.67	**0.0002**
Rhinorrhea	320	38.93	20	35.09	0.5647
Diarrhea	56	6.87	0	0	**0.0464**
Clinical diagnosis					
ARI[d]	331	40.27	32	56.14	**0.0186**
Common cold	125	15.21	13	22.81	0.272
Sinusitis	29	3.53	2	3.51	0.9939
Otitis media	14	1.70	0	0	0.3206
Pharyngitis	75	9.12	5	8.77	0.9288
Tonsillitis	25	3.04	0	0	0.1816
Laryngitis	13	1.58	2	3.51	0.2773
Tracheitis	11	1.34	2	3.51	0.1892
Bronchitis	46	5.67	13	23.21	<**0.0001**
Bronchiolitis	4	0.50	0	0	0.607
Pneumonia	15	1.82	2	3.51	0.372

[a] Numbers in strata do not add up to the total number of EV68-negative and EV68-positive patients because of missing or unknown values. One patient can have 1 or more clinical symptom(s).

[b] **Boldface** indicates significant result.

[c] Dyspnea was not included on the swab form from week 40/2000 through week 47/2009.

[d] The clinical diagnosis is influenza like illness (ILI) or another acute respiratory infection (ARI). In case of ARI, the specific diagnosis is reported in the table.

Table 4
Follow-up analysis of patients in 4 weeks post onset of disease; EV68-negative versus EV68-positive patients in weeks 35–40, 2010.

Characteristic[a]	EV68-negative patients No. = 76[b]		EV68-positive patients No. = 14[b]	
	No.	%	No.	%
Recovered				
Yes	55	94.83	13	100
No	3	5.17	0	0
Repeat consultation				
Yes (visit and/or by phone)	37	51.39	8	57.14
No	35	48.61	6	42.86
Death				
No	68	100	12	100
Hospitalization				
Yes	4	5.88	1	9.09
No	64	94.12	10	90.91
Complications				
Yes	10	15.38	2	16.67
No	55	84.62	10	83.33
Underlying conditions				
Yes	22	32.35	4	33.33
No	46	67.65	8	66.67

[a] χ^2 (or if ≥50% of cells have an expected count <5 Fisher's exact test); p-value for all variables ≥0.67.
[b] Numbers in strata do not add up to the total number of EV68-negative and EV68-positive patients because of missing or unknown values.

Fig. 3. Maximum clade credibility tree with 95% HPD intervals for the node times (in years; gray bars at nodes) and posterior probabilities for branching events (numbers at nodes).
The tree was inferred using partial VP1 sequences of Dutch EV68 viruses and of EV68 viruses from other regions in the world (for Japan all unique sequences included in the tree inference) available in GenBank by March 31, 2011, and using the year of specimen collection. Strain names consist of: laboratory number for Dutch strains or GenBank accession number|city/village or state, or unique name or number|2-letter country iso code|year of specimen collection. Dutch strains start with plain number for sentinel GP surveillance, V or VR for children cohort studies, or E for UMCG hospital derived strains. The geographic origin of non-Dutch strains is indicated by symbols; closed triangle for US, open triangle for Finland, closed diamond for Japan and open diamond for France. Underlined strain names indicate strains representative for the unique amino acid signature sequences that have been used in the amino acid sequence analysis. Major recent clusters are indicated by a vertical line at the right side and numbered.

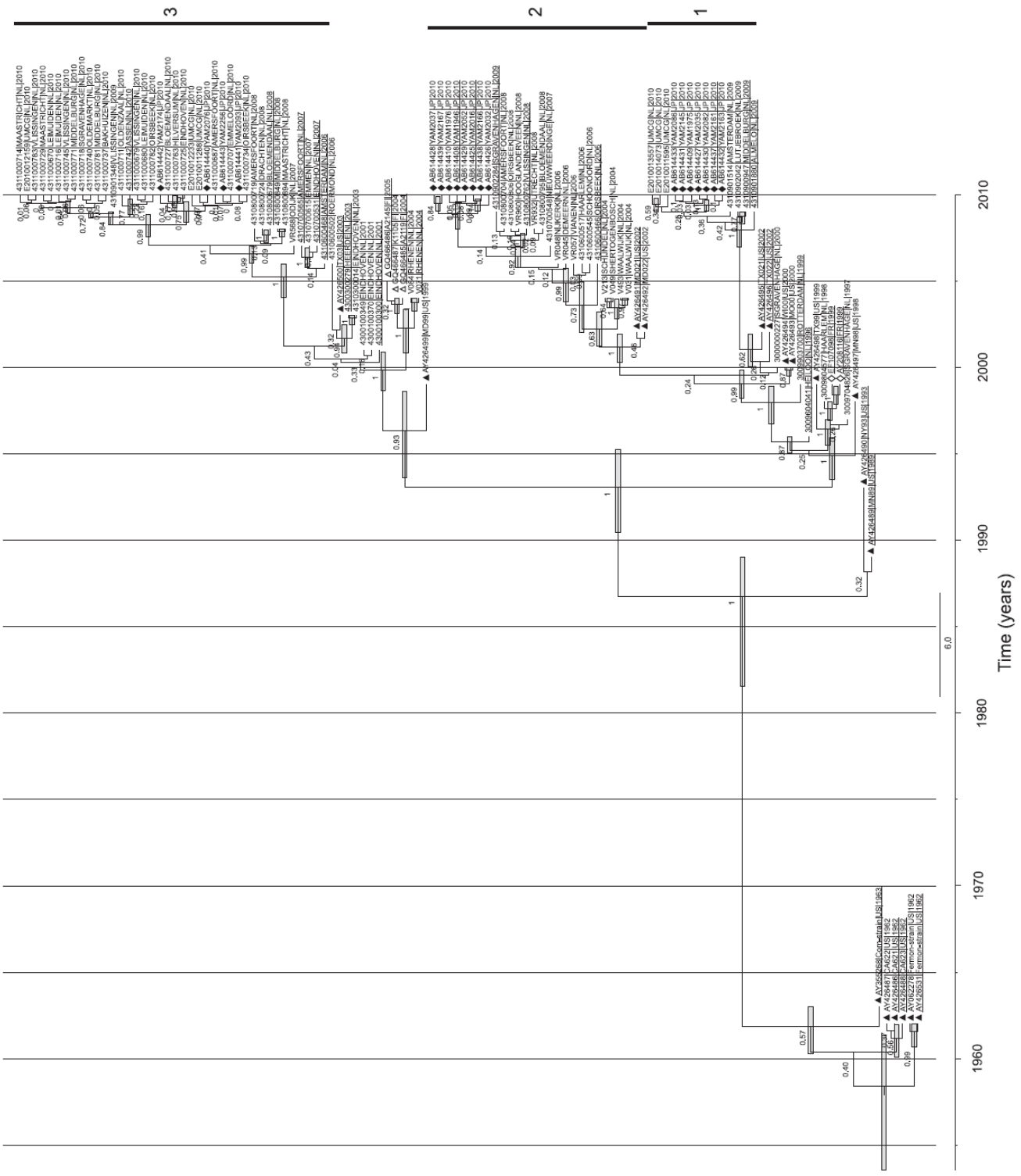

Fig. 4. Amino acid sequence analysis. All unique amino acid sequences of Dutch and international EV68 partial VP1 sequences available in GenBank by March 31, 2011 were included. The left part of the figure shows the maximum likelihood consensus bootstrap tree after 1000 iterations with percentage bootstrap support for branching events indicated at the nodes. Names of strains which are representative for unique amino acid signature sequences are followed by the subsequent years in which the signature sequences were found, and between brackets by the frequency in which the signature sequences were found in the dataset. Clusters as identified in Fig. 3 are indicated on the right of the tree. The right part of the figure shows the corresponding amino acid signature sequences. Numbering of amino acid residues is relative to the start of the VP1 reading frame of the Fermon strain of EV68. Amino acids common to the Fermon strain are indicated with a dot in the alignment. The putative BC and DE loops are indicated by boxes on the aligned amino acid sequences.

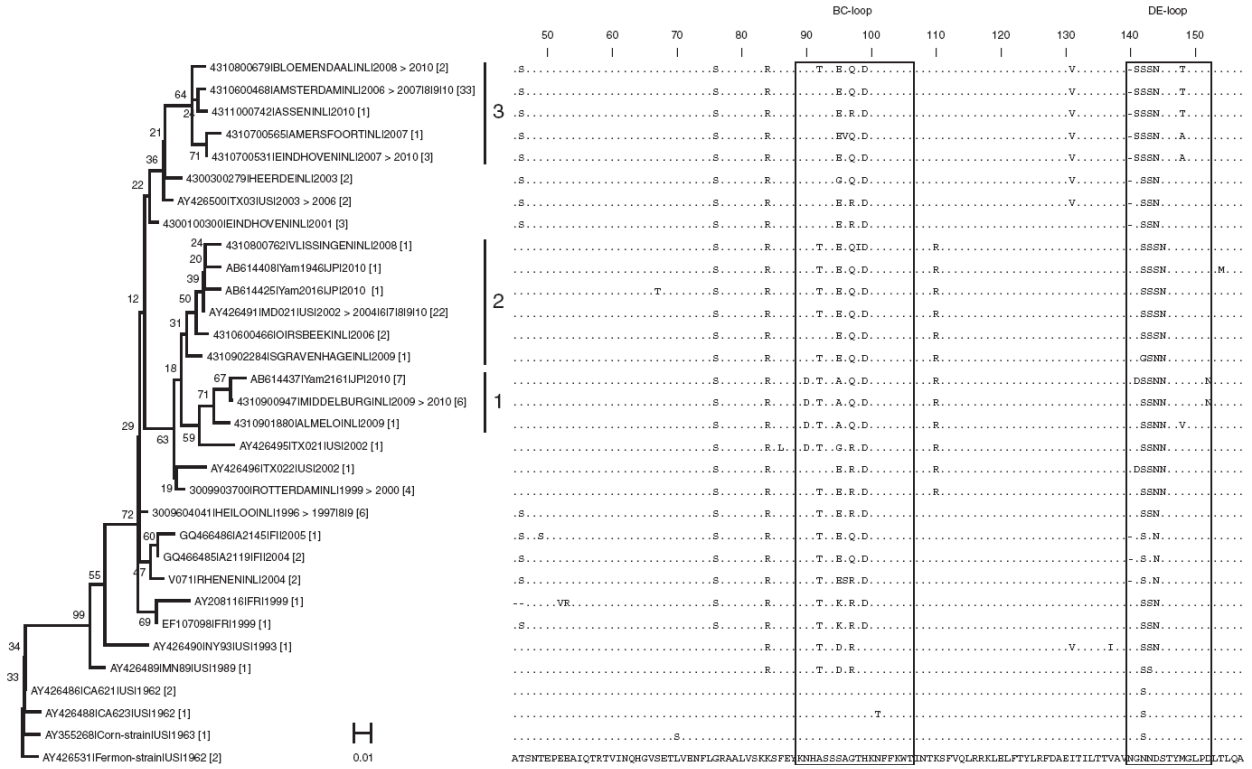

Table 5
Primers and probes in the 5′ noncoding region used in the nested (1994–2009) and real-time (2009–) RT-PCR protocols.

Specificity; period in use	Primer or probe name; Specification[a]	Sequence 5′-3′[b]	Match with EV68[b]	Reference[c]
Picornavirus specific				
1994–1996	Coxprim 1; OF	ACCTTTGTACGCCTGTT	100%	2
	Coxprim 2; OR	CACGGACACCCAAAGTA	100%	2
	Coxprim 3; IF	AAGCACTTCTGTTTCCC	1 or 2 mismatches; C or T, C	2
	Coxprim 4; IR	ATTCAGGGGCCGGAGGA	100%	2
Rhinovirus and enterovirus subset specific				
1996–2009	ncr 1; OF	CGGTAAYTTTGTACGCCAGTT	2 or 3 mismatches; C or T, C, T	1
	ncr 2; OR	ACACGGACACCCAAAGTA	100%	1
	ncr 3; IF	CAAGCACTTCTGTTTCCCCGG	1 or 2 mismatches; C or T, C	1
	ncr 4; IR	CATTCAGGGGCCGGAGGA	100%	1
	Rhinovirus; P	*Biotin*-AGCCTGCG_TGGCTGCC	1 gap, 2 mismatches; G, G	1
	Enterovirus; P	*Biotin*-GGCTGCGTTGGCGGCC	100%	1
Rhinovirus and enterovirus subset specific				
2009–	ncr 3; F	CAAGCACTTCTGTTTCCCCGG	1 or 2 mismatches; C or T, C	1
	ncr 2; R	ACACGGACACCCAAAGTA	100%	1
	Rhinovirus1; P	*FAM*-TcTTcRcAccCTGT-*BBQ*	1 mismatch; T	This study
	Rhinovirus2; P	*FAM*-TcTTSRcAccTTGT-*BBQ*	100%	This study
	Rhinovirus3; P	*FAM*-TcTTYRcAccATGT-*BBQ*	1 mismatch; T	This study
2010–	Enterovirus; P	*TXR*-CTGCGTTGGCGGCCTRCC-*BHQ2*	1 mismatch; T	This study
Enterovirus specific				
2009–	EV-F; F	CCCCTGAATGCGGCTAAT	100%	3
	EV-R; R	CAATTGTCACCATAAGCAGCCA	2 or 3 mismatches; A, A or T, T	3
	EV-Pr; P	*YY*-AACACGGACACCCAAAGTAGTCGGT-*BHQ1*	100%	This study[d]

[a] Specification: O = outer; I = inner; F = forward; R = reverse; P = probe.
[b] Positions that mismatch with non coding region sequences from EV68 collected in the time period 1962–2010 (GenBank ID: GU933068–GU933070, GU933077; AY426516–AY426530; AB569257–AB569276; AB601882–AB601885; EF107098; EU870491; EU870464; AF108187; AY062273–AY062274; AY426531) are underlined and explained in the "Match with EV68" column. Lower case nucleotide = locked nucleic acid (LNA) nucleotide.
[c] Reference: 1, Andeweg et al., 1999; 2, Kämmerer et al., 1994; 3, Noordhoek et al., 2008.
[d] The 3′ end of the original probe described by Noordhoek et al. (2008) was changed from T to G, and GT was added, based on recent available enterovirus 5′ noncoding region sequences and to accommodate our implementation of the protocol.