

Spectrum of Enterovirus Serotypes Causing Uncomplicated Hand, Foot, and Mouth Disease and Enteroviral diagnostic yield of different clinical samples

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Summary of main point of this article. Our study investigated the full spectrum of enteroviruses causing uncomplicated HFMD, and identified significant differences of epidemiology among EV-A71, CV-A16, CV-A6, CV-A10 and CV-A4. Evaluation of enterovirus diagnostic yield of different clinical samples can guide optimal sample collection strategies for virological diagnosis.

Running title: HFMD associated enterovirus epidemiology

ABSTRACT

Background. Hand, foot, and mouth disease (HFMD) represents a substantial disease burden in the Western Pacific region. We investigated the spectrum of causative enteroviruses of HFMD, and evaluated different clinical samples' diagnostic yield for enteroviruses.

Methods. We enrolled pediatric patients hospitalized for HFMD among six hospitals in Anhua County, Hunan Province, China between October 2013 and September 2016. Throat swabs and stool samples (or rectal swabs) were collected to detect the enterovirus serotypes by real time RT-PCR or nested PCR.

Results. Among the 2,836 patients only one developed severe illness. Seventeen serotypes were identified in 2,401 patients (85%), with the most frequently detected being CV-A16 (29%, 814), CV-A6 (28%, 784), EV-A71 (17%, 491), CV-A10 (4%, 114), and CV-A4 (2%, 53). Children were younger in CV-A6, CV-A10, and CV-A4 infections (median 12 months, IQR 12-24 months) than EV-A71 and CV-A16 infections (median 24 months, IQR 12-36 months, $p < 0.05$). Annual peaks of HFMD hospitalization occurred during April-June. The predominant enterovirus serotype shifted between CV-A16 and CV-A6 during the three years. Stool had a higher diagnostic yield (89%) than rectal (79%) and throat swabs (74%). Detection rates reached 93% when testing stools followed by throat swabs if stools were negative, and 89% when testing rectal swabs followed by throat swabs if rectal swabs were negative.

Conclusions. Our results provide a virological benchmark for future surveillance and diagnostics. Continuous comprehensive virological surveillance is essential, especially after implementation of the EV-A71 vaccine in China, to monitor serotype replacement and the impact of EV-A71 vaccine.

Key words. HFMD; enterovirus; diagnostic yield; virological surveillance.

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common childhood illness, caused by various enteroviruses [1]. HFMD represents a substantial disease burden in the Western Pacific region, particularly in mainland China [2]. Of the enteroviruses associated with HFMD, enterovirus 71 (EV-A71) and coxsackie virus A16 (CV-A16) are the most frequently reported [2]. Other enterovirus A species, including CV-A2-6, CV-A8, CV-A10, CV-A12, CV-A14, have also been identified to co-circulate during HFMD outbreaks [3-7]. Some studies have suggested that EV-A71 circulates in 3- to 4-year cycles in Japan and Malaysia [8, 9] and CV-A16 circulates in a cyclical pattern of every 2 to 3 years in Singapore, England and Wales [10, 11]. The predominant enterovirus serotypes responsible for HFMD shift between epidemic seasons, and in 2012 CV-A6 and CV-A10 emerged as increasingly prevalent serotypes in Europe and the Asia-Pacific [3, 4, 12-16]. However, systematic and comprehensive virological surveillance that fully investigates the entire spectrum of enterovirus serotypes associated with HFMD and monitor their shift patterns over multiple years has rarely been conducted. This is of particular importance for EV-A71 vaccine implementation.

Multiple choices of clinical samples were recommended for enterovirus diagnostics when available [17]. Due to the rather low detection rate of EV-A71 and CV-A16 from cerebrospinal fluid [18, 19] and the difficulty of collecting vesicle swabs and CSF samples, clinical samples consisting of throat swabs, stool and rectal swabs are the most commonly sent in for enterovirus diagnostics associated with HFMD [3, 6, 13, 16]. In planning future surveillance for HFMD and other enterovirus-associated disease, the effectiveness of screening different sample types and their diagnostic yield requires further evaluation.

To address these issues, we conducted comprehensive virological surveillance of HFMD in Anhua County, Hunan Province, mainland China to investigate the spectrum of enteroviruses associated with HFMD, to describe the pattern of enterovirus serotypes distribution over multiple epidemic seasons, and to evaluate the diagnostic yield of different clinical samples.

METHODS

Study population

This study was conducted among 3/4 county-level hospitals and 3/24 township level hospitals in Anhua County, located in southern China (Supplementary Figure 1) between October 2013 and September 2016. These hospitals were selected as they admitted 87% of the reported HFMD patients from Anhua County between 2010 and 2012. All patients aged ≤ 14 years who were hospitalized for HFMD at the six hospitals were enrolled after verbal consent was obtained from the patients' parent/guardian. A probable case of HFMD was defined as a patient with papular or vesicular rash on hands, feet, mouth, or buttocks, with or without fever. A confirmed case in this group was defined as one with laboratory evidence of enterovirus infection detected by real-time RT-PCR or nested PCR.

Data collection

A standardized form was used to collect data, including basic demographic information, date of illness onset, date of sample collection, types of samples, complications, and clinical outcome.

Specimen collection and testing

We used plastic shaft fiber swabs and a conical tube containing 3.5 mL of UTM viral transport medium (Yocon, Beijing, China) to collect throat swabs and stool samples (rectal swabs instead if

stool samples were unavailable) within 24 hours after enrollment. Swabs were placed in viral transport medium, and all samples were stored at -70 °C until testing.

In the first stage of testing, all the clinical samples from the patients enrolled in the first two months of surveillance (October - November 2013), were tested to preliminarily evaluate the enterovirus diagnostic yield of different clinical samples. In the second stage, we initially tested the clinical sample with higher diagnostic yield according to the preliminary evaluation. If a specific enterovirus serotype was identified, the other type of clinical sample from the same patient was not tested, otherwise, the other sample when available was used for further virological diagnosis.

Viral RNA was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and amplified using generic (pan-enterovirus) and specific (EV-A71, CV-A16, and CV-A6) primers and probes (Quant one step qRT-PCR kit, Tiangen, China). If a sample tested positive in the generic and negative in the specific RT-PCRs, a nested RT -PCR assay was used to amplify a portion of the VP1 region [20]. The laboratory procedures of real-time RT-PCRs and nested PCRs were provided in the supplementary file.

Data analysis

We described the profiles of age and gender, time series of HFMD patients by enterovirus serotype. The χ^2 test or Fisher's exact test was used to analyze categorical data, and multiple χ^2 comparisons was done with a Bonferroni-adjusted α value when relevant. Student's t test or ANOVA was used to analyze continuous data and the Bonferroni method for multiple comparisons was used if a significant difference between groups was noted. Data cleaning and

analyses were conducted using R statistical software (Version 3.2.5) and ArcGIS 10.2 (ESRI, Redlands, CA, USA).

Ethical approval

This study was approved by the ethical review committees at the Chinese Center for Disease Control and Prevention, WHO Regional Office for the Western Pacific, and the School of Public Health, Fudan University. Study participation only required patients' parents/guardians to provide verbal consent.

RESULTS

Overview

We enrolled 85% (2,836) of the 3,326 patients with a clinical diagnosis of HFMD during the three-year surveillance. The demographics were similar between the enrolled and un-enrolled patients (supplementary file). Only one 27-month old child with detection of EV-A71, had symptoms suggesting neurological involvement (frequent jittering and myoclonic jerks after 4 days of fever), all others had uncomplicated illness. The patient fully recovered after 5 days of supportive treatment including 2g/kg intravenous immunoglobulin.

Spectrum of enterovirus serotypes associated with HFMD

Overall, 6,188 clinical samples were collected from the enrolled 2,836 patients (Figure 1), including 2,827 throat swabs, 2,583 rectal swabs and 778 stool samples, with a median interval of 1 day between illness onset and sample collection (Supplementary Figure 2). We tested 883 (31%) throat swabs, 1,976 (77%) rectal swabs, and 778 stool samples (100%) for enteroviruses.

Enterovirus was detected in 2,517 patients (89%) via the pan-enterovirus PCR, and serotypes were successfully identified in 2,401 patients (85%). Seventeen enterovirus serotypes were

detected, with the most frequent being CV-A16 (29%, 814), CV-A6 (28%, 784), EV-A71 (17%, 491), CV-A10 (4%, 114), and CV-A4 (2%, 53) (Figure 1). Two serotypes were identified in 51 (2%) patients, including EV-A71 and CV-A6 in 23 patients, EV-A71 and CV-A16 in 19 patients, and CV-A16 and CV-A6 in 9 patients. Detection of EV-B serotypes was less frequent and consisted of E18 (10), CV-B5 (8), CV-B2 (6), E9 (3), CV-B3 (2), CV-A9 (2), E30 (1), CV-B4 (1), and E16 (1). Sequencing failed for 116 patients (4%) who tested positive in the pan-enterovirus PCR. The genotypic subgroup of EV-A71 (C4a) and CV-A6 (B1), and the gene group of CV-A6 (D) were identified based on the VP1 sequences,

Age and gender profiles by enterovirus serotype

The median age was 24 months (IQR 12-36 months). Most patients (90%, 2,561) were younger than 5 years, and 35 (1%) were aged under 6 months. Fifty-seven percent of the patients (1,628) were boys. The age distribution was similar between EV-A71 infections and CV-A16 infections (median 24 months, IQR 12-36 months for both, $p=1.000$). Children were younger in CV-A6, CV-A10, and CV-A4 infections (median 12 months, IQR 12-24 months for each) than EV-A71 and CV-A16 infections ($p<0.05$, Figure 2). There was no association with gender among different serotypes ($p=0.233$, Supplementary Figure 3).

Temporal pattern of enterovirus serotypes

An annual peak of HFMD hospitalization was observed between April and June, with lower epidemic activity in the second year between October 2014 and September 2015 (Panel A, Figure 3). EV-A71 infections accounted for 15% of the HFMD patients in the first year (October 2013 - September 2014), and gradually increased to 17% in the second year and 22% in the third year (October 2015 - September 2016). CV-A16 predominated in the epidemic seasons of the first and the third years, but was infrequently detected in the second year (Panels B and C, Figure 3). The annual number of hospitalization from CV-A6 infections was comparable across the three years,

and CV-A6 was predominant in the epidemic season of the second year and every winter season between 2013 and 2015. Notably, the proportions of EV-A71, CV-A16 and CV-A6 were highly consistent among all the enrolled HFMD patients in the third year, ranging from 22% to 23%. CV-A4 co-circulated in the first and third year, but did not circulate in the second year. All serotypes of enterovirus A species had peak activity between April and July (81% of EV-A71, 82% of CV-A16, 68% of CV-A10, 93% of CV-A4, 84% of CV-A8, 83% of CV-A5, 100% of CV-A2) except CV-A6 (only 37%).

Virological diagnosis by sample type

Enterovirus detection frequencies were 73% (66/90) for throat swabs, 83% (53/64) for rectal swabs and 89% (34/38) for stool samples, among 90 patients from the preliminary evaluation of diagnostic yield. After then, an adapted strategy of sample selection for enterovirus diagnosis was used, in which we tested stool samples (or rectal swabs if no stool sample was available) first and only tested throat swabs if stool and rectal swab were negative. To avoid the bias induced by the sample selection strategy, we only included the 1,875 patients for whom rectal swabs were tested first to estimate the detection proportions of rectal swabs (including the 90 patients admitted October-November 2013), and included the 133 patients for whom throat swabs were tested first to calculate the detection proportions of throat swabs (also including the 90 patients).

The overall proportion for enterovirus detection was 89% (679/759) for stool samples alone, higher than throat swabs alone (74%, 99/133, $p<0.001$) or rectal swabs alone (77%, 1448/1875, $p<0.001$). When stratified by the interval between illness onset and sampling, stool samples also showed higher detection proportions (Figure 4). Detection frequencies decreased as the interval between sample collection and illness onset increased for stool samples ($p=0.008$), for rectal swabs ($p=0.016$), and for throat swabs in particular ($p<0.001$), while it was relatively stable for stool samples collected within 3 days after illness onset (Figure 4). The diagnostic yield only

slightly increased if stools were tested first followed by throat swabs for negative samples (93%, 693/746). Detection frequencies reached 89% (1,662/1,875) if rectal swabs were tested first followed by throat swabs if rectal swabs were negative.

We compared the virological diagnosis results by type of sample among the 90 patients for whom multiple types of samples were tested simultaneously and hospital admission occurred in the first two months. The results were identical for 50 (79%) of 63 HFMD patients with both throat swabs and rectal swabs tested, identical for 28 (76%) of 37 patients with both throat swabs and feces tested. The inconsistent results were caused by the different sensitivity of enterovirus detection for different type of samples.

DISCUSSION

Principle findings

This study provides a comprehensive description of the enterovirus serotypes associated with uncomplicated HFMD over three years in Anhua County, Hunan Province, China. Of the 2,836 patients with HFMD enrolled, 80% (2,256) were associated with CV-A6, CV-A16, EV-A71, CV-A10 and CV-A4 infections. The age was lower in patients with infections of CV-A6, CV-A10 and CV-A4 than in patients with EV-A71 or CV-A16 infections. HFMD showed an annual peak of activity in spring and early summer, with the predominant enterovirus serotype shifting between CV-A16 and CV-A6 during the surveillance period. Stool samples had a higher diagnostic yield (89%), followed by rectal swabs (79%) and throat swabs (74%), and detection frequencies for each single type of sample were higher during the early days after illness onset. The diagnostic yield would increase if combination of stools and throat swabs, or combination of rectal swabs and throat swabs were tested.

Strength and comparison with previous studies

All children except one in this study had mild illness and were admitted to hospital because of a low hospitalization threshold applied by local physicians that was designed to capture all potential rapidly deteriorating cases, secondly because many parents requested hospital admission to ease their concerns about HFMD, and thirdly because of rural healthcare insurance (new rural co-operative medical system), providing higher reimbursement for inpatient than outpatient care in mainland China [21]. This enabled us to capture the uncomplicated HFMD cases systematically and comprehensively in multiple hospitals, as opposed to the sentinel surveillance in which five patients with mild, probable HFMD visiting hospital outpatient clinics in each county were sampled every month, as per national guideline [4, 5, 14, 15, 22].

The annual epidemic activity of HFMD varied substantially during the 3-year period of surveillance. A shift among the circulating enterovirus serotypes was also observed across the three epidemic seasons. During the epidemic seasons with predominant circulation of CV-A16 and EV-A71 (the first and third years), more cases were admitted than during CV-A6 seasons (the second year). We could refer more incidence of HFMD during epidemic seasons with circulation of CV-A16 and EV-A71, since the same hospitalization threshold for HFMD was used during the study period. This is consistent with the national surveillance data that reported fewer HFMD cases in the years (2013 and 2015) when non-EV-A71/ CV-A16 serotypes (most often CV-A6) were dominant in China [14, 15, 23]. The varying epidemic intensity can potentially be attributed to the difference of transmissibility, infectious periods and pathogenicity of diverse enterovirus serotypes. Difference in incidence may have arisen from changes in host population susceptibility over the study period, children social contact [24], and/or the climatic drivers [25]. The enterovirus serotypes EV-A71, CV-A16, CV-A10, CV-A4 had obvious seasonality during April and June, while CV-A6 also commonly circulated during the cooler seasons, which was also

reported in previous studies [4, 26]. The drivers of HFMD epidemic activity and seasonality of enteroviruses are not fully understood and need further study.

Our age profile of HFMD accords with other previous reports which only documented the age among EV-A71, CV-A16 and CV-A6 infections [4, 7, 14, 15]. Of note, CV-A6, CV-A10 and CV-A4 infections had younger age than EV-A71 and CV-A16, and higher percentages of infants younger than 6 months were found in CV-A6, CV-A10 and CV-A8 infections. This infection pattern of new emerging viruses may result from possible younger age of infection and reduced maternal immunity that might otherwise limit their spread in very young children. Unfortunately, there is only very limited seroepidemiological studies on non-EV-A71/CV-A16 serotypes of enteroviruses A except two reports on CV-A6 [27, 28]. The seropositivity rate of CV-A6 was significantly higher compared to that of CV-A16 and EVA71 in infants aged 6-11 months in 2012 in Jiangsu Province, China [28]. More work should be done to study the age specific prevalence and incidence of infections of the predominant enterovirus serotypes based on serology, including maternal antibodies, which will help to understand the age distribution of infections with different enterovirus serotypes.

Detection frequencies of enteroviruses from different sample types in our study were comparable to the 73% (201/274) for throat swabs and 92% (1608/1748) for stool samples described in two previous studies [4, 15], and higher than that in other reports (80% for stool samples, 61% for rectal swabs, and 36-63% for throat swabs) [4, 11, 19, 29]. Diagnostic yields of enteroviruses were dependent on viral load in the throat and gut, timing of sample collection, and type of samples. The timing of sample collection and availability of multiple samples may have contributed to the high diagnostic yield in our study. Studies have suggested longer persistence of enterovirus shedding in the stool than in the throat [30-32]. Excretion of enterovirus was

identified for up to 6 weeks in 6 of 12 patients in stool, and in 4 of 12 patients for 1-2 weeks in the throat [30]. EV-A71 was detected in 43% and 71% of HFMD patients through throat swabs and stool respectively during 9-12 days after illness onset [31]. We also found the detection proportion of enterovirus decreased faster in throat swabs than in rectal swabs or in stools.

Enteroviruses are commonly detected in healthy children's stool samples (10% in cross-sectional study and 22%-51% in prospective follow-up studies) [33-36]. The 51 patients (2%) with detection of two serotypes and 34 EV-B (1%) infections in our study should therefore be interpreted with caution. This may particularly apply to those detected in stools or rectal swabs, because we could not exclude asymptomatic carriage or persistent shedding from a past episode of HFMD. Diagnosis of enterovirus co-infections and EV-B associated with HFMD would be more robust if such results were detected from sterile sites.

LIMITATIONS

This study subjects to several limitations. First, 15% of the hospitalized patients with HFMD were not enrolled due to refusal to provide clinical samples. However, the demographics and disease severity were not significantly different between enrolled and un-enrolled groups. Thus, we believe no significant subject selection bias would be induced. Second, due to the difficulty of collecting vesicle swabs and absence of an indication for lumbar puncture, we used the clinical samples from the non-sterile sites for enterovirus diagnosis in this study. Third, the diagnostic yield could not be evaluated for testing the throat swab first followed by stools or rectal swabs due to the sample selection strategy. This alternative sampling strategy would, however, be unlikely to improve the high enterovirus detection rates achieved in the study.

CONCLUSIONS

Our study provides robust and comprehensive analysis of the full spectrum of the enteroviruses associated with uncomplicated HFMD in Anhua County, Hunan Province, China between 2013 and 2016. We recorded significant differences of age profiles, epidemic activity and seasonality among CV-A6, CV-A16, EV-A71, CV-A10 and CV-A4. Our results will serve as a virology baseline before EV-A71 monovalent vaccine immunization against which future virological features can be compared. Also, the evaluation of enterovirus diagnostic yield of one sample type and combination of different sample types will provide valuable data to guide sample collection strategies for future virological diagnosis in public health and in clinical practice. Continuous comprehensive virological surveillance is essential, especially in the post EV-A71 vaccine era in China, to monitor the potential serotype replacement and the potential ecological impact of monovalent EV-71 vaccine.

Authors' contribution

H. J. Y. conceptualized, designed and supervised the study. L. D. G., G. Z. Q. H. L., F. F. L., B. B. D., Z. Y. C., W. J. X., L. Y., H. L., Y. Z., L. L., X. W. M., Q. L., and K. W. L. coordinated and participated data collection. G. Z., Y. H. Z., J. L., Z. H. C., L. L. W., and K. L. performed the real time PCR or nested PCR. P. S., R. A., and H. R. D. provided the technical support for the laboratory tests and were responsible for the quality control. L. D. G., Q. H. L. carried out the statistical analysis and drafted the manuscript. H. J. Y., P. W., P. W. H., and B. J. C. provided guidance on the data analysis. P. W. H., B. J. C., P. S., R. A., H. R. D. and H. J. Y. revised the manuscript. All authors contributed to review and revision and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Potential conflicts of interest

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Figure legends

Figure 1. Flowchart of patients enrollment and virological surveillance of HFMD, Anhua county, China, October 2013- September 2016.

Figure 2. Age profile of laboratory-confirmed HFMD by enterovirus serotype, Anhua county, China, October 2013- September 2016.

Figure 3. Probable and laboratory-confirmed HFMD in Anhua county, China, October 2013- September 2016.

A: Time series of weekly probable and laboratory-confirmed patients with HFMD.

B: Time series of weekly laboratory-confirmed patients with HFMD by enterovirus serotype.

C: Monthly proportions of laboratory-confirmed patients with HFMD by enterovirus serotype.

Figure 4. Evaluation of throat swabs, rectal swabs and feces in enterovirus diagnosis among HFMD patients in Anhua county, China, October 2013- September 2016.

Figure 1.

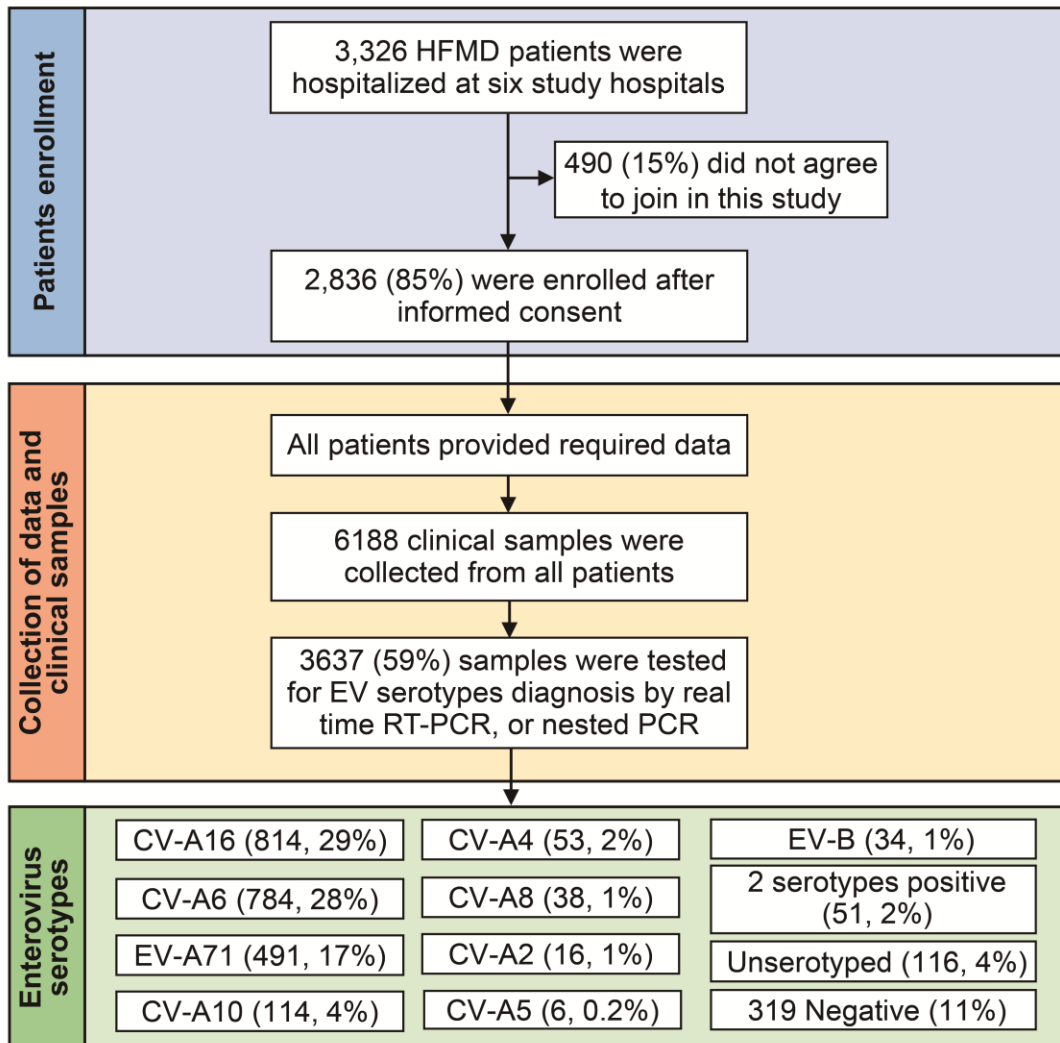


Figure 2.

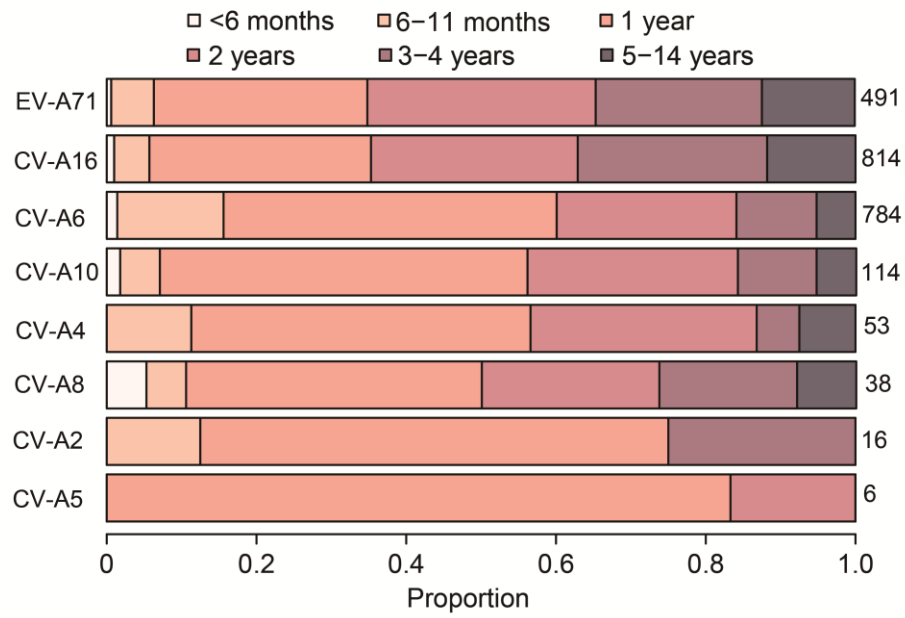


Figure 3.

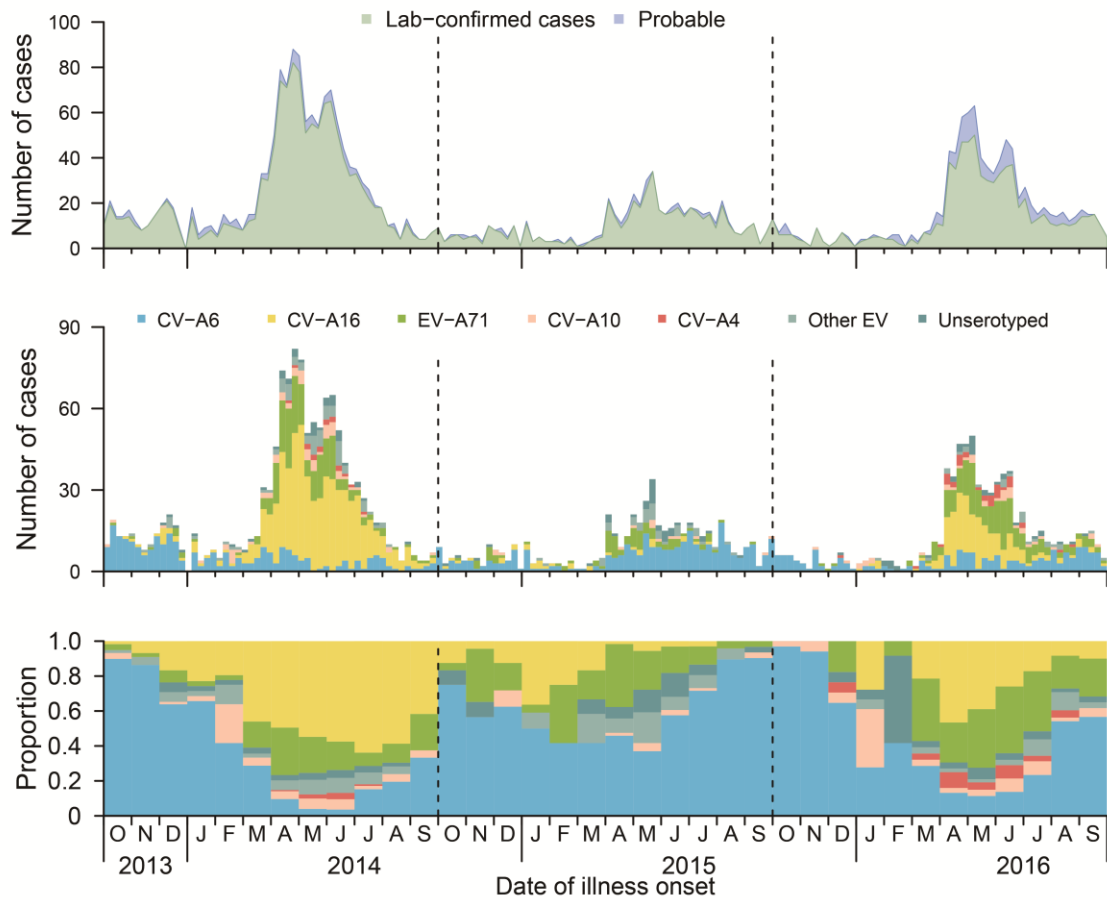


Figure 4.

