Seroepidemiology and Mathematical Modelling of Non-Polio Enteroviruses

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To my family, who supported and encouraged me

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ABSTRACT

Enteroviruses are spread fecal–orally or via respiratory transmission and cause a wide range of diseases. They generally cause mild and self-limited illness but have emerged to cause outbreaks with more severe disease manifestations than previously known. The principal public health needs for enterovirus surveillance include outbreak detection, response and monitoring of enteroviruses associated with severe disease. However, the burden of enterovirus infections is not adequately described due to inadequate systemic surveillance. Here, serum neutralizing antibodies to EV-D68, EV-A71, CVA6 and EV-D111 were measured in samples collected in the UK and four African countries to estimate population-level exposure.

Overall, the serology data showed high antibody levels and seroprevalence that reached 100% in adults indicating extensive exposure and ubiquitous virus circulation. Clinical detection of enteroviruses in the UK showed biennial and annual occurrence of EV-A71 and CVA6, respectively. CVA6 detections were marked by an increasing trend from 2006 to 2017. Model fitting of the seroprevalence data showed possible existence of age-specific transmission or relative risk of infection. The increase in virus detection or clinical occurrence without parallel increase in FOI could be attributed to a change in virus characteristics or pathogenicity leading to pronounced clinical disease even without a change in virus transmissibility. This thesis demonstrates the application of serology and mathematical modelling to estimate pathogen prevalence. The data and inferences will be beneficial for guiding future policies focused on enterovirus epidemiology and disease burden. Certainly, better documentation of enterovirus true prevalence and collective impact on human health is warranted.

Chapter 1: Background

Picornaviruses

Viruses assigned to The *Picornaviridae* family possess a non-enveloped, single, positive-stranded RNA genome packed into a 30 nm capsid. The family includes many human and animal pathogens, including poliovirus, hepatitis A virus, and foot-and-mouth-disease virus [1, 2]. Picornavirus genomes vary between 7 and 8.8 kilobases in length and share a conserved organization. The 5' end of the genome is linked to the small viral protein VPg and contains a long untranslated region composed of cis-acting elements, a 5' terminal domain involved in replication and an internal ribosomal entry site (IRES) necessary for translation [1, 3]. The overall organization of polyproteins starts with the structural capsid proteins (1A–1D or VP4–VP1) located at the amino-terminal portion of the polypeptide, followed by the non-structural proteins (2A–2C and 3A–3D) [4] (Figure 1). For some genera, the VP0 capsid precursor protein is not cleaved into VP4 and VP2, and 2A and 3B may exist in more than one copy [2, 5].

Mutation and recombination generate genetically diverse genomes contributing to picornavirus evolution [6, 7]. Members of the family are currently classified into 68 assigned genera including *Enterovirus, Parechovirus, Hepatovirus, Cardiovirus, Kobuvirus, Cosavirus and Salivirus,* with a total of 158 species [8].

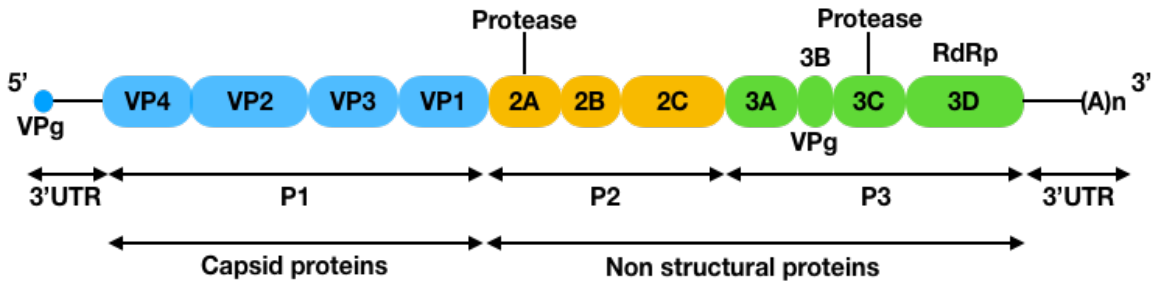


Figure 1 *Genome organization of viruses in the Picornaviridae family. There is a VP0 protein (not shown) that is a precursor of the capsid proteins.*

Enteroviruses

Enteroviruses (EV) are spread through the fecal–oral route or via respiratory transmission, and from their primary sites of replication in the gastrointestinal or respiratory tract, they can disseminate and infect other tissues and organs, including the central nervous system [9]. The lack of a lipid envelope confers stability on exposure to human gastric acid, and they can survive at room temperature for several days. Enteroviruses have also been detected in ground water and hot spas [3]. They may be acquired prenatally but most infections are acquired intrapartum or post-partum, from family contacts or by transmission in the nursery. Enteroviruses use different endocytic routes for entry depending on the serotype and cell type [3]. Receptor binding and/or pH changes in the endosomal system induce virus uncoating, releasing the viral genome from the capsid into the cytoplasm through the endosomal membrane [9].

The viral RNA is translated into a single large polyprotein proteolytically processed by viral proteinases 2Apro, 3Cpro and 3CDpro into capsid proteins VP1-VP4, replication proteins 2A–2C and 3A–3D and some stable and functional cleavage intermediates [9]. Genome replication starts with the synthesis of a negative-strand copy to generate a double-stranded RNA intermediate. The negative strand serves as a template for the synthesis of new positive strands [9]. The positive strands serve as templates for further translation and replication or form new virions. Viral proteinases 2Apro and 3Cpro also cleave host proteins to optimize virus translation, replication and spread and to suppress antiviral cellular responses. They induce host shut-off, disrupt nucleocytoplasmic

transport, interrupt type I interferon and stress pathways and disrupt the cellular cytoskeleton [5, 9].

Taxonomy

Enteroviruses infecting humans are assigned to four species, *Enterovirus A–D*, each with numerous serologically distinct viruses (serotypes) (Table 1). Coxsackie A viruses are genetically highly heterogeneous and distributed across EV-A, EV-B and EV-C species. EV serotypes show >25% nucleotide sequence divergence in the VP1 gene, while variants of the same serotype show <25% divergence and these genetic thresholds are now used to define newly discovered types in the absence of antigenic characterization [10]. Several enteroviruses have been detected in non-human primates e.g., EV-A92, simian virus 5, baboon enterovirus A13 and simian enterovirus SA5, while others have been isolated from both humans and apes (e.g., EV-A119, EV-D111) [10].

Table 1. Division of *Enteroviruses* into species and types. Table adopted from [10].

Species	**Name / abbreviation of types**
Enterovirus A (n=25)	Coxsackievirus (CV): CVA2 -> CVA8, CVA10, CVA12, CVA14, CVA16 Enterovirus (EV): EV-A71, EV-A76, EV-A89 -> EV-A91, EV-A114, EV-A119 -> EV-A121 * EV-A92, EV-A22 -> EV-A125 (formerly simian virus 19, 43, 46 and baboon enterovirus A13
Enterovirus B (n=63)	CVA9 CVB1 -> CVB6 Echovirus I: E1 -> E7, E9, E11 -> E21, E24 -> E27, E29 -> E33 EV-B69, EV-B73 -> EV-B75, EV-B77 -> EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B111 * EV-B110, EV-B112, EV-B113, EV-B114 (formerly simian virus SA5)
Enterovirus C (n=23)	CVA1, CVA11, CVA13, CVA17, CVA19 -> CVA22, CVA24

	EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116 -> EV-C118 Poliovirus (PV): PV1 -> PV3
Enterovirus D (n=5)	EV-D68, EV-D70, EV-D94, EV-D111 * EV-D120

* non-human enteroviruses

Enterovirus infections and clinical manifestations

Enterovirus infections are associated with a wide variety of clinical manifestations, including non-specific febrile illness; exanthems; severe respiratory illness [11]; herpangina; hand-foot-mouth disease (HFMD); hemorrhagic conjunctivitis; myocarditis and pericarditis; neurologic disease, including meningitis, encephalitis, and acute flaccid paralysis (AFP); chronic and/or disseminated infection in immunocompromised patients, and perinatal infections. AFP is characterized as polio-like, with sudden onset of limb weakness that is not the result of trauma [12-14]. Enteroviruses generally cause mild and self-limited diseases, but they have been found to affect neonates differently, and often more severely than older children. Infections in newborns usually present with fever, poor feeding, lethargy, irritability, with or without a rash, and occasionally can lead to sepsis, myocarditis, and hepatitis with coagulopathy [15].

Enterovirus myocarditis in newborns can present with respiratory distress, congestive heart failure, shock, arrhythmia, and infarction. Mortality ranges between 30-50%, and residual dysfunction, including chronic calcific myocarditis, dilated cardiomyopathy, and ventricular aneurysms may occur [12, 15]. Pneumonitis in neonates may occur within hours of birth, with rapid progression to severe pulmonary hypertension, and is associated with echoviruses 6, 9, 11, 7, and 22, and coxsackie B viruses [16, 17].

Treatment

Specific antiviral therapy is not available for enterovirus infections. Treatment is supportive, for example, ventilator assistance (non-invasive or mechanical ventilation) in EV-D68 infections. In neonatal sepsis and meningitis, administration of hyper immune plasma is used but high concentrations of antibody are needed to alter the disease course [13]. A variety of molecules have been identified that bind enterovirus capsids or interfere with viral proteins involved in enterovirus replication. For example, Pleconaril, is a viral capsid inhibitor with broad antiviral activity against enteroviruses and rhinoviruses [18]. Pirodavir and vapendavir exhibit activity against EV-A71 and polioviruses [19]. However, clinical experience with these treatments is limited by antiviral activity, poor bioavailability, intrinsic resistance by some circulating strains, and pharmacoeconomic considerations [12, 20].

Enterovirus detection and diagnosis

Enteroviruses can be detected in stool, cerebral-spinal fluid (CSF), nasopharyngeal aspirates and swabs, vesicular fluid, bronchoalveolar lavage (BAL), blood, conjunctival swabs, biopsy specimens (including nail tissue) and urine [21]. The suitability of specimen will vary by age, and according to the time from the symptom onset and clinical manifestations. In aseptic meningitis, encephalitis or myelitis, viral loads are usually higher and detectable for a longer period in stool, blood and respiratory samples compared to CSF; and up to 2 months in newborns with extensive involvement [22, 23]. Respiratory specimens are recommended for acute flaccid myelitis (AFM) cases to detect EV-D68 infection, which has only rarely been detected in CSF or stool, but detection in these specimens must be interpreted cautiously as enteroviruses colonize the throat and gut for

weeks to months. Conjunctival swabs allow for detection of EV-D70 or CV-A24 in acute hemorrhagic conjunctivitis, and vesicular lesions, throat and stool swabs are useful for CV-A6, EV-A71 and CV-A16 detection in HFMD cases [21, 24].

PCR assays targeting the conserved 5'UTR have a higher sensitivity and specificity and a short turn-around than viral culture and are commonly suitable for all enteroviruses. Serotype-specific assays and commercial kits are available [25]. Virus isolation is not recommended for routine diagnosis but can be used for virus characterization or confirmation of PCR-negative samples for suspected new variants [21]. Serotype-specific virus neutralization assays are sometimes used but are time-consuming and laboratory-intensive as they require different virus isolates. Specific IgM (acute infection) and IgG (past infection) assays have limited clinical utility due to the cross-reactivity between the different serotypes and are not recommended for routine diagnosis [21].

Pathogen emergence and re-emergence

Pathogen emergence and subsequent spread have been attributed to socioeconomic drivers (e.g., population density, antibiotic use and agricultural practices), ecological and environmental conditions (e.g., wildlife host species richness) [26]. Viruses have a higher capacity to adapt to new hosts and evolve, allowing immune and therapeutic escape, efficient infection and transmission [27]. Cross-species host switches of enzootic and epizootic viruses are also sources of new epidemics or pandemics [28]. Host population size and density, which can be thought of comprising the 'effective susceptible population size' for emerging viruses, also have a major impact on successful pathogen transmission [29]. Population size is a crucial transmissibility factor, which could increase or decrease

the transmissibility risk, and contacts are affected by a changing age distribution of the population through feedback of ageing on the force of infection [30]. For infections where transmission, infection (including contact patterns relevant for transmission), and disease processes are age-dependent, demographic shifts contribute to changes in disease burden even in the absence of changes in transmission and infection [31].

Population immunity dynamics and disease emergence risk

Acquired immunity can suppress pathogen emergence. Immunity from pre-emergent exposures can suppress transmission such that emergence hazard rates eventually decline. For instance, bridge communities like bush meat hunters are thought to acquire immunity to zoonotic pathogens before pathogen adaptation for human transmission, which consequently affects emergence, offsets infection risk and impedes emerging epidemics [32]. The potential evaporation of local herd-immunity barriers through population mixing coupled with frequency- and density-dependent transmission may influence the likelihood of emergence [32, 33]. It is postulated that the emergence of HIV, dengue, Lassa fever and hantavirus was associated with social-ecological disruptions that altered bridge communities [29].

Effective susceptible population size plays a major role in successful disease and pathogen emergence. Enterovirus incidence dynamics and outbreaks were determined to be largely driven by serotype-specific long-term immunity and demographic transitions [34]. Introduction of 'novel' influenza viruses from animals into a population with low or inexistent immunity, has resulted in highly transmissible pandemic viruses [35]. Herd immunity had a major impact on circulation and risk of arboviruses, with major outbreaks produced upon introduction into naïve populations (e.g., ZIKV and CHIKV in Americas) and

smaller, periodic outbreaks or no transmission occurring once herd immunity limits transmission efficiency or drives transmission to extinction [36].

Frequent pathogen exposure decreases the susceptible population, reducing the proportion of successful introductions and percolation probability i.e., probability of transmission chains emerging into an epidemic given introduction and adaptation. The lack of immune stimulation due to reduced pathogen circulation induces an immunity debt, posing greater likelihood of community-transmitted infections due to a growing susceptible population and a decline in herd immunity [37, 38]. Recent reemergence of EV-D68 infections is a good example of reduced antigen stimulation due to COVID-19 control measures [39].

Where susceptible individuals likely contact other susceptible individuals more than would be expected by chance, an introduced pathogen has a higher probability of producing uninterrupted chains of transmission than in a setting where contact is homogeneous [40]. The effective reproductive number increases with increasing susceptibility clustering, in turn increasing the relative probability of an outbreak. Age-specific heterogeneity in contact patterns between susceptible individuals has been demonstrated in clusters of low measles and pertussis vaccination or herd immunity [40]. Assessing herd immunity is therefore essential for routine risk monitoring of future epidemics.

Pathogen processes and increased susceptibility to infection

Another component of disease emergence risk is the emergence of a pathogen with novel characteristics resulting in sudden disease flare-up within the same host population. Multiple biological features determine virus transmissibility and successful emergence and

include low host mortality, ability to establish long-term chronic infections, being non-segmented or non-enveloped, and not being transmitted by vectors [41]. However, genome length, genome type, and recombination frequency seemingly have little predictive power to establish productive infections in humans [41]. SARS-CoV-2 adaptation in humans has resulted in variants emergence due to selective pressure and ongoing virus evolution. Shaped by fitness for replication and transmission, and escape from antibody-mediated immunity, the variants have impacted the spread of the SARS-CoV-2 and the efficacy of vaccines [42, 43].

Adaptive mutations in chikungunya virus and emerging lineages mediated enhanced transmission permitting widespread transmission and large epidemics in previously unexposed populations [44, 45]. Influenza viruses continuously explore new antigenic landscapes, and the evolution of seasonal strains shape the global epidemic dynamics. Antigenic drift contributes to annual disease burden via reinfection of previously infected or vaccinated individuals and is also attributed to poor vaccine effectiveness [46, 47].

The upsurge of EV-D68 infections in the last decade was associated with novel divergent subclades [48] as well as re-emergence of ‘old’ sub-clades with novel mutations [49]. Recurrence of EV-A71 outbreaks was linked to genotype change resulting in antigenic cluster shifts [50]. Intra-genotypic evolution in EV-A71 non-structural genes has been attributed to changes in virus infectivity and/or antigenicity by augmenting viral adaptability and fitness. This could explain why a circulating genotype with limited capsid protein diversity causes outbreaks after persistence in the population for years [51].

Serology for pathogen surveillance

Immune responses and pathogen presence drive pathogen transmission. Analysis of host immune responses at a population level is fundamental for understanding immunity dynamics and insights from serological surveillance are valuable for policy making and public health decision planning [52]. Preparedness for emerging pathogens relies on both pathogen surveillance and immunological surveillance to guide rapid development of diagnostic, therapeutic, and preventative tools [53]. Serological surveys are useful for assessing the trajectory of susceptible individuals who together with cases determine the shape and size of the pandemic, and the immune individuals who drive the dynamics of herd immunity [53, 54]. Often, the true number of infections is higher than the officially reported cases due to various factors, including testing capacity, type of tests used, testing strategies, health-seeking behavior, false-negative tests, and incomplete case reporting [55-57]. Therefore, the reported cases based on clinical identification only represent a small proportion, and asymptomatic and mild infections might only be identified through seroepidemiological studies [52].

Serological assays have been used to quantify the magnitude of transmission of pathogens, estimate the infection fatality rate, assess the effect of interventions e.g., lockdown measures, and understand antibody responses upon infection and vaccination [58, 59]. The assays allow antibody quantification and functionality (e.g., virus neutralization), they can be setup relatively easily and inexpensively, and work with minimally invasive sample types e.g., saliva and blood spots for SARS-CoV-2 [58, 60]. Although impractical to test the entire population, well-designed serosurveys are useful for determining pathogen prevalence [61-63].

Serology surveys have been used to evaluate autochthonous circulation of emerging diseases with high potential for epidemic spread, e.g., MERS-CoV [64, 65], avian influenza [66], and pathogenic arboviruses [67]. Elevated seroprevalence for such viruses indicates widespread circulation correlating with an increased risk of infected but asymptomatic populations. Serosurveys in different settings, are vital in understanding the extent of population immunity and trajectories of future transmission. Seroprevalence data have been useful for assessing geographical risks associated with reemergence or resurgence of mumps and poliomyelitis outbreaks [68].

Mathematical modelling for infectious diseases

Mathematical representation and descriptions of processes associated with dynamics of infections have been central to infectious disease epidemiology. Models describe system dynamics through mathematical equations and link biological transmission processes and infection dynamics at individual and population levels [69]. Mathematical models allow estimation of the magnitude of transmission, or duration of infection by identifying combinations of parameters that result in projected numbers of cases (or deaths) that match the observed numbers [70]. Models have contributed to vaccination programs by determining optimal age range for vaccination, timing of vaccination, how best to use limited vaccine supplies; and by introducing herd immunity thresholds, models were used to predict potential impact of vaccine protection and future uptake [71, 72]. Transmission models were used to evaluate the relative impact of control measures and public health interventions during the Ebola outbreak and COVID-19 pandemic [73-76].

Mathematical models can elucidate properties of pathogens such as R_o and generation time [69], and have been used to compare mechanisms of spread and persistence and identify competitive interaction and cross-protection between pathogens [77]. Models were used to improve situational awareness and forecast the course of epidemics, such as Ebola [78], and more recently, the future trajectory of the COVID-19 pandemic [79, 80]. They were used to study pandemic preparedness and mitigation strategies for seasonal influenza [81]; for unravelling drivers of MERS-CoV transmission [82]; and for informing prevention of the spread of antimicrobial resistance [83]. In the 2016–2017 Zika epidemic, modeling provided critical early insights on the risk of microcephaly [84]. Models were used to decipher the impact of weather and climate on Chikungunya invasion in the Americas [85] and latitudinal differences in the dynamics and timing of local RSV epidemics [86].

Mathematical models take many forms depending on biological knowledge of processes involved, data available and purpose. Individual-based models comprise individual entities with own traits such as age, location, sex, immune status, risk profile and behavior pattern, described in interaction with each other in a contact network and with a pathogen [87]. Compartmental models describe individuals aggregated into compartments where everyone has the same average characteristics, and interaction is typically random. They do not describe the disease history of single individuals, but rather the time evolution of the compartments [87, 88].

A standard compartmental model is the susceptible-infected-removed (SIR) model whose dynamics are governed by a system of ordinary differential equations (ODEs) to capture counts of infected and susceptible people at each timepoint [89]. The model describes the infection spread in a homogeneously mixing population where a susceptible

individual becomes infected at a rate βI/N and remains infectious for a duration 1/γ before recovering or removal [88]:

$$\frac{dS(t)}{dt} = -\alpha S(t)I(t)$$

$$\frac{dI(t)}{dt} = \alpha S(t)I(t) - \beta I(t)$$

$$\frac{dR(t)}{dt} = \beta I(t)$$

where $\alpha > 0$ and $\beta > 0$ represents transmission and recovery rates, respectively.

The SIR model is also extendable to include an *exposed* or *latent* compartment, loss of immunity (SIRS), demographic transitions, discrete time, spatial structure, contact networks and heterogeneity configurations in susceptibility and infectivity [31, 90-94]. Other compartmental models include the SIS model where the pathogen does not induce lasting immunity [88]. Mathematical models can have mechanistic parts based on assumptions about biological mechanisms involved, and phenomenological parts where a statistical or presumed relation between variables is included [87, 95]. Techniques such as Markov Chain Monte Carlo (MCMC), particle filters, and approximate Bayesian computation, have become established tools for parameter estimation from data in infectious disease models [96]. The need to include uncertainty in model parameters and/or structure has increased the popularity of Bayesian approaches [97].

Using serology with mathematical models

A pathogen's effective transmission potential is linked to the current individual- and population-level exposure or herd-immunity. While insights such as case fatality ratio can be obtained from incidence data, serology helps resolve uncertainty around infection

prevalence and define better denominators for measures such as infection fatality ratio and transmission-related parameters e.g., basic (R_o) and effective (R_e) reproduction numbers [52, 98]. Also, due to non-availability of proper clinical surveillance data and the inherent limitation to capture unapparent infections despite substantial disease burden, age-stratified seroprevalence profiles are more realistic for force of infection determination and invaluable in quantifying transmission in a population [99]. Serological data are also useful for dissecting disease properties such as behavioral and seasonal changes in transmission or age-specific heterogeneities in immunity, and in contrast to traditional case-based surveillance, it is possible to narrow down the range of plausible epidemic scenarios by calibrating the model to empirical observations of susceptible depletion [99].

Serological assays have been instrumental in mathematical modelling studies to infer infection histories and describe dynamics of immune responses and duration of protection [100]. Serological data have been used widely with mathematical models to generate insights into infection dynamics. Examples include estimation of influenza age-specific infection fatality [101]; for quantifying the (cost-)effectiveness of reducing transmission with non-pharmaceutical interventions during the COVID-19 pandemic [102]; evaluation of the impact of epidemic cycles on parameter estimation for mumps and rubella infections [103]; and for assessing spatial heterogeneity in dengue endemicity [104].

Thesis overview

Emerging enteroviruses were added to the WHO Blueprint list of priority diseases in 2018. However, non-polio enteroviruses are not included in the WHO global virus surveillance guidelines. The capacity for enteroviruses to spread widely, display changes in pathogenicity and cause outbreaks in already exposed populations exemplifies their unpredictable epidemic potential and the reason why these viruses require attention in the post-polio eradication era. My DPhil studies are organized into four chapters as follows:

Chapter 2: Retrospective seroepidemiology studies of three non-polio enterovirus serotypes known to have emerged to cause clinical disease and one serotype considered as an emerging epidemic threat.

Objective: estimate age-specific seroprevalence and population-level exposure to EV-D68, EV-A71, CVA6 and EV-D111.

Chapter 3: Analysis of clinical virology data to describe occurrence of enteroviruses in England, UK.

Objective: to examine incidence, seasonality, and trends of EV-A71 and CVA6 detections.

Chapter 4: Mathematical modelling of seroprevalence data to reconstruct changes in virus transmission.

Objective: to fit catalytic models to EV-A71 and CVA6 age-stratified seroprevalence data to estimate force of infection and infer rates of acquisition of seropositivity.

Chapter 5: Quality assessment for harmonization of enterovirus neutralization assays.

Objective: to evaluate performance and variability of virus neutralization assays for antibodies to EV-D68 and echoviruses.

Chapter 2: Seroepidemiology studies

Summary

Neutralizing antibody levels are a correlate of immune protection. Four seroepidemiology studies were conducted by measuring serum neutralizing antibodies to EV-D68, EV-A71, CVA6 and EV-D111 to estimate extents of past infection and virus prevalence. EV-D68 is mostly associated with respiratory disease and has a recent causal role in acute flaccid myelitis. EV-A71 infection presents commonly as HFMD and rarely as neurological disease. CVA6 affects a broader demographic and results in more severe atypical HFMD manifestations, and EV-D111 was recently detected in children with acute flaccid paralysis.

Archived samples collected in 2006, 2007, 2011 and 2017 were used and serology methods were common to all enteroviruses. Of the 1586 serum samples tested, 93%, 74% and 80% were seropositive for EV-D68, EV-A71 and CVA6, respectively. Seroprevalence increased over time and with age reaching 100% in adults. However, antibody levels declined with age (>21 years) particularly for EV-A71 and CVA6 suggesting waning immunity. Geometric mean titers (GMT) were highest for EV-D68 and comparable between EV-A71 and CVA6, but always higher for CVA6. Sera from human and non-human primates showed extensive exposure to EV-D111 (>50%).

Overall, the studies show considerable exposure and breadth of population immunity to enteroviruses, although the declining titers in adults could have implications in maternally transferred immunity.

2.1 Serology methods

Samples

1573 residual sera collected in 2006 (n=514), 2011 (n=498) and 2017 (n=561) were obtained from the SeroEpidemiology Unit archive collection of the Public Health England (PHE), now UK Health Security Agency (UKHSA), UK (Table 1). This archive is an opportunistic collection of residual clinical samples from laboratories throughout England. Individuals were divided into seven age groups, and we aimed to get 100 samples from each (<6 months, 6-11 months, 1-5 years, 6-10 years, 11-20 years, 21-40 years and >40 years). All samples were anonymized and unlinked from any patient identifying information, while retaining information on age, gender, date of collection, sample type and contributing laboratory.

The 2011 cross-section corresponded to the approximate timing of large EV-A71 outbreaks most notable in Vietnam and China [105, 106] in addition to emergence of CVA6 infections associated with atypical HFMD phenotypes [107, 108]. The 2006 timepoint was selected to precede these recorded events and the 2017 cross-section measured the population immunity post-CVA6 emergence period. The 2017 time point also corresponded to recorded EV-A71 outbreaks in Spain and elsewhere in Europe in 2016 [109-111]. Collectively, these selected timepoints reflected changed activity of both viruses and hence enabled us to measure their effects on population immunity.

For the EV-D111 seroepidemiology study, serum samples had been collected from human populations in Cameroon (n=100), Democratic Republic of Congo (n=100), South Africa (n=100), and BurkinaFaso (n=100) (Table 1), four countries considered to have risk factors that influence exposure to enteroviruses from wild animal populations [112, 113].

The EV-D111 seroepidemiology study also included sera from non-human primates (NHP) (n=32) collected in two wildlife sanctuaries in Cameroon from chimpanzees (*P. troglodytes*), gorillas (*Gorilla gorilla gorilla*) and several Old World Monkeys, for veterinary welfare purposes [114]. Enterovirus infections have frequently been detected in NHPs [115]. 227 samples from the 2006 and 2017 cross-sections in England, UK, were also tested for anti-D111 neutralizing antibodies. Here, the UK population was considered a low-risk control group.

Ethical approval for the use of the UK sera was obtained from the UKHSA's Research Ethics and Governance Group. For the African samples, approval had been obtained as part of the Global Viral Forecasting Initiative, which was set up as an early warning system to track emergent pandemics and to monitor the interface between animals and human beings [116, 117]. NHP sample shipments had been approved by the Cameroon Ministry of Forestry and Fauna and Limbe Wildlife Centre [114].

Table 1 Description of samples used for the seroepidemiologic studies.

Country	**Category**	**Mean age (range)**	**Collection year**
UK	General population	35 y, (0-92)	2006, 2011, 2017
BurkinaFaso	Blood donors	25 y, (18-56)	2007
Cameroon	General population	39 y, (35-45)	2007
Democratic Republic of Congo	Military population	39 y, (21-71)	2007
South Africa	Blood donors	25 y (18-62)	2007

Control sera

Horse anti-EV-D68 serum (Fermon 1962 prototype strain, ATCC) and anti-EV-A71 human serum of known neutralizing antibody titer (National Institute for Biological Standards and Control (NIBSC) 14/140) were used as positive control sera for EV-D68 and

EV-A71, respectively. There was no commercially available material for CVA6 and EV-D111 and instead, pooled adult sera that inhibited virus replication at a dilution >1:32 were used as positive controls. Inactivated horse serum (ATCC) was used as negative control. There was no human negative control serum available.

Virus strains

A genotype B3 EV-D68 strain isolated in 2016 was obtained from the National Institute for Health and Welfare, Helsinki, Finland, for neutralizing antibody testing. A genogroup B4 EV-A71 strain isolated in Singapore (5865/SIN/000009, GenBank accession no. AF316321) was obtained through a DNA-launched infectious clone as described in [118]. EV-D68 and EV-A71 viruses were propagated in rhabdomyosarcoma cell line (RD; American Type Culture Collection, ATCC). Two CVA6 strains isolated in 2008 and 2016 were obtained from the National Institute for Health and Welfare, Helsinki, Finland. The CVA6/2008 isolate was obtained during a HFMD outbreak in Finland [108], and the CVA6/2016 isolate was a contemporary clinical strain. CVA6 viruses were propagated in TE32 or 130T cells obtained from the National Institute for Biological Standards and Control (NIBSC, UK). The EV-D111 isolate was obtained from the European Virus Archive [119] and had been cultured in RD cells from stool samples of children with acute flaccid paralysis [120].

The 50% tissue culture Infective dose ($TCID_{50}$) of virus stocks was determined by end-point dilution in a 96-well format using the Reed and Muench method as described below. For all virus and antibody titration assays, Dulbecco's Minimum Essential Medium (DMEM, Sigma-Aldrich) containing fetal bovine serum (FBS, Sigma-Aldrich) and Penicillin-

Streptomycin (10000 U/ml, Sigma-Aldrich) was used as described below. A virus control and an uninfected cell control were included for each batch of tests.

Virus titration

Virus titers or potency were determined by $TCID_{50}$ as follows:

1. For a 96-well plate, check cell culture viability and ensure 95-100% confluency using a light microscope.
2. Thaw virus aliquots on ice and prepare assay diluent solution: 2% DMEM-FCS (1x DMEM, 2% FCS, 1% Penicillin and Streptomycin; ThermoFisher and Gibco).
3. Perform ten-fold dilutions of the virus isolate starting with a 1/10 dilution, for example by adding 100µl neat virus to 900µl of diluent. Change tips between each dilution.
4. As shown in Figure 1 below: (i) dispense 100µl assay diluent into column 11 and 12 (cell control), (ii) dispense 100µl of the lowest virus dilution into row H, column 1:10. Repeat with the next lowest dilution in row G, column 1:10, continue filling until row A1:A10.

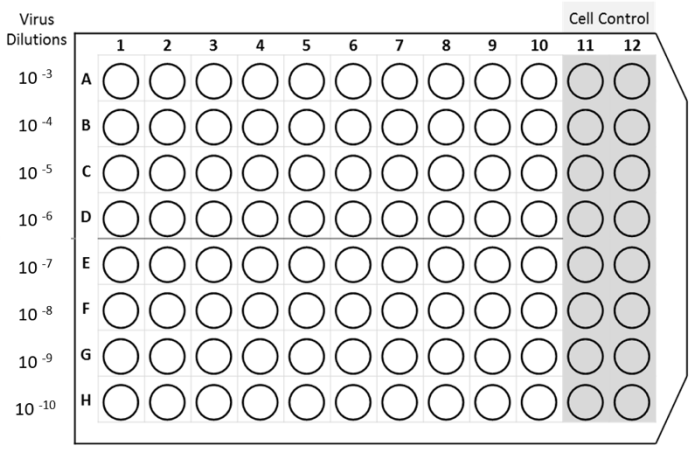


Figure 1 Plate layout for determining virus TCID50

5. Resuspend cells in appropriate volume of 10% DMEM-FCS to a cell count of 1-2 x 10^5 per ml. Add 100µl of cell suspension to all the wells in the plate.

6. Incubate plates in a CO_2 incubator at 33°C for EV-D68 and at 37°C for EV-A71 and CVA6 4-5 days.
7. After 4-5 days, check for CPE/virus growth under the light microscope and calculate the virus endpoint/TCID50 by the Reed-Muench method as below.

Using Figure 1 as a reference plate layout, the virus endpoint/TCID50 would calculated as: $\log_{10}$ 50% end point dilution = $\log_{10}$ of dilution showing CPE next above 50% - (difference of logarithms * logarithm of dilution factor).

Difference of logarithm, also known as 'proportionate distance' = [proportion CPE at dilution next above 50% - 50%] / [proportion CPE next above 50% - proportion CPE next below 50%]

For example:

log10 virus dilution	Wells with CPE (A)	No CPE (B)	Cumulative total with CPE I	Cumulative total without CPE (D)	Total I = C + D	% with CPE: C / E
-4	8	0	21	0	21	100%
-5	7	1	13	1	14	92.8%
-6	5	3	6	4	10	60%
-7	1	7	1	11	12	8.3%

Difference of logarithms = [60-50] / [60-8.3] = 0.193;

$\log_{10}$ 50% end point dilution = -6 – (0.193 x 1) = -6.193; the 50% end point dilution = $10^{-6.193}$; and the titer of the virus = $10^{6.193}$ $TCID_{50}$/ml

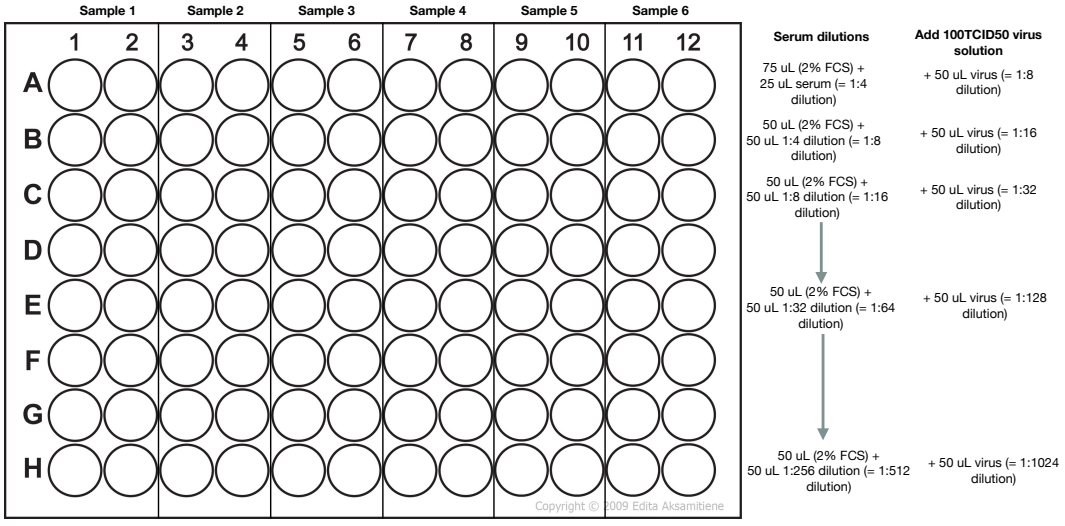
The EV-D68, EV-A71, CVA6 and EV-D111 virus titers were determined as 5.1 x 10^6 $TCID_{50}$/ml, 7.1 x 10^8 $TCID_{50}$/ml, 3 x $10^{7.6}$ $TCID_{50}$/ml, and 2.1 x $10^{5.9}$ $TCID_{50}$/ml respectively.

Virus neutralizing antibody assays

The neutralizing antibody titers were determined as follows:

1. Check cell culture viability and ensure confluency is 95-100% using light microscope.

2. Thaw virus aliquots on ice and prepare relevant volume of 2% DMEM-FCS diluent solution.
3. Sera were tested in duplicate, with the 96-well plate in the 12x8 (wide) orientation. Dispense 50µl of assay diluent into all wells from row B to H (B1 to H12) and 75µl into all wells in row A. Pre-dilute sera (25µl) using the assay diluent to a starting concentration of 1:4, i.e., 1st sample goes into wells in columns 1 and 2, 2nd sample into columns 3 and 4, and so on.
4. Dilute sera 2-fold from top to bottom, down the plate by removing 50µl from row A into row B, mixing up and down, and transferring 50µl into the next row. Repeat until the last row is reached and discard the 50µl, leaving 50µl.
5. For serum neutralization Prepare 100 $TCID_{50}$/50µl virus solution using the assay diluent and dispense 50µl of the 100 $TCID_{50}$/50µl virus solution into each well. This doubles the dilutions, where 1:4 becomes 1:8, 1:8 becomes 1:16 and so on. See illustration below for the setup.



6. For back titration of the viruses: prepare a 5-step log_{10} dilution (1:10) of the 100 $TCID_{50}$/50µl virus solution and dispense 100µl of each dilution to 8 wells (columns 2:6) in a new plate. Add 100µl of the 100 $TCID_{50}$/50µl virus solution in column 1 (Figure 2).
7. Add 100µl of assay diluent to cell control wells – column 12 (Figure 2).
8. Prepare positive and negative controls using the assay diluent: (i) dilute positive serum stock (horse anti-EV-D68) to 1:8192 in appropriate volume and (ii) negative serum stock to 1:16. Into columns 10 and 11, dispense 50µl of the diluted controls (positive and negative, respectively) and 50µl of the 100 $TCID_{50}$/50µl virus solution.

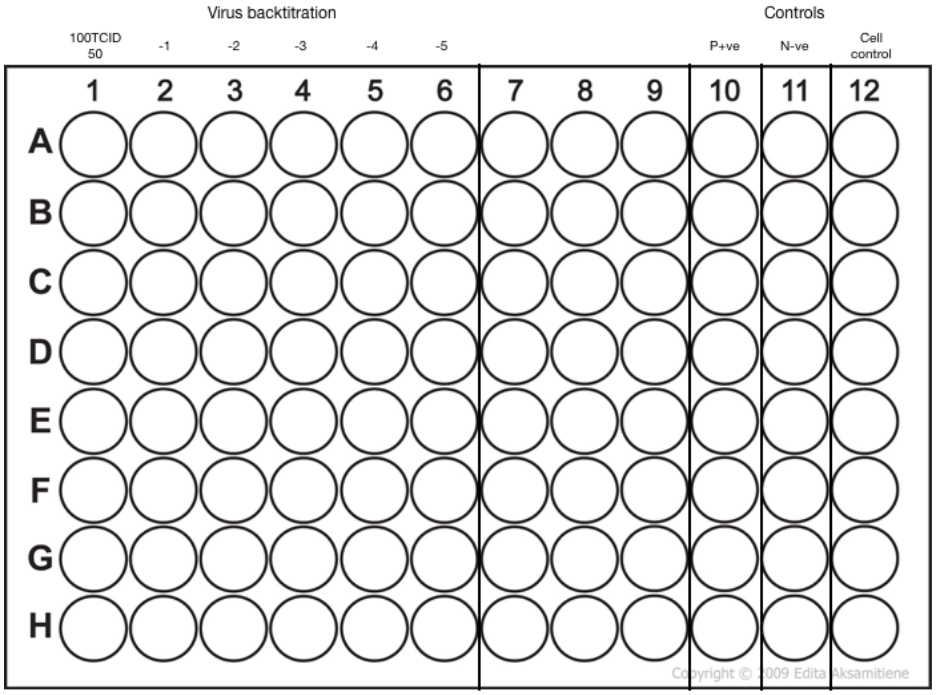


Figure 2 Plate layout for the back titration, positive and negative controls, and cell control.

9. Incubate the plates at 33°C for 1.5 hours.
10. After 1.5 hours, prepare cell suspension in 10% DMEM-FCS. Add 100µl of cell suspension to all plates/wells and incubate in a CO_2 incubator at 33°C for 5 days.

11. After 5 days, check CPE/virus growth under the light microscope and calculate or record the serum antibody titer: the highest dilution which protects 50% of the cultures against 100 $TCID_{50}$ of challenge virus. Antibody titers were expressed as reciprocals (example titer of 1/256 = 256).

Adherent cells were stained with 0.5% crystal violet in 25% methanol. For this, the supernatant was gently aspirated from wells, followed by a single rinse with 100µl PBS. Cells were then fixed with 4% Formaldehyde (Sigma-Aldrich) for 10 minutes and then rinsed with 100µl PBS before adding 50µl crystal violet solution. After adding crystal violet, the plates were incubated at room temperature for 20 minutes then rinsed with tap water.

Evaluation of batch-to-batch variation

Batch to batch variation or batch effects were evaluated by visually checking the antibody titer distribution of serum samples and controls from each batch of samples processed. There was no distinctive inter-batch variation (Figure 3) and the controls occasionally varied by a single dilution factor, which could be attributed to freeze-thaw effects. The serum samples were not tested in any specific order or preference, and since the same samples were not tested in multiple batches it cannot be concluded that there were no batch effects based on antibody titers data alone.

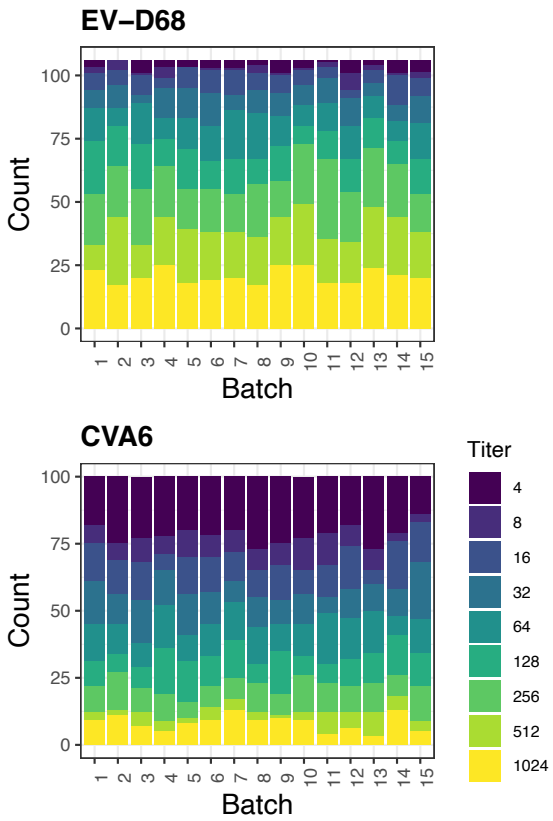


Figure 3 Distribution of serum neutralizing antibodies for each batch of samples processed.

2.2 Statistical analyses

Seroprevalence values were calculated with a 95% confidence interval based on a binomial distribution using the Wilson method (http://vassarstats.net/prop1.html). Comparisons of seropositivity in different age groups were done using Chi-square or Fisher exact test. Antibody titers were analyzed using the Kruskal-Wallis rank-sum test to determine if overall distributions' medians significantly differed among groups, and subset comparisons were performed using the Kolmogorov-Smirnov test. Geometric mean titers (GMT) were calculated for each year and for each age group and compared using the Mann-Whitney U or Kruskal-Wallis test, as appropriate. *P*-values <0.05 were considered statistically significant. All the statistical analyses were computed in R 4.1.2 (R Core Team, 2021).

2.3 Enterovirus D68 seroepidemiology

Introduction

Enterovirus D68 (EV-D68) was first isolated in California in 1962 from four children with pneumonia and bronchiolitis and has been detected almost exclusively in respiratory tract specimens. EV-D68 is mainly associated with respiratory infections and a major factor of the acute flaccid myelitis (AFM) epidemiology [121, 122]. It is acid-labile and differs from most enteroviruses, sharing more physiochemical properties with human rhinoviruses [123, 124]. EV-D68 preferably binds α2-6 sialic acid instead of α2-3, the receptor of the other enteroviruses, giving it affinity for the upper respiratory tract [125]. It also differs from other enteroviruses in temperature sensitivity, growing in cell culture at 33°C, which is the temperature of the nose, rather than at 37°C [123].

EV-D68 has very rarely been reported in stools and has a short C-terminal α-helix in the VP3 protein that is not present in other enteroviruses [123]. Viraemia is detected in pneumonia patients a median of 2 days after symptom onset, suggesting hematogenous migration similar to poliovirus [126]. Four primary genetic clades (A-D) identified based on the VP1 gene are distributed globally with a most recent common ancestor of all clades estimated in the mid-1990s. Clades A, B and D are further divided into subclades A1, A2, B1, B2, and B3, and D1 and D2 [49, 127, 128].

VP1 is one of the more variable proteins and contains receptor binding sites and putative neutralizing epitopes, such as the hypervariable BC- and DE-loops. Different subclades differ at several positions in these loops, which have been shown to have elevated amino acid substitution rates [122, 129]. Compared to the historical prototype Fermon and Rhyne strains, all the modern EV-D68 isolates lost a 24 RNA base region in the

5’UTR between the IRES and the polyprotein ORF [127]. Emergence of strains or genetic lineages with different antigenicity was attributed to increased EV-D68 detections in recent years [125].

Diagnostics to confirm EV-D68-associated neurological invasion/complications in adults and children are challenging, since viral RNA is rarely detected in cerebrospinal fluid (CSF). Either the virus is rapidly neutralized and cleared from the central nervous system (CNS) or is present in neural tissue but not released into the CSF [130]. Virus quantification in human spinal cord or brain tissue to analyze an association of tissue virus concentration with neurological complications severity is only feasible postmortem [131]. Timing of specimen collection probably explains at least part of the low virus detection in sterile sites as most patients with neurological complications report the onset of fever and upper respiratory illness, a median of 5 days before onset of limb weakness, meaning that up to half of specimen collection is occurring >7 days after initial illness onset [132]. Nonetheless, EV-D68-specific antibodies have been detected in the CSF in neurological complications, confirming CNS invasion [133, 134].

Clinical presentation of EV-D68 infections ranges from mild to severe upper and lower respiratory complications requiring hospitalization, intensive care and mechanical ventilation; central nervous system disease and, rarely, death [135, 136]. EV-D68 infection is characterized by sudden onset of the disease, and symptoms include fever, wheezing, cough, influenza-like illness and sore throat [11]. Pneumonia and asthma exacerbation represent the main complications [135]. For instance, many of the hospitalized children in the 2014 outbreak in the USA had difficulty breathing and respiratory distress, largely without fever [137, 138].

Children represent the most symptomatic infections and more severe disease occurs in patients with underlying conditions such as prematurity or chronic diseases, immunocompromised and with a history of asthma or wheezing [137, 139-141]. Healthy adults can get infected although typically show a milder range of respiratory symptoms. While infections in adults are rarely reported, the clinical impact in adults was documented in Hongkong, where EV-D68 strains of subclade A2 were detected in elderly patients with pneumonia and underlying comorbidities [142]. As no specific antiviral therapy is available, treatment is mainly supportive. No vaccines are commercially available, and conventional infection control measures for contacts and droplets in community and healthcare settings are recommended [135].

EV-D68-induced neurological disease

EV-D68-induced respiratory disease has been temporally associated with the emergence of AFM, a paralytic disease occurring predominantly in children and resembling poliomyelitis. AFM exhibits as acute onset of flaccid limb weakness, with imaging showing spinal cord grey matter lesions [121, 143]. However, the inability to detect EV-D68 in the CNS and other affected tissues in most AFM cases, including infrequent detection in cerebrospinal fluid, leaves the association without direct proof of causality [131]. Nonetheless, accumulating clinical, immunological, and epidemiological evidence points to EV-D68 as the major causative agent of recent seasonal childhood AFM outbreaks [144, 145].

EV-D68-associated AFM cases were reported in 14 countries between 2006 and 2018 as described in [131, 146-153]. EV-D68 detections were also reported in children <5 years old with AFM in seven countries in West Africa in 2016 [154]. Increasing awareness and

testing for EV-D68 in Europe led to 31 EV-D68 associated AFM cases reported in 2016, more than 10 times compared with 2014 [131]. AFM occurred predominantly in children, with a mean age of onset between 3–8 years old, and majority were immunocompetent and had completed standard childhood immunizations, including the inactivated poliovirus vaccine [148]. The majority experienced fever and upper respiratory illness in the week prior to the onset of limb weakness but the severity of respiratory disease did not correlate with the development of paralysis [131, 148].

Delayed respiratory sampling following presentation with AFM decreases the frequency of enterovirus D68 detection, as seen in 2014 when the virus was identified in 47% of respiratory specimens collected within 7 days, 20% of specimens collected between 7 and 14 days, and 0% of 17 specimens collected more than 14 days after onset of respiratory symptoms [155]. The pattern of respiratory symptoms and virus detection before onset of neurological symptoms in AFM shows a temporal relationship between EV-D68 and AFM that suggests causality [122, 131]. For instance, the prevalence of EV-D68 from 2009 to 2018 in USA significantly correlated with temporal occurrence of AFM cases [156].

In distinction to poliomyelitis, EV-D68 associated AFM patients were more likely affected in upper limbs rather than lower limbs, consistent with injury to cervical rather than lumbar spinal motor neurons, although some developed widespread limb involvement [144]. Some children developed respiratory failure due to paralysis and required mechanical ventilation, and other symptoms included facial weakness and dysphagia consistent with brainstem involvement [144, 157]. AFM patients in peak years of EV-D68 circulation were more likely to have upper limb involvement compared with patients in nonpeak years providing clues about viral pathogenesis [158]. Diagnosis of AFM

includes the acute onset of flaccid limb weakness plus confirmatory imaging evidence of spinal cord lesions spanning more than one vertebral segment in extent, and the spinal cord lesions should be largely restricted to the gray matter, distinguishing AFM from acute flaccid paralysis (AFP), an umbrella term that encompasses any onset of acute limb weakness [144].

Genome analysis of sub-clade B1 strains isolated from respiratory samples of AFM patients in 2014 showed five polyprotein coding changes sharing sequence homology with neurovirulent poliovirus, EV-A71, or EV-D70 [159], plus 21 nucleotide substitutions involving both coding and non-coding regions that differed significantly from non B1 strains [160]. EV-D68 strains associated with AFM cases from 2014 through 2016 mapped to sub-clade B3 and emerged from a common ancestor of the 2012-2014 sub-clade B1 [156, 161-163]. Sub-clade B3 strains from AFM cases in 2016 and 2018, unlike earlier strains, lacked homology with other neurotropic enteroviruses [144]. Subclade D1 strains were associated with AFM in 2018 in France [164]. Sialic acid-independent strains have been identified that bind to the neuron-specific receptor ICAM5 present in CNS tissue [165]. The possibility of functional receptors in the olfactory nerve endings in the upper nasal cavity epithelium provides another potential portal of CNS entry [131].

An area of uncertainty on the apparent relationship between EV-D68 and AFM is the serologic evidence of high seroprevalence indicating that EV-D68 has been a common infection for decades [166, 167]. The occurrence of disease among populations with apparently high prevalence of serum neutralizing antibody raises questions about whether serum antibodies are protective against EV-D68 infection [130, 166, 168]. Alternatively, serum antibodies resulting from prior exposure to EV-D68 are insufficient to prevent re-infection, and mucosal antibodies or other immunity components are more closely

correlated with protection from disease [130]. Another possibility is whether serum antibodies or prior homologous or heterologous viral exposure could increase the risk for AFM through antibody-dependent enhancement as previously hypothesized for coxsackie B viruses and EV-A71 infection [169]. That is, primary infection antibodies fail to neutralize the virus during re-infection and instead facilitate infection of monocytes through Fc receptors resulting in increased viral replication and higher risk for severe disease [130].

Epidemiology and genetic diversity

Until recently, reports of respiratory disease due to EV-D68 were rare. Between 1970 and 2005 only 26 clinical isolates of EV-D68 were reported in the USA [124]. Minor outbreaks were also described, with 699 EV-D68 cases confirmed in Europe, Africa, and southeast Asia between 1970 to 2013 [11]. From late 2000s, small outbreaks evidenced by increasing genetic diversity were observed in Japan, Philippines, France, Italy, UK, the Netherlands and USA, implicating EV-D68 as an emerging respiratory pathogen [11, 127, 170-173]. In 2012, EV-D68 associated AFM cases occurred in California [174]. Retrospective phylogenetic analyses of VP1 sequences between 2008 and 2014 showed frequent presence of long branches, indicative of limited global surveillance and detection, as well as the presence of in-situ evolution in individual locations [127, 175].

In autumn 2014, a large-scale outbreak of EV-D68 resulting in an upsurge of hospitalizations and admissions to intensive-care units related to severe respiratory infection and neurological complications mainly in children, was first noted in the USA and Canada, with subsequent reports from other countries [11, 138, 176-178]. 2287 EV-D68 infections and 1153 AFM cases were reported between September and December in 49 US states and 700 EV-D68 cases were confirmed in Canada, including eight cases of AFM [137].

This unprecedented outbreak resulted in enhanced surveillance. Subsequently, two AFM cases were reported in Chile, and 408 EV-D68 infections were reported across Europe, including three children with complications of AFM in France and Norway [179-184]. 25 AFM cases were reported in China, Taiwan, and Thailand and attributed to EV-D68 infection [11]. Retrospectively, four lineages of A1, A2, B1 and B3 subclades were found to have circulated in Hongkong between 2010-2014 earlier than their reported emergence in the USA, suggesting that the 2014 and 2016 epidemics in the USA may have originated from Asia [142].

The 2014 EV-D68 strains in the USA showed considerable genetic diversity manifesting as two distinct lineages indicative of multiple introductions, and an inter-subclade recombination event between the B1 and B2 subclades [138, 185]. These two distinct lineages were not localized to a specific region or clinically distinctive, i.e., severe illness was associated with both lineages [138]. EV-D68 strains circulating in New York, USA, in the 2014 outbreak had functional mutations which could alter viral proteases' cleavage efficiency, leading to altered pathogenicity and increased rate of viral replication and transmission [178]. No significant association was found between EV-D68 genetic variation and demographic and clinical variables, suggesting that host factors likely played a major role in determining disease severity [185].

Since 2014, outbreaks occur in a seasonal, biennial pattern (in even years) of late summer to early fall (July–October), peaking August-September in the USA, Europe and China [48, 130, 152, 156, 186, 187]. In 2016, subclade B3 strains were detected in severe respiratory infections in the USA and Europe (France, Sweden, Denmark, Spain, Italy and the Netherlands) [109, 161-163, 188-190]. An upsurge of EV-D68-related pneumonia in Beijing in 2016 comprised of a new subclade D3 which co-circulated with subclade B3 and

had 52 amino acid polymorphisms different from subclade D1 [48]. Six common mutations (M291T, V341A, T860N, D927N, S1108G, and R2005K) associated with neurovirulence in the 2014 subclade B1 outbreak strains were not present in B3 isolates from 2016, suggesting that either the genetic residues responsible for neurovirulence have yet to be identified or that convergent evolution resulted in multiple paths to neurovirulence [191].

The absence of EV-D68 in 2015 and its resurgence in 2016 suggests continued periodic circulation and the biennial seasonality reflects the need for a new susceptible cohort to accumulate and reach a threshold to support virus transmission, or the time required for new antigenic variants to arise [130, 192]. EV-D68 was not detected in AFM cases during the nonpeak years of 2015 and 2017, suggesting that it plays a role in the increase in AFM cases every 2 years but less so during nonpeak years [158].

In 2018, 238 AFM cases were reported in 42 states in the USA [130, 193]. The 2018 EV-D68 outbreak in the USA was milder in terms of respiratory illness but the frequency of severe neurologic manifestations was higher than in 2014 [152]. The 2018 strains associated with AFM belonged to subclade B3 and formed a distinct lineage from the 2014 and 2016 strains [152, 156]. EV-D68 subclade D1 strains were detected in 2018 in patients with acute respiratory infections in Italy, France and Brazil [49, 194, 195]. In the USA, EV-D68 detections decreased in 2019 and 2020, lower than anticipated for 2020 likely due to the COVID-19 mitigation measures [193].

Contrary to the expected seasonality, EV-D68 strains of the B3 and A2/D subclades was detected in five European countries in 2019, a season of expected low circulation. The patients were primarily children (median age 4 years), majority required hospitalization and five had severe neurologic manifestations [196]. More recently, a rapid increase in EV-D68 infections was reported from eight European countries in 2021, and presumably

stimulated by the widespread reopening after COVID-19 lockdowns [39]. EV-D68 strains (clade D and sub-clade B3) were detected in wastewater samples in UK in 2021 mirroring the EV-D68 detections in clinical samples from various European countries in the same year [197].

Although EV-D68 circulation has been detected worldwide, little has been reported in Africa due to lack of systematic surveillance – inclusion of fever in case definition likely underestimates prevalence as not all EV-D68 infections result in fever [198]. In 2014, EV-D68 subclade A1 viruses were detected in Senegal [199] and in 2016, subclade B3 viruses were detected in respiratory illness and AFP mostly in children under 5 years [200]. Genetically distinct EV-D68 strains of clade A and subclade B2 were reported in South African patients with respiratory illness in 2014 [201]. Earlier (2001-2011) EV-D68 strains detected in South Africa belonged to clades A, B and C and were detected in severe acute respiratory illness but none in AFM/AFP cases [202]. EV-D68 strains of clades A, B and C were also reported in children (<13 years) in Kenya between 2008 and 2011 [203].

Enterovirus D68 circulation in UK

In UK, 17 EV-D68 cases were reported between November 2009 and December 2010 in patients with respiratory tract infections. One was fatal but none had AFM [175]. A retrospective study indicated that EV-D68 was only detected in 14/1875 enterovirus positive samples during 2004–2011 [204]. In another study, EV-D68 detection increased from 0.4% (September 2013–January 2014) to 0.8% (September 2014–January 2015) and the strains circulating between 2012 and 2015 were genetically like those detected in 2014 in USA [205]. Between November 2015 and November 2016, 75 EV-D68 infections, including seven AFM cases, were also reported in UK [206].

In Scotland, five EV-D68 associated AFM cases in were reported in children in 2016 with EV-D68 being detected in nasopharyngeal aspirates [207]. All cases presented with significant neurological symptoms, and two required intensive care support because of respiratory paralysis. At 18-month follow-up, two cases remained ventilator-dependent with the other cases requiring ongoing community rehabilitation [207]. From 2015 to 2018, 220 EV-D68 cases were reported in Wales in children of median age 2.5 years. 94% were hospitalized in intensive care and all had respiratory symptoms and two had AFM [208, 209]. More recently, nine EV-D68 infections were identified between January 2018 and January 2019 during an increase in reports of acute flaccid paralysis cases in UK. These occurred primarily among pre-school children presenting with acute respiratory tract illness and neurological symptoms, and approximately half reported global limb paralysis requiring respiratory support [210].

Study objective

EV-D68 antisera and human intravenous immunoglobulin containing neutralizing antibodies were shown to protect against infection and AFM-related paralysis [211]. This highlights the need for comprehensive surveillance of anti-EV-D68 neutralizing antibodies. This study aimed to estimate EV-D68 seroprevalence in England, UK, to infer levels of exposure and prevalence of neutralizing antibody immunity in the general population.

Enterovirus D68 seroprevalence in 2011 in England, UK

An antibody titer of ≥4log2 (≥1:16 dilution) was considered seropositive for EV-D68 neutralizing antibodies according to a previous study [212]. In 2011, the overall seroprevalence was 94% (95% confidence interval [CI], 92.1–96.1), increasing from 82% in children 1-5-years old to 98.7% in individuals 21-40-years old, before reaching ubiquity or 100% in adults older than 40 years. Seroprevalence was varying in children aged 0.5 months to 10 years old but narrowed in older individuals (>30 years) (Table 2). The high seroprevalence in the <6-months-olds is likely due to trans-placentally acquired maternal antibodies, consistent with the high titers in adults. The decline of maternal antibodies is seen as low antibody titers and seropositivity rates in the 6-12-month age group (Table 2). The proportion of samples with moderate to high (≥1:64) neutralizing antibody titers increased linearly with age, with adults older than 40 years having the highest titers. Geometric mean titers (GMT) of seropositive samples also increased with age, matching the increasing seropositivity, and ranged from 66 in 1-5-year-olds to 434 in >40-year-olds (Table 2).

Table 2 EV-D68 seroprevalence and geometric mean titers in England, UK, 2011.

Age group	Positives/No. samples	Seroprevalence (95% CI) *	GMT
<6mo	69/78	88.5% (78.7 – 94.3)	97.6
6-11mo	21/26	80.8% (60.0 – 92.7)	63.1
1-5y	38/46	82.6% (68.1 – 91.7)	65.9
6-10y	45/49	91.8% (79.5 – 97.4)	122.7
11-20y	75/76	98.7% (91.9 – 99.9)	204.7
21-40y	74/75	98.7% (91.8 – 99.9)	380.9
>40y	154/154	100% (97.0 – 100)	434.4
Total	476/504	94% (92.1 – 96.1)	

*(CI: binomial confidence interval)

The 2011 data were then combined with previously published serology data from 2006 and 2017 cross-sectional surveys [213] for comparison and to infer dynamics of acquisition of infection over the period 2006-2017. For the three timepoints combined, the overall seroprevalence was 93% (95% confidence interval (CI) 92-94.4); increasing from 89% (86.8–92.1) in 2006 to 94% (92.1–96.1) in 2011 and 95% (93.5–97) in 2017. Consistently at each timepoint, seroprevalence decreased in the 6-12-months age group then gradually increased with age, approaching 100% in the adult population (individuals older than 20 years) (Figure 4, Table 3).

At the titer cut-off of 1:16, the proportion seropositive at ages 1-5-years was ranging between 0.65 (95% CI 0.54–0.75) in 2006 and increased to 0.92 (95% CI 0.84–0.97) in 2017. Age-stratified seroprevalence in 2011 was higher than in 2006 but lower compared to 2017 (Table 3), suggesting a decrease in the mean age of infection through the study period, potentially consistent with increasing transmission in the last decade. Overall, infections were acquired at early age, with high incidences in the 0.5-2-year and 3-4-year age ranges.

Table 3 Summary of EV-D68 seroprevalence in England, UK, in 2006 and 2017.

Age group	2006		2017	
	Pos/No.	Seroprevalence (95% CI)	Pos/No.	Seroprevalence (95% CI)
<6mo	9/12	75% (42.8 – 93.3)	45	91.1% (77.9 – 97.1)
6-11mo	2/6	33.3% (6 – 75.9)	21	57.1% (34.4 – 77.4)
1-5y	62/94	66% (55.4 – 75.2)	86	91.9% (83.4 – 96.4)
6-10y	36/42	85.7% (70.8 – 94.1)	51	98% (88.2 – 99.9)
11-20y	104/110	94.5% (88.0 – 97.8)	99	98% (92.2 – 99.7)
21-40y	118/120	98.3% (93.5 – 99.7)	149	98.7% (94.7 – 99.8)
>40y	132/132	100% (96.5 – 100)	115	100% (96.0 – 100)
Total	463/516	89% (86.8 – 92.1)	541/566	95% (93.5–97)

(mo: months, y: years, Pos: positives)

Table 4 Geometric mean titers (GMT) of EV-D68 seropositive samples, 2006 to 2017.

Age group	2006		2011		2017	
	No.	**GMT (95% CI)**	**No.**	**GMT (95% CI)**	**No.**	**GMT (95% CI)**
<6mo	12	62.2 (21.8-177.2)	78	97.6 (68.2-139.6)	45	77.6 (49.7-121.1)
6-11mo	6	9.5 (4.8-18.7)	26	63.1 (30.4-131)	21	30.9 (16.1-59.4)
1-5y	94	26.3 (20.4-33.9)	46	65.9 (39.7-109.6)	86	77.0 (55.9-106.1)
6-10y	42	53.8 (35.7-81)	49	122.7 (84.1-179)	51	175.0 (117.7-260)
11-20y	110	115.0 (85.9-154)	76	204.7 (147.2-284.7)	99	280.4 (211-372.5)
21-40y	120	226.8 (179.2-286.8)	75	380.9 (271.3-534.7)	149	265.1 (216.2-325)
>40y	132	317.5 (258.5-389.8)	154	434.4 (359.3-525.2)	115	253.7 (204.3-315)

A marked increase in titers was seen for children >5 years of age in 2011 and 2017 compared to 2006 (Figure 5), as expected with increasing rates of EV-D68 exposure and seroconversion in the younger age ranges. Frequencies and titers of EV-D68 neutralizing antibodies differed substantially among the three timepoints for young children (age categories 0.5–1 year, 1–5 years and 6–10 years) (Kruskal-Wallis test, $p < 0.05$) (Figure 5). In the three sampling years, the proportion of samples with moderate to high neutralizing antibody titers (≥1:64) increased linearly with age, with adults older than 40 years having the highest titers.

Consistent with the seropositivity rates, the geometric mean titers for seropositive samples in 2011 were higher compared to 2006: children younger than 10 years who were seropositive had a GMT 2.5-fold higher in 2011 compared to similar ages in 2006 (Table 4). The mean titers for individuals aged 1-20 years old were lower in 2011 compared to 2017, and collectively, the higher titers and seroprevalence in this age range in 2017 demonstrates greater infection rates in the years between 2011 and 2017.

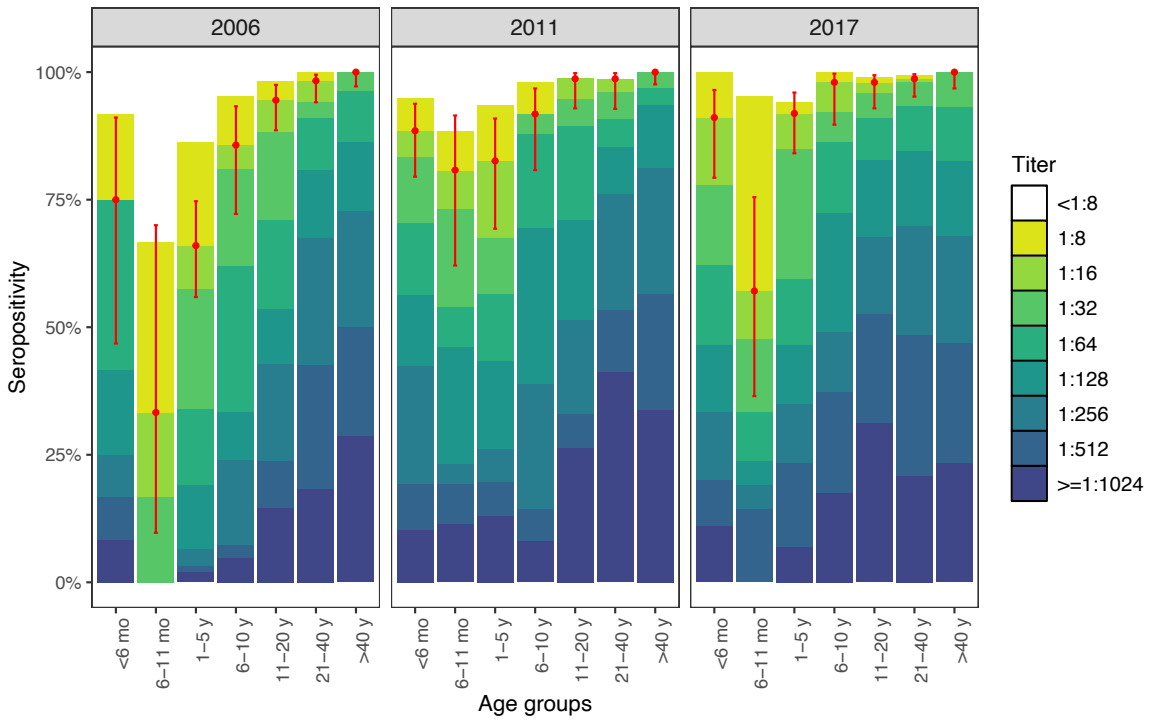


Figure 4 *Seroprevalence of EV-D68 by different age categories. Results are expressed as percentage of samples displaying neutralizing antibody titers from <1:8 to ≥1:1024 (colored bars). Point estimates of the percentage seropositive are shown in red and error bars indicate 95% confidence intervals of the proportions. Samples were scored seropositive if virus neutralization was achieved at serum dilution of ≥1:16. (mo: months, y: years)*

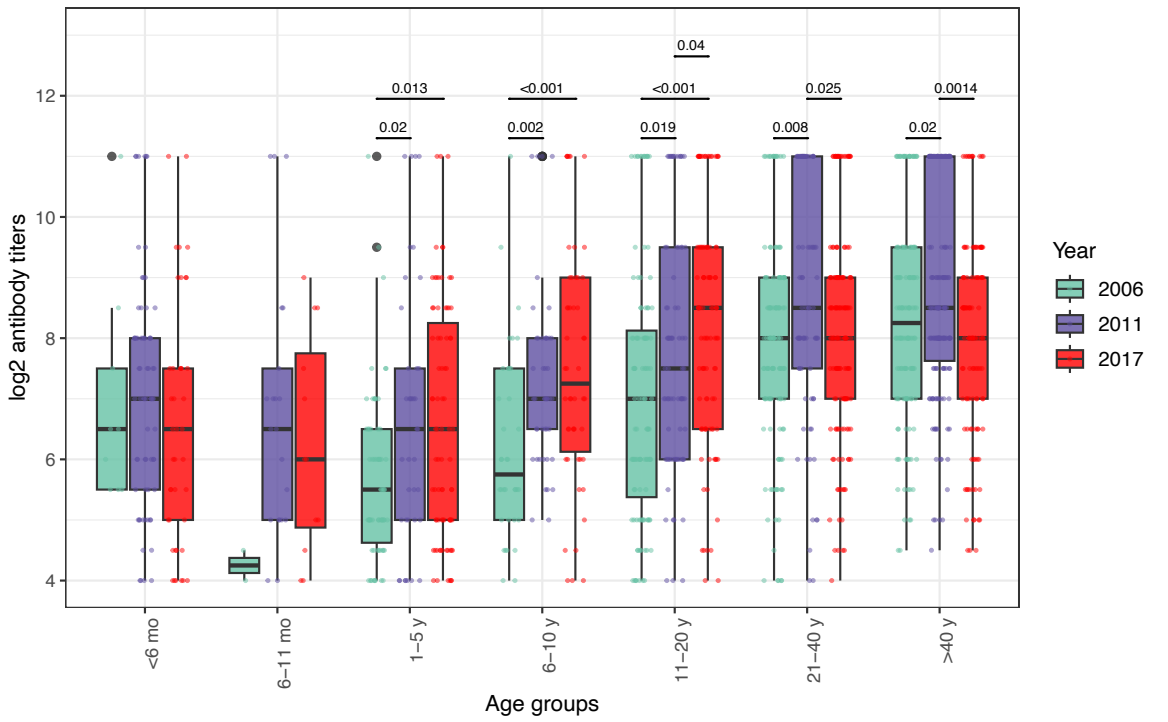


Figure 5 *Boxplots showing the distribution of EV-D68 log2 neutralizing antibody titers of seropositive samples by age group and year of sampling. (mo = months, y = years). The horizontal lines and numbers on top of the boxplots indicate statistical significance level and are results of Kruskal-Wallis tests for antibody titer comparisons.*

Additional analysis was performed on a subset of women ('Gender' = 'Female') aged 19-45 years (n=260), who would be of childbearing age or are caregivers. The age range was based on the average age of first-time mothers in England (Office for National statistics, UK). Immunity levels in women of child-bearing age could be an indicator of neonatal passive immunity through maternal transfer, which can mitigate the risk for severe infections in the early postnatal period [214, 215]. Women also form majority of child minders and regularly interact with children and high seroprevalence in this age group (herd immunity) would form a protective layer ('cocoon') against transmission in the pediatric population who are most susceptible to disease. Parental 'cocooning' has been

used against pertussis in newborns [216] and cocoon vaccination was used against COVID-19 and Influenza in the immunosuppressed patients [217, 218].

For EV-D68, all samples except four (98%) in the age group 19-45y were seropositive (antibody titer ≥1:16) and higher titers in this age group occurred in the 2011 samples (Figure 6). The mean titers in this age group were 290.6, 479.9 and 304.3 in 2006, 2011 and 2017, respectively, suggesting high prevalence of infections.

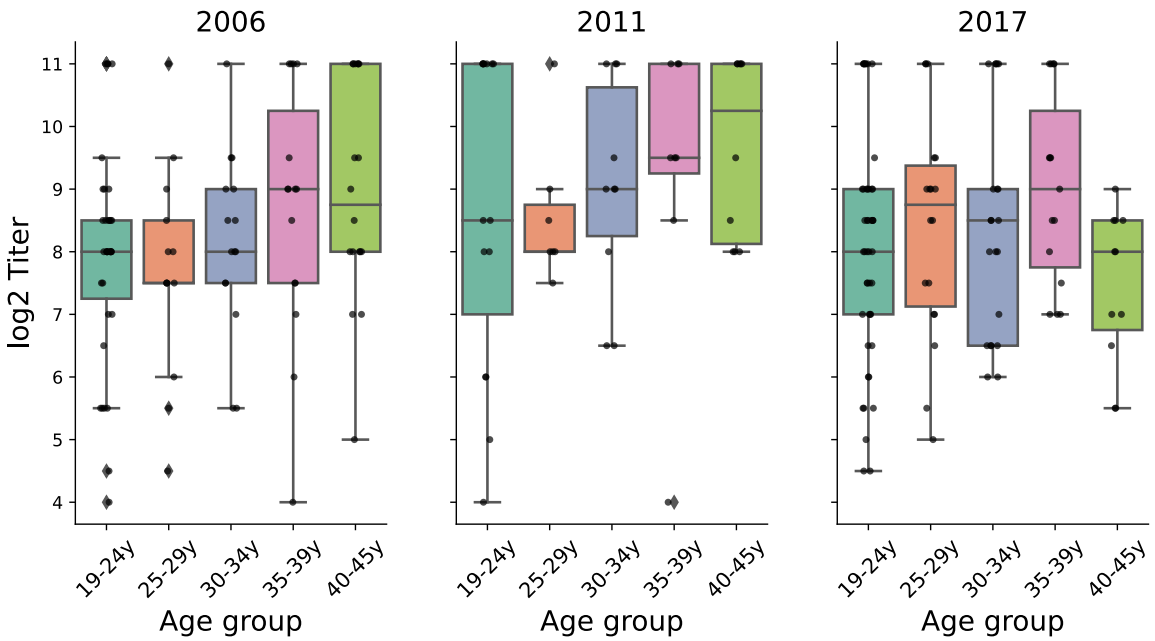


Figure 6 *Distribution of EV-D68 log2 neutralizing antibody titers in seropositive samples from women aged 19-45 years old. (y = years)*

Discussion

This study measured neutralizing antibodies in the UK population against a genotype B3 2016 strain. The overall antibody prevalence in UK was remarkably high in the three sampling timepoints suggesting that EV-D68 circulation has been widespread in recent years. Seroprevalence and GMT increased with age reaching ubiquity in adults, and this has been reported elsewhere including Taiwan (increase from 43% in 2-year-olds to nearly 100% in individuals 12 years of age and older) [219]; China (seropositivity decreased to 10% at age 1 before increasing to 92% in 11-15-year-olds) [220-223]; USA (increase from 60% in 2-5-year-olds to 99% in >50-year-olds) [224, 225]; in Malaysia (seroprevalence was 32% in children versus 89% in adults) [212]; and the Netherlands (GMT increased from 20 in 1-10-year-olds to 530 in >41-year-olds) [226].

A study of sera collected from pregnant Finnish women in 1983, 1993, and 2002 had similarly showed 100% seroprevalence in adults [227]. Seroprevalence and GMT increase with age indicates that children are highly susceptible to EV-D68 infection and transmission rate is high in younger age classes (1-10 years). Seroprevalence in UK also increased over time, i.e., higher in 2011 compared to 2006 and higher in 2017 compared to 2011 and 2006, in comparable age groups. This resembles other cross-sectional studies e.g., in Taiwan (increase from 2006 to 2008) [228]; the Netherlands (increase from 2006/7 to 2015/16) [226]; China (from 2004 to 2011, and 2012 to 2017) [220, 221]; and in Japan from 2014 to 2016 [229].

In China, seropositivity had increased from ~50% in 2004 to ~90% in 2009 in children <5 years old [220], analogous to the 66% to 92% increase in UK. Children in the UK born after 2014 (6-month to 3-year-olds sampled in 2017) had higher titers than comparably aged individuals in 2011 and 2006, implying higher community circulation of EV-D68

viruses in recent years. Similar observations in seroprevalence after the 2014 outbreak were made in USA, where higher antibody titers were found in samples collected in 2017 compared to 2012 [225]. The increase in GMT in 2017 compared to 2006 and 2011 in UK is analogous to data from China that showed higher GMT in 2017 compared to 2012 [221]. Increases in seroprevalence over time in multiple countries is concomitant with change or emergence of novel EV-D68 subclades.

Global dispersion and evolution of EV-D68 has been characterized by rapid mixing among countries, rapid evolution of surface proteins, and divergent and bimodal age distributions of different subclades [230]. It was estimated that EV-D68 undergoes waves of diversification about 1 year prior to outbreaks, and emergence and expansion of EV-D68 subclades has been attributed to apparent increase in infections and changes in clinical disease [230, 231].

Continuous antigenic evolution of EV-D68 subclades was identified in immunoreactive epitopes or antibody binding sites in the C-terminus of VP1 and VP2 [230]. Different subclades differ in the putative neutralizing epitopes also shown to have elevated amino acid substitution rates and under positive selection [129]. Substantial differences in neutralization of strains from various subclades have been documented and changes in the dominant circulating subclade, genetic and antigenic evolution, all together could result in imperfect immunity and subsequently frequent re-exposure and antibody boosting, hence the increasing seroprevalence over time [224]. Model-based analysis of the EV-D68 seroprevalence data from UK suggests an increase in virus transmission mostly driven by an increase in infections in children aged 12-23 months old [232]. This increase in transmission is consistent with reported clinical data showing higher and increasing numbers of EV-D68-associated respiratory illness in UK in the last decade [205, 206, 208].

The correlates of immunity to EV-D68 are poorly understood, and although infection appears to elicit long-lasting strain-specific immunity as evidenced by the high prevalence of neutralizing antibodies, outbreaks still occur [166]. Neutralizing antibodies against outbreak strains were already present years earlier in multiple populations before the outbreaks, including UK, USA, China, and in majority of individuals older than two years [166]. If EV-D68 is the main cause of recent increases in AFM, the occurrence of disease in populations with apparently high prevalence of antibodies raises doubts whether serum antibodies are protective against infection [130]. Either the current serologic assays are flawed, or children with AFM represent a small percentage of immunologically naïve population [130], or increased force of infection resulted in the higher seroprevalence observed.

A recent publication determined a difference in 1:64 antibody titer between acute and convalescent sera, implying that the protective levels of anti-EV-D68 maybe around 1:64 [221]. At the antibody titer of ≥1:64, the proportion of seropositive children aged 1-5 years (the age group mostly at risk of disease) in the UK samples was 0.29 (95%CI 0.19–0.40) in 2006, 0.49 (0.41-57) in 2006 and 0.51 (0.40–0.62) in 2017, suggesting low protection from disease or potential outbreak.

Nevertheless, human neutralizing antibodies were recently shown to protect mice against respiratory infection and neurologic disease [211] and IVIG was also shown to reduce paralysis in infected mice and decrease spinal cord viral loads [233]. Commercially available IVIG preparations have been shown to contain high neutralizing antibody titers to different EV-D68 strains [234], signifying that neutralizing antibodies are a mechanistic correlate of protection against AFM and are candidates for clinical use. Still, there is

insufficient data on the relationship between serostatus and risk for EV-D68 (re-)infection. Further studies are critical for understanding immune correlates of protection.

This study, and the studies that follow in the next sections, focused on serum antibodies. However, it is known that mucosal immunity and secretory antibodies play a significant role in regulating infection outcome since (entero)virus interactions with the host are often primarily at the respiratory and oral mucosa. For deployment of immune cells and the production of immunoglobulins, mucosal immunity is by far the largest component of the entire immune system and provides protection at the main sites of infectious threat. Determining antibody responses of the mucosal immunity can be expected to provide valuable information distinct from, and complementary to, the determination of serum antibody responses.

In conclusion, EV-D68 circulates widely in UK as to elicit neutralizing antibodies in nearly all individuals older than five years, and this ubiquitous spread predated recently recognized virus outbreaks.

2.4 Enterovirus A71 seroepidemiology

Introduction

Enterovirus A71 (EV-A71) was first described in 1969, although it was shown to have been circulating in the Netherlands in 1963. It was later associated with severe neurological disease in the 1970s [235, 236]. EV-A71 is transmitted predominantly via the oral-fecal route, but also through contact with virus-contaminated oral secretions, vesicular fluid, surfaces, and respiratory droplets [237]. EV-A71 uses human scavenger receptor class B, member 2 (hSCARB2) as the major receptor. Other 'attachment receptors' such as P-selectin glycoprotein ligand-1 (PSGL-1), annexin II (Anx2), vimentin, nucleolin, heparan sulfate (HS) proteoglycan, sialylated glycan, fibronectin, and prohibitin support viral attachment to the cell surface [238].

EV-A71 viruses are classified into 7 genogroups: A–G. Genogroup A includes the EV-A71 prototype strain BrCr isolated in California in 1969 and no other circulating strains have since been observed in this genogroup. Genogroups B and C are the most common and are differentiated into subgenogroups B0–B5 and C1–C5 [239-242]. Subgenogroups B4, B5, and C4 are mainly restricted to countries in South-East Asia, whereas C1 and C2 circulate in Europe and the Asia-Pacific region. Genogroup D and the newly proposed genogroup G appear to be indigenous to India, whereas E and F were recently discovered in Africa and Madagascar, respectively [239-242]. Genogroup D isolates share a recent common ancestor with the members of genogroup E, while the isolates of genogroup F evolved from a recent common ancestor shared with the members of the genogroup B. More recently, genetically distinct EV-A71 strains were identified from Pakistan via environmental sampling and proposed as genogroup H [243].

EV-A71 became endemic in the Asia-Pacific countries since early 2000s, resulting in unprecedented increase in infections attributed to the circulation of genogroups B and C [244]. Globally, the shift from genogroup B to C occurred simultaneously where the B viruses were predominant in the 1970s and 1980s, and genogroup C emerged in the late 1980s and replaced B in Asia and Europe. Genogroup switching over time has been documented during HFMD outbreaks in Vietnam, Malaysia, Taiwan, and Japan, and the Netherlands [235, 245-247]. Subgenogroup C3 is restricted to Korea and other genotypes were reported to have attained long-term geographic endemicity including C4 in China, C5 in Vietnam, and B5 in Taiwan, Vietnam and Malaysia [248, 249]. C5 emerged in Vietnam in 2012, became dominant in the following year and was involved in HFMD outbreaks in Vietnam in 2013 and 2017 [249]. Subgenogroups B2 and C2 were detected in AFP cases in Brazil between 2005 and 2017 [250].

In Europe, sporadic introductions of B4, B5 and C4 were observed, and multiple events of virus spread involving subgenogroups C1 and C2 have occurred since the mid-1990s, with two waves of C2 infections also occurring in 2010 and 2013 [251]. In 2007, 2010, and 2013, European countries experienced multiple virus introductions of C1 and C2 within the same year, and the spread of C2 strains in 2007 occurred when C1 transmission rates were low [251]. Frequent importation and exportation events were reported to fuel trans-continental transmission and epidemiological patterns of EV-A71 lineages [248, 251, 252], and contemporary outbreaks are caused by different lineages and less likely by sequential transmission of a single lineage between countries [245].

Most EV-A71 infections occur as uncomplicated hand, foot and mouth disease (HFMD), and disease manifestations range from gastroenteritis and respiratory infections to severe neurological disease, particularly in young children [253, 254]. HFMD is

characterized by 3-4 days of fever, development of a rash in the mouth, hands, feet and buttocks [253]. EV-A71 has also been linked to herpangina, characterized by an abrupt onset of fever and sore throat and can cause aseptic meningitis and brainstem encephalitis [253]. Neurological complications are frequently accompanied by severe cardiopulmonary symptoms attributed to pulmonary edema [253]. Affected children typically present with a short febrile illness accompanied by subtle neurological signs and tachycardia, which rapidly develop into acute cardiac impairment and often fatal, pulmonary edema or hemorrhage [253]. Other features include non-specific rashes, exacerbation of asthma, bronchiolitis, and pneumonia, and young children were mostly affected and often at a higher risk of developing severe complications [254].

A wide range of experimental EV-A71 vaccine approaches have been studied including formaldehyde-inactivated virus, EV-A71 virus-like particles (VLP), VP1 recombinant protein, VP1 DNA vaccine and passive immunization with sera from infected individuals. Formalin-inactivated EV-A71 virus was found to be more immunogenic and may offer cross-protection between genogroups [255-258]. There are currently three inactivated, whole EV-A71 vaccines licensed and commercially available in China [259].

Vaccine trials have shown high efficacy, safety, and sustained immunogenicity [260-262]. One trial showed 94.8% efficacy against EV-A71-associated HFMD or herpangina, and 100% efficacy against EV-A71-associated hospitalization and neurologic complications [263]. Vaccine effectiveness in HFMD patients was 100% for severe cases and ~80% for mild cases [264]. No data on vaccine uptake are available yet. Vaccine efficacy was shown by observation of significant decline in EV-A71-related HFMD four months into the postvaccine period and 52% fewer severe HFMD cases than predicted [265].

In EV-A71 epidemics, acute neurological disease occurring concurrently with HFMD varies widely, signifying variability in pathogenicity of EV-A71 strains. It has been suggested that dermatotropism and neurotropism characteristics of EV-A71 infection are not rigidly linked [254]. Early EV-A71 epidemics in 1970s and 1980s in the Asia-Pacific region (Hong Kong, Singapore, China and Australia) were largely associated with HFMD and a low prevalence of acute neurological disease [254]. An epidemic in Japan in 1978 resulted in a large number of HFMD cases in addition to aseptic meningitis, acute cerebellar ataxia and AFP [266]. In the late 1990s, several large outbreaks and high-level epidemic activity of EV-A71 were reported in Malaysia, Taiwan, Australia and Singapore, and these were associated with HFMD and herpangina, with a small proportion having neurological complications and rapidly fatal neurogenic pulmonary oedema associated with severe brainstem encephalitis [254].

Previous reports in Asia associated neurological disease with genotype replacement, or circulation of specific genotypes which has not been reported in Europe. Certain EV-A71 genotypes e.g., B3, B4, B5, seem to be geographically restricted to Asia, and it's believed there is clinical and epidemiological evidence for different biological behavior of EV-A71 genotypes regarding the risk of CNS disease [246]. For instance, B5 infections in Malaysia were associated with a greater incidence of CNS disease suggesting that genotypes do indeed vary in their propensity to cause neurological disease [244].

In the European region, the first reports of EV-A71 epidemics came from Bulgaria in 1975 and Hungary in 1978, where cases of aseptic meningitis, encephalitis and AFP were mostly reported with only four HFMD cases being documented in Hungary [267, 268]. In Brazil in 1988-1990, EV-A71 was reported to cause significant acute neurological disease [269], and in Canada in 1998, EV-A71 was associated with undifferentiated febrile illness,

HFMD and mild respiratory infections, in addition to acute neurological disease [254]. EV-A71 genogroups B4 and C2 were identified in nearly 200,000 HFMD cases in Taiwan between 1999 and 2003 [270], and in 2008 in Singapore, genogroup B5 was predominantly detected in nearly 30,000 HFMD cases [271]. In China, EV-A71 was detected in 93% of 13.7 million HFMD cases reported between 2008 and 2015 [272], and in Vietnam, severe and widespread epidemics were observed following the introduction of genogroups C4 in 2011 and B5 in 2012 resulting in >300000 HFMD cases and 225 fatalities during 2011–2013 [273]. Re-emergence of genogroup C4 in Vietnam in 2018 was associated with over 53,000 HFMD hospitalizations [274], and in Japan, EV-A71 was detected in up to 82% HFMD cases and 10% aseptic meningitis cases from 2012 to 2018 [275].

Clinical infections in recent years differ from earlier epidemics. Subgenogroups C4 and B5 viruses caused a HFMD outbreak with a high fatality rate (92%) among children of age <3 years in Cambodia between 2012 and 2013. In this outbreak, many severe cases experiencing cardiopulmonary failure did not present the typical rash usually observed in classic HFMD [276]. In China in 2012 and 2013, patients with EV-A71 infection were significantly older, had a longer mean hospital stay and higher incidence of neurologic complications than patients with CVA6, CVA10 or CVA16 [277]. EV-A71-infected patients also had a much higher incidence of fever, and neck rigidity and more likely to have abnormal numbers of white blood cells in CSF [277].

In the EU and the European Economic Area (EEA) region, between 2015 and 2017, EV-A71 infections of sub-genogroups C1, C2 and C4 were reported mostly in Spain (248/738) and Germany (151/738). 45% of infections were in children aged 1-5 years; 65% had neurological symptoms and 10% presented with HFMD [206]. In Africa, EV-A71

genogroups C2 and E were detected in children aged 1-3 years with acute flaccid paralysis in several countries between 2013 and 2016 [278-280].

No genogroup has been conclusively associated with an increased risk of acute neurological disease, although genotype replacement particularly with C4, was associated with explosive EV-A71 outbreaks and severe illness in China and Vietnam. A switch from C5 to C4 in 2011 coincided with another explosive outbreak in Vietnam, resulting in > 200,000 hospitalizations and > 200 fatal cases between 2011 and 2012 [281]. In contrast, the replacement of C4 by B5 in 2013 in Vietnam coincided with a decrease in the numbers of reported cases and the proportion of cases with severe illness in subsequent years [246]. The re-emergence of C4 after an absence of 6 years was linked to severe HFMD in Vietnam in 2018 [274, 282], an in the Netherlands, a shift from genogroup B to C coincided with a change in clinical presentation and the average age at infection [235].

EV-A71 circulation in UK

There is limited data on the epidemiology and molecular evolution of EV-A71 within the UK. The first molecular epidemiological study reported 32 isolates of genogroup C collected over a period of 8 years (1998-2006) and associated with mild illness, including HFMD, neurological symptoms, and one fatality [283]. EV-A71 viremia was also reported in blood donors in Scotland [284] and 102 EV-A71 strains were collected in local hospital laboratories in England and Wales between 2004 and 2011 [204]. Between January 2015 and December 2017, 132 EV-A71 cases including six AFM cases were reported to have occurred in the UK [206].

Study objective

EV-A71 infections remains uncommon in the UK. The clinical prominence and association with neurological disease in recent global outbreaks and epidemics may have resulted from an increased incidence in previously unexposed and susceptible populations. To investigate this, age stratified seroprevalence of EV-A71 were determined in representative cross-sections of the UK population in 2006, 2011 and 2017.

Enterovirus A71 seroprevalence in England, UK, 2006-2017

An antibody endpoint titer of ≥3log2 (≥1:8 dilution) was considered seropositive for EV-A71 neutralizing antibodies as used in previous EV-A71 seroprevalence studies [285]. The overall seropositivity rate of EV-A71 was 74% (95% confidence interval [CI] 71.8-76.2) and seroprevalence in the three cross-sectional timepoints was comparable: 71% (95% CI 66.8-75.0) in 2006, 73% (69.1-77.0) in 2011 and 77% (73.8-80.9) in 2017. Age-specific seroprevalence of EV-A71 neutralizing antibody titers in each timepoint was lowest in children aged 6–11 months and gradually increased with age (Table 5, Figure 7). There was a linear increase in the seropositivity (chi-squared test for trend, $p < 0.001$) with increasing age category. The seropositivity rate for the >40y age group increased from 77.1% in 2011 to 91.3% in 2017 (Fisher exact test, $p = 0.003$).

Table 5 Summary of EV-A71 seroprevalence in England, UK, from 2006 to 2017.

Age group	2006		2011		2017	
	No.	**Seroprevalence (95% CI)**	**No.**	**Seroprevalence (95% CI)**	**No.**	**Seroprevalence (95% CI)**
<6mo	8/12	66.7% (35.4-88.7)	55/74	74.3% (63.3–82.9)	28/44	63.6% (47.7–77.2)
6-11mo	2/6	33.3% (10–70)	11/25	44% (26.7–62.9)	4/22	18.2% (7.3–38.5)
1-5y	41/92	44.6% (34.3–55.3)	24/46	52.2% (38.1–65.9)	50/82	61% (49.6–71.4)
6-10y	26/42	61.9% (45.7–76)	34/69	69.4% (55.5–80.5)	34/51	66.7% (52–78.9)
11-20y	82/110	74.6% (65.2–82.2)	62/76	81.6% (71.4–88.7)	84/98	85.7% (76.8–91.7)
21-40y	96/120	80% (71.5–86.5)	61/75	81.3% (71.1–88.5)	130/149	87.3% (80.6–92)
>40y	110/132	83.3% (75.6–89)	118/153	77.1% (69.9–83.1)	105/115	91.3% (84.2–95.5)
Total	365/514	71% (66.8-75)	365/498	73% (69.1-77)	435/561	77% (73.8-80.9)

(mo: months, y: years)

Table 6 Geometric mean titers of EV-A71 seropositive samples. (mo: months, y: years)

Age group		2006		2011		2017
	No.	GMT (95% CI)	No.	GMT (95% CI)	No.	GMT (95% CI)
<6mo	12	112.4 (37.6-335.8)	74	22.4 (17.9-28.1)	44	29.7 (17.9-49.2)
6-11mo	6	45.5 (19-67.6)	25	33 (9.8-110.4)	22	12.3 (5.4-28.2)
1-5y	92	90.5 (44.2-185.1)	46	313.3 (180-545.4)	82	57.3 (33.6-97.6)
6-10y	42	113.5 (56.3-229)	69	73.8 (41.8-130.1)	51	172 (94.8-312)
11-20y	110	151.5 (98.7-232.8)	76	67.3 (47.9-94.4)	98	64.8 (42.3-99.1)
21-40y	120	42.1 (31.6-56)	75	51.3 (37.4-70.2)	149	55.2 (41.5-73.5)
>40y	132	29.3 (22.8-37.5)	118	36.4 (29.5-44.9)	115	31.2 (23.8-41)

The proportion of samples with moderate (1:64 to 1:256) and high (≥1:512) neutralizing antibody titers increased with age from 1 to 20 years but decreased thereafter, with most samples from adults >20y having titers ≥1:256 (>85%). For example, in 2006, the proportion of individuals with high titers decreased from 30% in the 11-20y age group to 6.7% in the 21-40y group and to 3.8% in adults older than 40 years. A similar trend of declining titers was observed in 2011, where the proportion with high titers dropped from 12% (11-20-year-olds) to 9.3% (21-40-year-olds) to 2% (>40-year-olds); and in 2017, from 19.3% (11-20-year-olds) to 11.4% (21-40-year-olds) and to 5.2% (>40-year-olds).

Infants (<6 months old) who were seropositive in 2006 had a 5-fold higher geometric mean titer than the same age group in 2017, while the seropositive children aged 6-11 months old in 2006 had a geometric mean titer that was 3.6-fold higher than the same age group in 2017. Similarly, children aged 1-5 years who were seropositive in 2011 had a 5.5-fold higher GMT than a comparable age group in 2017 (Table 6). EV-A71 titers were mostly right-skewed in 2017 and the median titers were greater in 2006 and 2011 for 6-10y and 1-5y age groups, respectively. Significant differences in titers of seropositive samples were found among children of age <6 months and 1-5 years old (Kruskal-Wallis test, p = 0.014

and p = 0.0026, respectively), and also among individuals aged 11-20 years old (p = 0.0067) (Figure 8).

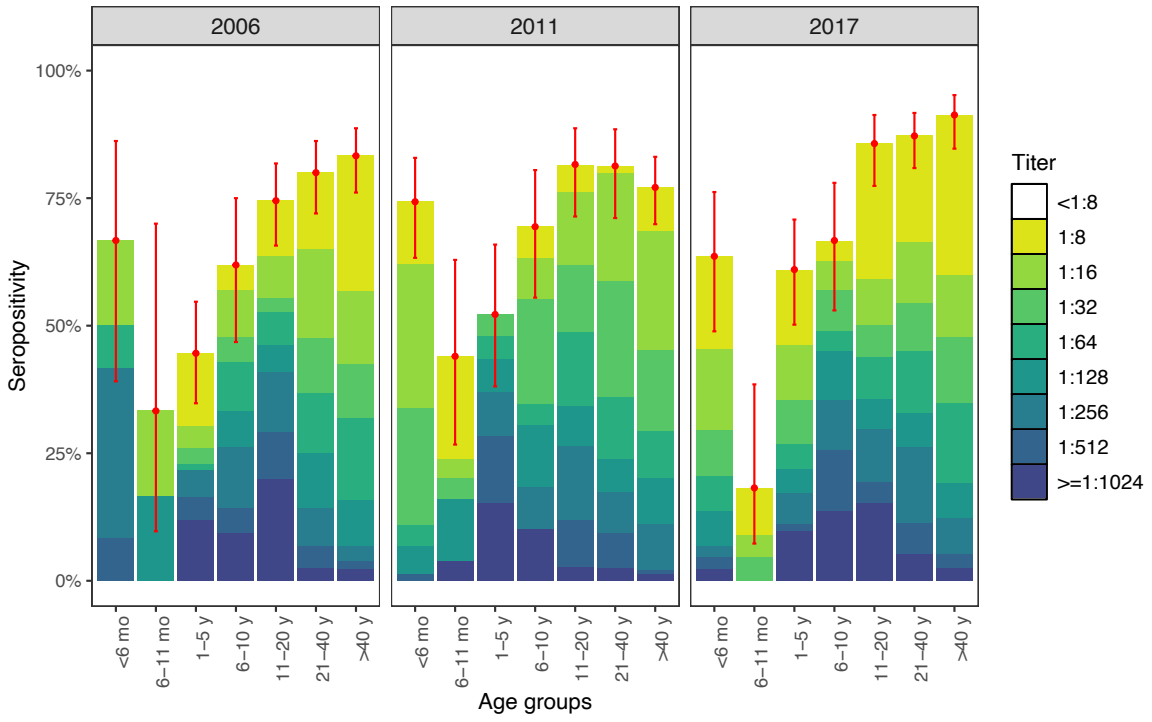


Figure 7 *Seroprevalence of EV-A71 by different age categories. Results are expressed as percentage of samples displaying neutralizing antibody titers <1:8 to ≥1:1024 (colored bars). Point estimates of the percentage seropositive are shown in red and error bars indicate 95% confidence intervals of the proportions. Samples were scored seropositive at endpoint serum dilution of ≥1:8.*

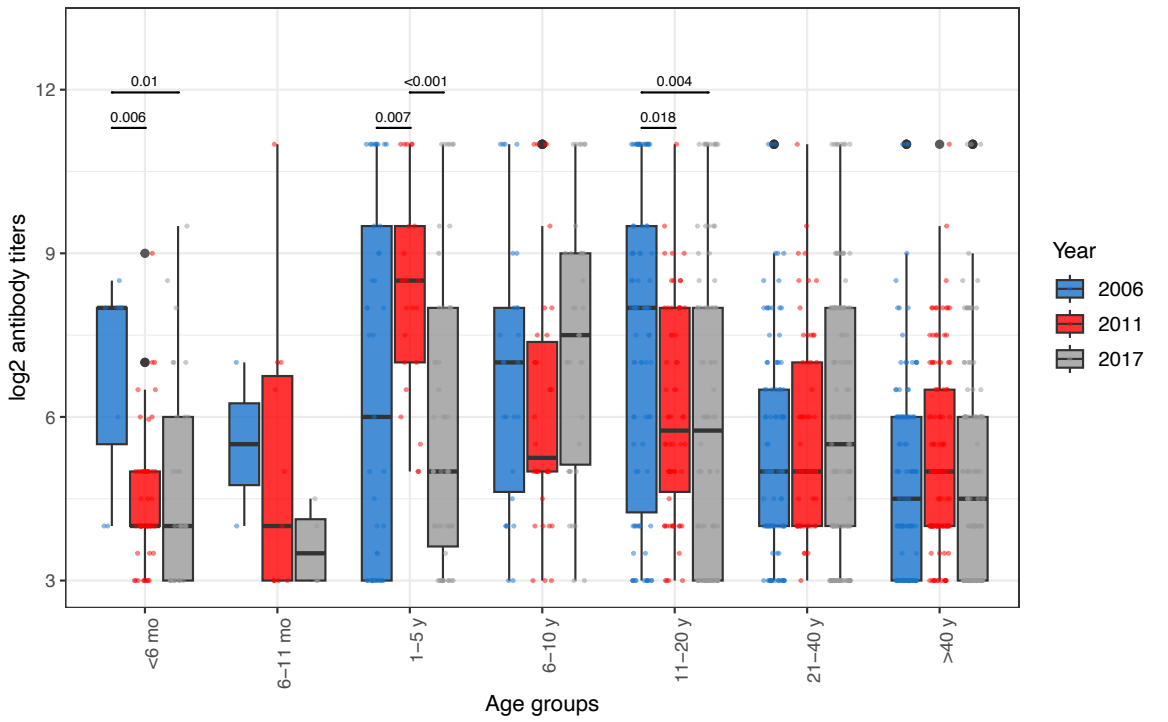


Figure 8 *Boxplots showing the distribution of EV-A71 log2 neutralizing antibody titers of seropositive samples by age group and year of sampling. (mo = months, y = years). The horizontal lines and numbers on top of the boxplots indicate statistical significance level and are results of Kruskal-Wallis tests for antibody titer comparisons.*

For EV-A71, 225/260 (86%) of women in the age group 19-45y were seropositive (had antibody titer ≥1:8) although majority had moderate levels of neutralizing antibodies titers between 1:32 and 1:256 and only a few (27%) had high levels (≥1:512) (Figure 9). The mean titers in this age group were higher in 2011 compared to 2017 and 2006: 214.2, 197.9, and 102.1, respectively.

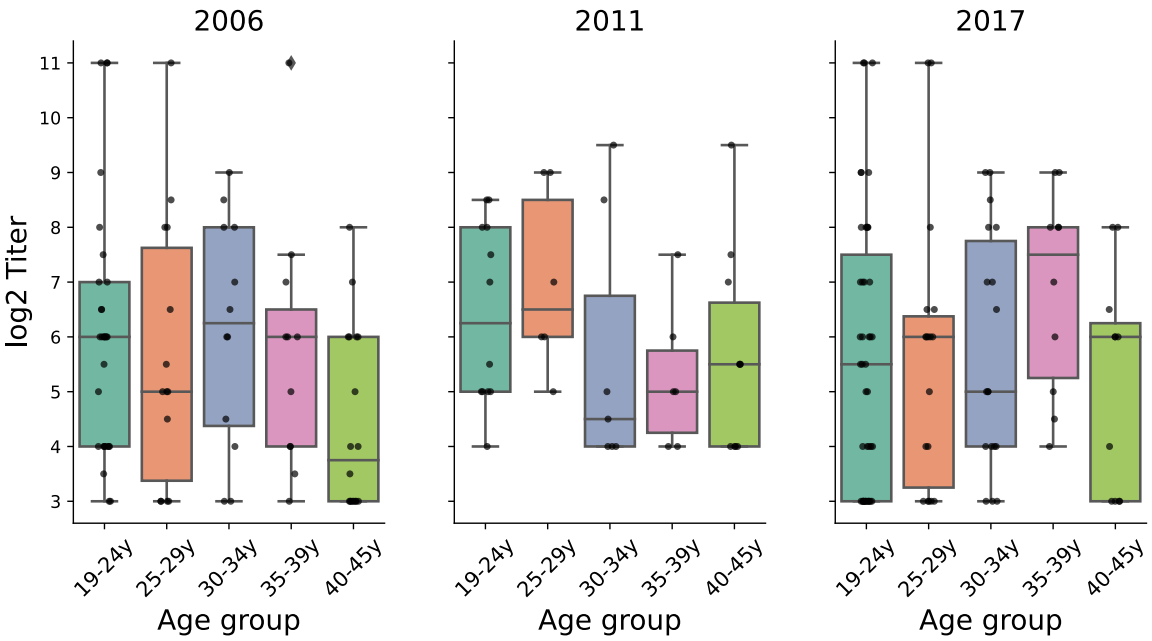


Figure 9 *Distribution of EV-A71 log2 neutralizing antibody titers in seropositive samples from women in the 19-45y age group during the three timepoints. (y = years)*

Discussion

This study measured anti-EV-A71 neutralizing antibodies in serum of individuals across England, UK. Children aged 6-11-month had the lowest EV-A71 seroprevalence indicating clearance of maternally conferred immunity**.** Antibody detection frequencies and titers increased steadily with age indicative of continuing exposure and infection throughout childhood. The EV-A71 seropositivity rate observed in England among pre-school children 1-5 years was 52% for all the three cross-sections combined and higher than seroprevalence observed in a comparable age group in Germany in 1997-1998 (48.4%) [286]. This was also consistent with other studies and within the seroprevalence rates reported in the Netherlands from 2010 to 2014 (range, 50%-83%) [287] and in China [288]. This observation suggests high prevalence of infections in this age group. For the 6-10-year-olds, the seroprevalence in the UK was higher (range, 62%-70%) than that observed in Germany in two separate studies (49% and 56.4%) [286, 289]. Seroprevalence estimates in adults >21 years old were similar to those reported in the Netherlands (75%-84%) and Germany (>75%) [286, 287].

The number of individuals with high antibody titers declined with age (Figure 7), similar to the EV-A71 seroprevalence reported in 2006 in Germany [289], denoting reduced exposure and/or waning immunity with age. This also suggests that reinfection with the same strain is uncommon (homologous immunity) and that the circulating EV-A71 strains or lineages have limited antigenic diversity. Of the several existing EV-A71 subgenogroups, C1 and C2 are the most commonly circulating in Europe and presumably or by extension, in the UK [251]. It might be that antigenic similarity (thus neutralizing antibody cross-reactivity) is high within the C1 and C2 circulating strains. Evolution of EV-A71 lineages with novel antigenic properties while assuming antigen persistence and probability of infection

independent of age [290], would result in continual exposure/infection and enhanced antibody titers which is not seen here. Instead, we observed declining antibody titers with age suggesting limited genetic and antigenic diversity in the circulating strains or age-dependent transmission.

High mean titers were mostly present in the 10-20-year-olds, akin to observations made for the 10-14-year-olds in the Germany study [289], an additional indication that EV-A71 primarily circulates in and infects children. The subsequent decline in titers but not frequencies of seropositivity in the older population denotes that re-exposure is uncommon and could also reflect the differences between acute serological responses post-infection in the younger population and the homeostatic antibody levels in the older population that become established years after infection [291].

Related to this, the >4-fold attrition in mean neutralizing antibody titers in the 21–40-year age group may furthermore create the low mean titers of maternally-derived antibodies observed in infants <6 months of age, particularly in 2011 and 2017 (Table 6). This may underpin the high incidence of EV-A71 diagnosis reported in England in the 0–3-month infants [292], when they are most susceptible to severe infection outcomes. A previous study suggested that if mothers show higher anti-EV71 antibodies, the high titers transferred vertically and were detectable in infants [288]. Immunity to EV-A71 infection is mainly dependent on humoral factors, i.e., neutralizing serum antibodies, although enterovirus infections also induce T-cell immunity [293].

It is not clear whether low or no detectable neutralizing antibodies increases susceptibility to infection. Even with lower antibody titers, the elderly population seems protected since the incidence in this age group is almost nil. Neutralizing antibody titer of 1:32 has been suggested as an immune correlate of protection [260, 294]. In the UK sample

set (excluding children <1 year old), 56% in 2006, 68% in 2011 and up to 57% in 2017 had antibody titers of ≥1:32 (Figure 7), indicating moderate levels of protection against EV-A71-associated disease. A large proportion of children aged 6-11 months old in 2011 and 2017 had low antibody levels and would be at high risk of disease: only 23% and 6% had titers ≥1:32, respectively (Figure 7). Therefore, in addition to natural boosting, vaccine induced protection could be more beneficial to these groups.

2.5 Coxsackievirus A6 seroepidemiology

Introduction

Coxsackievirus A6 (CVA6) is a member of the species *Enterovirus A* and has recently emerged as a major cause of HFMD, gradually replacing EV-A71 and CVA16 as the predominant pathogen in HFMD epidemics [295-297]. CVA6 is considered as pathogenic as EV-A71 and CVA16 and can cause aseptic meningitis and brainstem encephalitis [298]. Based on the VP1 gene, CVA6 is segregated into four genotypes, A, B, C, and D, and genotype D is further divided into the five subtypes D1-D5 [299]. Genotypes E1 and E2 were recently identified in China [300, 301]. Genotype A is composed of the prototype strain and genotypes B and C are made of a few sporadic isolates [302, 303]. Transmission of subtype D3 was associated with CVA6-related HFMD outbreaks worldwide. Subtypes D2 and D3 mainly circulate in mainland China and detection of D2 declined from 2013 [302].

Before 2008, CVA6 infections globally were seldomly reported and commonly associated with HFMD, sporadic meningitis cases and herpangina [124, 304, 305]. For instance, only four CVA6 detections were reported in Finland in eight years (2000-2007) [306], and 13 detections were reported in healthy children in Norway from 2001 to 2003 [307]. CVA6 was identified among the major causes of herpangina and HFMD in Taiwan between 2004 and 2009 [308], and in a large HFMD outbreak in Singapore in 2008 [271]. In Finland, in 2008, emergence of new genetic variants of CVA6 became associated with a nationwide HFMD outbreak with atypical clinical symptoms of onychomadesis (nail shedding) [108, 305]. Cases of onychomadesis after onset of HFMD were also reported in 2007-2008 in Spain, Greece, China and Japan [309-312]. An outbreak of CVA6 HFMD associated with onychomadesis occurred in Taiwan in 2010, and the patients presented

with more widespread skin lesions and profound tissue destruction. The CVA6 strains circulating in Taiwan in 2010 differed from those circulating before 2010 in clinical manifestations and in the VP1 genetic sequences [313].

Since 2008, CVA6 outbreaks and CVA6-associated HFMD epidemics have been reported widely [314-317]. CVA6 was frequently detected in a HFMD/herpangina outbreak in France in 2010 [318], and it was the predominant pathogen in a large HFMD outbreak in Thailand in 2012 [319], and in 23% of HFMD patients in Vietnam during 2011-2015 [105]. Emergence of CVA6 in Singapore in 2010 as the predominant circulating enterovirus was associated with a change in the annual pattern of HFMD epidemics from bimodal to unimodal [285]. In the USA in 2011-2012, CVA6 was detected in 75% of HFMD patients [320], and between 2010 and 2012, several outbreaks and sporadic cases of HFMD occurred in different regions of Spain where CVA6 was the most frequently detected enterovirus [321, 322]. Phylogenetic analysis of CVA6 strains associated with HFMD and other mucocutaneous symptoms in Spain during 2006-2020 showed that strains clustered by year suggesting multiple introductions of distinct variants, and that strains from 2010 clustered differently from earlier strains indicating altered epidemiology and pathogenicity post-2008 [323].

Atypical CVA6-associated HFMD

Typical signs of HFMD are low-grade fever, vesicles on the buccal mucosa and tongue, and peripherally distributed cutaneous lesions, usually on the palms and soles and sometimes on the buttocks or genitalia [324]. Formation of bullae and scabs are not typical symptoms of HFMD, while herpangina, is clinically characterized by a vesicular exanthem of the tonsillar fauces and soft palate [324]. Generalized vesicular and bullous lesions

associated with enterovirus infections were very rarely reported before 2008. CVA6-associated atypical HFMD (an enanthem or exanthem characteristic of HFMD with unusual morphology or extent of cutaneous findings) is frequently associated with crusted lesions, an eczema-form rash affecting the arms, trunk, buttocks, and legs, onychomadesis (nail loss), and a greater disease severity than HFMD associated with other EV-A serotypes [325, 326]. Widespread vesiculobullous and erosive lesions, “eczema coxsackium,” petechial/purpuric eruptions, and delayed onychomadesis and palm and sole desquamation, were reported in an outbreak of CVA6-associated HFMD in children. The exanthem had a perioral, extremity, and truncal distribution in addition to involving classic HFMD areas such as palms, soles, and buttocks [326].

Atypical presentation of CVA6-associated HFMD was reported in Boston in 2012, characterized by atypical seasonality (late winter rather than summer and early autumn) and emerging clinical phenotype (perioral lesions and peripheral vesicles on the dorsum rather than palmar/plantar surface of hands and feet) that could be confused with herpes simplex or varicella-zoster virus infections [327]. Atypical seasonality of CVA6-associated HFMD was also reported in China [300].

Atypical HFMD caused by a new CVA6 lineage and characterized by high fever, vesiculobullous eruptions on the calves and backs of the hands, and onychomadesis was reported in an infant aged nine months [324]. CVA6 was recently detected in atypical HFMD vesiculobullous exanthema in ambulatory patients in the Amazon, Brazil [328]. Atypical HFMD can also mimic herpes zoster infection [329]. A cluster of CVA6 infections occurred in January and February 2014 in Edinburgh, UK, and the hospitalized cases presented with a rash diagnosed as eczema herpeticum by dermatologists and initially leading to clinical diagnoses of severe, disseminated herpes simplex virus infections [330].

The erythematous rash involved the face, trunk, and limbs, and was followed by the appearance of vesicles and bullae. Sequence analysis of the VP1 gene showed that the CVA6 strains associated with the outbreak were genetically distinct from other previously characterized CVA6 strains, including those associated with previous outbreaks of aHFMD since 2008 and all earlier isolates [331].

Atypical HFMD is not limited to infants and children and has been reported in adults. In one report, 24% of affected patients were >18 years old [320, 332]. A case of an adult patient with pre-existing eczema presenting with CVA6-associated HFMD and atypical lesions distributed in areas of eczematous skin was reported in Malaysia [333]. In Texas in 2012 to 2013, a wide array of disease presentation of CVA6-associated HFMD in adults was reported including delayed onychomadesis and desquamation of the palms and soles, peri-oral erythematous papules (without lip or buccal involvement), and cutaneous findings presenting at different stages, with variation in color, morphology and distribution [334]. Two adults with widespread cutaneous lesions related to atypical CVA infection were reported in Connecticut, USA [335].

Recombination in CVA6 and clinical disease

Recombination is an important mechanism of genetic evolution in enteroviruses, generating novel strains that may emerge to cause severe disease [6]. CVA6 strains/variants have also been categorized as recombinant forms (RF), RF-A to RF-M, based on genetic divergence in the $3D^{pol}$ non-capsid coding region. Recombination breakpoints were frequently observed between the 2A-2C regions and the 5′UTR region [336]. Temporal reconstruction of the recent evolutionary history of CVA6 showed that RF-A, the most frequently detected RF, was the ancestral form of recombinant forms -E, -F, -

G and -H which have emerged more recently following separate recombination events at various times in the past 5–10 years [331, 336]. RF-F and -H appear to have originated through single recombination events with breakpoints in the 2A/2B region and multiple recombination events could also be inferred for RF-G and -E. A breakpoint was detected in the VP3 and VP1 genes of RF-E variants in Taiwan, likely indicating intra-serotype recombination [331]. Amino acid substitutions from RF–A to –J in the VP1 receptor-binding sites (DE and HI loops) may influence the pathogenicity of RF–J strains, and the 3Dpol sequence variation among RF–A, –J, and –L may explain their dissimilar pathogenic properties [302].

A recombinant CVA6 monophyletic lineage was identified in a HFMD outbreak in China in 2012-2013 and accounted for 22% of infections. Recombination analyses showed that the 2C gene was probably derived from CVA4. Clinical observation showed that the recombinant lineage led to a more generalized disease [337]. RF-J strains were first identified in China in 2013 and likely resulted from two recombination events estimated to occur in 2011 [338]. RF-J, -L, and -M were identified in Hongkong between 2013 to 2015, with the 3D region being a common recombination site [299]. The RF-L variant resulted from CVA6 and CVA4 recombination, while the RF-M was detected in an acute encephalitis case and had resulted from recombination between CVA6 and the EV-A71 C4 genogroup [299]. RF–L was thought likely to have caused severe HFMD in China in 2017 to 2018 [302].

Recombinant forms are characterized by rapid emergence, variable penetrance into the sampled virus population and relatively rapid extinction, within years rather than decades, based on average recombination half-lives [336]. The evolutionary phenomenon of noncapsid polymorphism in the same subtype of CVA6 might explain the rapid spread of CVA6 within a short period of time [302]. Associations between RFs and clinical

presentations in HFMD have been observed [299, 331, 336, 339], suggesting the potential role in pathogenicity, for new variants to emerge and modify disease outcomes. For instance, RF-A was associated with atypical HFMD in Finland in 2008, and then subsequently appeared across Asia and Europe in 2013-2014 [336]. RF-H was associated with the aHFMD cases in Edinburgh in 2014 [331]. RF-A and RF-G showed a strong association with aHFMD that contrasted with presentations of herpangina of RF-B and -E; and RF-H was associated with eczema herpeticum, a presentation unusual or absent for other recombination groups [331].

CVA6 circulation in UK

From 2004 to 2011, 72 CVA6 strains were collected in local hospital laboratories in England and Wales [204], and between January 2015 and December 2017, 613 CVA6 cases were reported in the UK [206]. CVA6 was reported as the predominant enterovirus in a mixed pediatric and adult population with HFMD between January 2014 to December 2016, and some of these CVA6 infections presented with eczema herpeticum-like vesicular or more florid rashes, and others presented with more typical HFMD [340]. However, the lack of active prospective clinical and microbiological surveillance data makes it difficult to establish CVA6 epidemiology in UK.

Study objective

The emergence of CVA6 as major cause of HFMD and with atypical clinical presentation, challenges the current efforts, including vaccines, to reduce HFMD burden and presents a developing public health threat. The emergence of new genetic variants (recombinant

forms) linked to outbreaks emphasizes the need to improve the epidemiology of this emerging pathogen and to characterize CVA6 population immunity are key to elucidate its transmission dynamics.

Coxsackievirus A6 seroprevalence in England, UK, 2006-2017

An antibody endpoint titer of ≥3log2 (≥1:8 dilution) was considered seropositive for CVA6 neutralizing antibodies as used in [285]. The seropositivity for CVA6 was 80% (95% confidence interval [CI] 78.2-82.3) overall; and 82% (78.7-85.3), 78% (74-81.8) and 80% (76.7-83.3) in 2006, 2011 and 2017, respectively. Seroprevalence linearly increased with age (chi-squared test for linear trend, $p < 0.001$) (Figure 10) (Table 7) and the seropositivity rates were comparable among age groups (Fisher Exact test, $p > 0.05$). Significant differences in CVA6 antibody titers were observed among seropositive children of age <6 months old (Kruskal-Wallis test, $p < 0.001$), 1-5 years ($p = 0.005$) and 6-10 years ($p < 0.001$). Neutralizing antibody titers were significantly lower in 2011 for seropositive individuals (titer ≥8) older than 21 years (Figure 11).

Table 7 Summary of CVA6 seroprevalence in England, UK, from 2006 to 2017.

Age group	2006		2011		2017	
	No.	**Seroprevalence (95% CI)**	**No.**	**Seroprevalence (95% CI)**	**No.**	**Seroprevalence (95% CI)**
<6mo	9/12	75% (42.8–93.3)	38/58	65.5% (52.7–76.4)	28/42	66.7% (50.4–80.0)
6-11mo	3/5	60% (17–92.7)	12/22	54.5% (34.7–73.1)	11/22	50% (28.8–71.2)
1-5y	61/87	70.1% (59.2–79.2)	25/39	64.1% (48.4–77.3)	55/82	67.1% (55.7–76.8)
6-10y	31/42	73.8% (57.7–85.6)	28/36	77.8% (61.9–88.3)	39/51	76.5% (62.2–86.8)
11-20y	86/110	78.2% (69.1–85.3)	61/67	91% (82–96)	80/97	82.5% (73.1–89.2)
21-40y	107/120	89.2% (81.9–93.9)	62/74	83.8% (73.8–90.5)	134/149	89.9% (83.7–94.1)
>40y	121/132	91.7% (85.2–95.6)	120/147	81.6% (74.6–87.1)	100/114	97.7% (79.9–92.9)
Total	418/508	82% (78.7-85.3)	344/443	78% (74-81.8)	447/557	80% (76.7-83.3)

(mo: months, y: years)

Table 8 Geometric mean titers of CVA6 seropositive samples. (mo: months, y: years)

Age group	2006		2011		2017	
	No.	GMT (95% CI)	No.	GMT (95% CI)	No.	GMT (95% CI)
<6mo	12	155.2 (86.7-277.7)	58	27.7 (18.9-40.4)	42	30.1 (17.5-51.5)
6-11mo	5	32 (1.6-631.5)	22	55.4 (17.2-178.6)	22	18.7 (10.8-32.3)
1-5y	87	100.8 (61.6-164.9)	39	69.5 (31.4-154.1)	82	228.5 (152-343.7)
6-10y	42	133.8 (79.2-226)	36	52.5 (30.2-91)	51	284.8 (186.3-435.4)
11-20y	110	121.5 (89.3-165.2)	67	63.3 (46.1-86.8)	97	84.1 (58.7-120.3)
21-40y	120	65.7 (49.3-87.3)	74	40.5 (30-54.5)	149	90.5 (70-116.9)
>40y	132	58 (44.4-75.9)	147	28.6 (23.1-35.3)	114	65.1 (49.7-85.3)

The proportion of infants <6 months old with titers ≥64 was significantly higher in 2006 (75%) (Fisher Exact test, $P < 0.001$) compared to 2011 (17%) and 2017 (14%). For adults over 40 years old, the proportion with moderate titer levels was significantly lower in 2011 (27.8%, Fisher Exact test: $P < 0.001$) compared to 2006 (49.2%) and 2017 (51.7%). Geometric mean titers were highest in seropositive children of age 1-10 years in 2017, and more than 5-fold higher in 2006 for the <6-month-olds (Table 8).

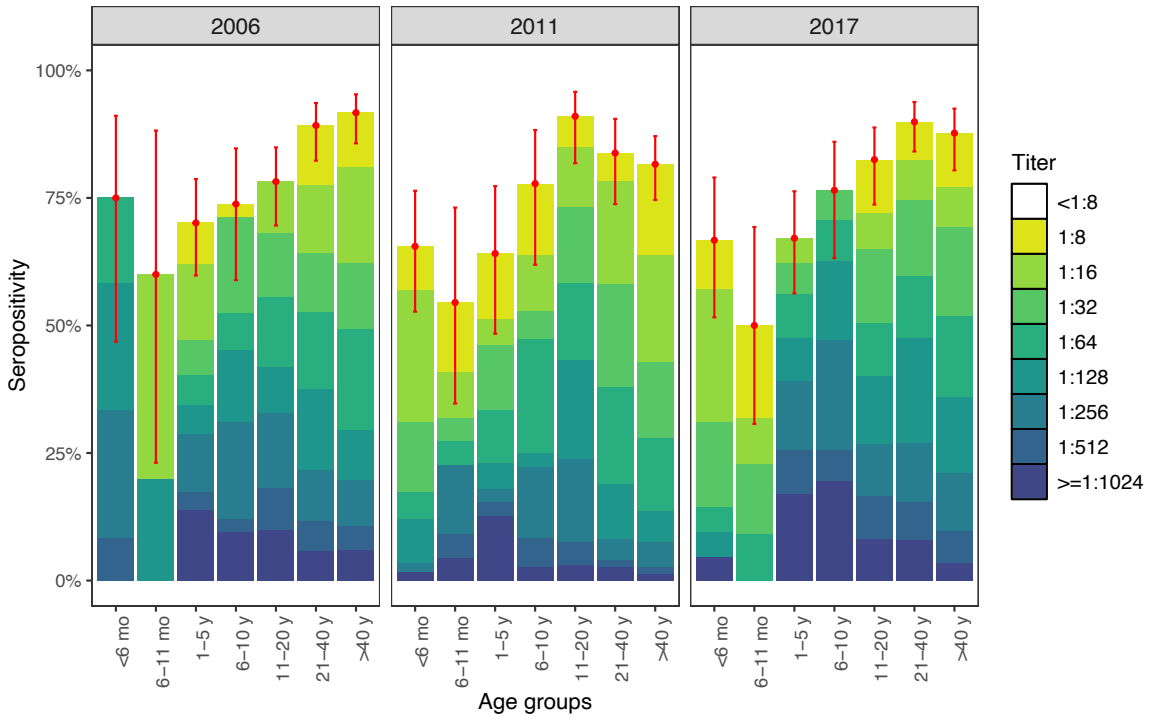


Figure 10 *Seroprevalence of CVA6 by different age categories. Results are expressed as percentage of samples displaying neutralizing antibody titers <8 to ≥1024 (colored bars). Point estimates of the percentage seropositive are shown in red and error bars indicate 95% confidence intervals of the proportions. Samples were scored seropositive if neutralization was achieved at serum dilution of ≥1:8.*

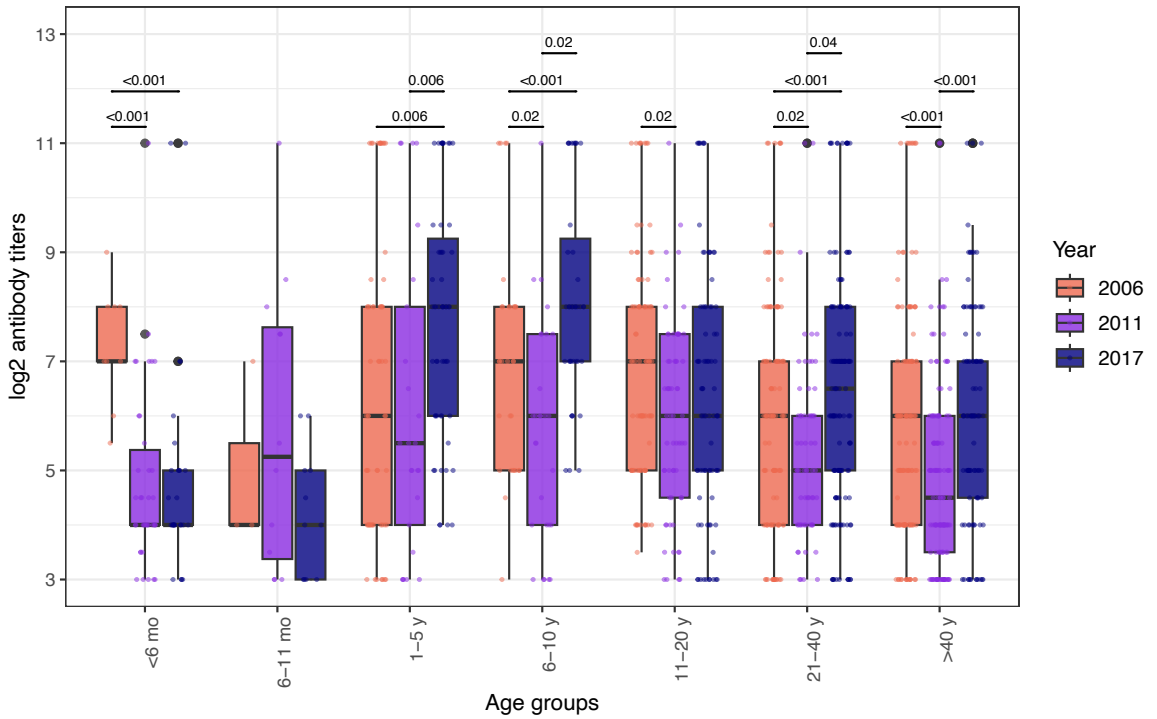


Figure 11 *Boxplots showing the distribution of CVA6 log2 neutralizing antibody titers of seropositive samples by age group and year of sampling. (mo = months, y = years). The horizontal lines and numbers on top of the boxplots indicate statistical significance level and are results of Kruskal-Wallis tests for antibody titer comparisons.*

231/260 (88%) women of age 19-45 years were seropositive for CVA6 (antibody titer ≥1:8) but only 35% had antibody levels ≥1:1024. Geometric mean titers for the 231 individuals

were lower in 2011 compared to 2006 and 2017, i.e., 148.3, 207.1 and 274.6, respectively (Figure 12).

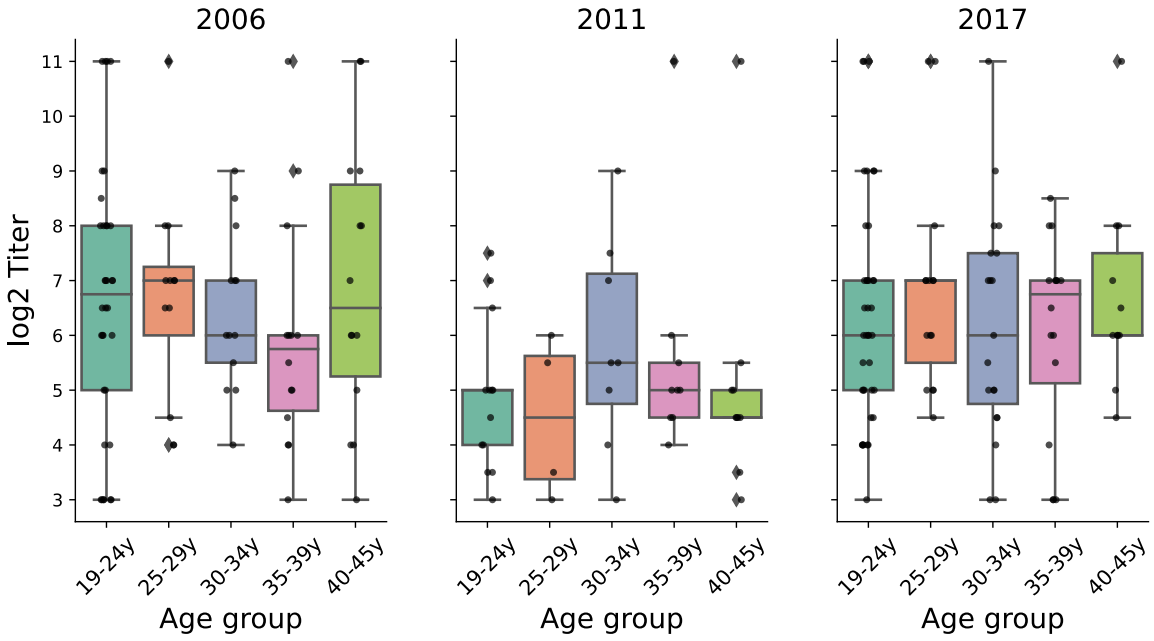


Figure 12 *Distribution of CVA6 log2 neutralizing antibody titers in seropositive samples for the 19-45y age group across the three sampling timepoints. (y = years)*

Discussion

CVA6-associated enterovirus outbreaks are potentially more widespread, severe, and infections vary from classical HFMD and are atypical in clinical presentation. This study measured population levels of anti-CVA6 serum neutralization antibodies in 2006, 2011 and 2017. Like EV-A71, the minimum CVA6 seroprevalence estimates were observed in the 6–11-month age group following clearance of maternal antibodies and the neutralizing antibody titers increased steadily with age indicating ongoing exposure and infection throughout childhood. Similar observations were made in Taiwan, where CVA6 seroprevalence increased from 51.9% in the 1–6-year-olds to 63.6% in the 7–12-year-olds [285]. In all the three sampling timepoints studied here, the proportion of individuals with high antibody levels (≥1:512) decreased with age (Figure 10).

The decrease in antibody levels with age was also seen in Taiwan, from 65% seroprevalence in children of 1-6 years to 30% in adolescents 13–17 years) [285]. The GMTs in the UK samples were higher among children of age 1–10 years and declined with age (Table 8). A similar declining trend was seen in Taiwan substantiating that children are most susceptible to infections and infrequent exposure occurs in adults. The lack of seroepidemiology studies on CVA6 limits comparison of our data with other settings. One other seroepidemiology study found increasing seropositivity from 24.4% in infants 6-11 months to 47% in children 1-3 years old [341].

CVA6 seroprevalence in younger children of age 1-10 years was higher than EV-A71 seroprevalence in the same age group. The seroprevalence data presented here show that CVA6 circulated widely before the emergence of atypical HFMD in 2008, with seroprevalence approaching 90% in adults over 40 years of age in 2006. This discounts the idea that the increased incidence of CVA6-associated HFMD simply reflects a change in

incidence and the existence of a widely susceptible population. The global emergence of CVA6 since 2008 was instead linked to an increase in pathogenicity of CVA6 around 2010 [34]. This study shows high overall prevalence (80%) of CVA6 infections among the UK population.

2.6 Combined analysis of EV-D68, EV-A71 and CVA6 seroprevalence

Serology data for EV-D68, EV-A71 and CVA6 were combined to assess antibody specificity. Overall, 1507 samples had been tested for all three viruses. At the antibody titer cutoffs of ≥1:16, ≥1:8 and ≥1:8 for EV-D68, EV-A71 and CVA6, respectively, 921/1507 (61%) samples were seropositive for the three enteroviruses and 23/1507 (0.015%) were seronegative for the three enteroviruses (Table 9). The 23 seronegative samples came from children aged <1y (n=9) and 1-5 (n=14) years. Of the 921 samples, 294 (32%) were collected in 2006, 270 (29%) in 2011 and 357 (39%) in 2017, and in each sampling year, the number of samples with multiple seroreactivity was higher for individuals 11 years of age or older.

Table 9 Three-way cross tabulation of seroprevalence of EV-D68, EV-A71 and CVA6 out of 1507 samples tested.

		CVA6 seronegative	CVA6 seropositive
EV-D68 seronegative	**EV-A71 seronegative**	23	31
EV-D68 seronegative	**EV-A71 seropositive**	14	23
EV-D68 seropositive	**EV-A71 seronegative**	88	236
EV-D68 seropositive	**EV-A71 seropositive**	171	921

Chi-squared tests of independence on overall seroprevalence showed that the three enteroviruses were significantly associated with each other (p <0.001, Table 10), which indicates common sources of infection. There was a significant difference in antibody titers of EV-D68 seropositive individuals when comparing between CVA6 seropositive and seronegative groups; and between EV-A71 seropositive and seronegative groups (p <0.001, Kruskal-Wallis tests in Table 10 and chi-square tests in Table 11). There was no significant

difference in antibody titers in other comparisons (*p*-value[b] in Table 10; Table 11), which might suggest that EV-D68 generates a distinct serologic response, while EV-A71 and CVA6 have shared immune response characteristics.

Table 10 Pairwise comparison of serology data for assessing neutralizing antibody specificity. *p*-value[a] is derived from Fisher's exact tests comparing seroprevalence among the different serotypes, and *p*-value[b] is derived from Kruskal-Wallis tests comparing antibody titer distributions between the different serotypes. Seronegative samples were excluded in the Kruskal-Wallis tests.

	EV-D68 (-)	**EV-D68(+)**	**CVA6(-)**	**CVA6(+)**	**EV-A71(-)**	**EV-A71(+)**
EV-D68 (-)	-	-	37 (40.6%)	54 (59.4%)	54 (59.3%)	37 (40.7%)
EV-D68 (+)	-	-	259 (18.3%)	1157 (81.7%)	324 (22.9%)	1092 (77.1%)
p-value[a]	-		<0.001		<0.001	
p-value[b]	-		<0.001		<0.001	
EV-A71 (-)	54 (14.3%)	324 (85.7%)	111 (29.4%)	267 (70.6%)	-	-
EV-A71 (+)	37 (3.3%)	1092 (96.7%)	185 (16.4%)	944 (83.6%)	-	-
p-value[a]	<0.001		<0.001		-	
p-value[b]	0.56		0.82		-	
CVA6 (-)	37 (12.5%)	259 (87.5%)	-	-	111 (37.5%)	185 (62.5%)
CVA6 (-)	54 (4.4%)	1157 (95.5%)	-	-	267 (22%)	944 (78%)
p-value[a]	<0.001		-		<0.001	
p-value[b]	0.96		-		0.66	

Table 11 *P-values* from Chi-squared tests of independence*. Neutralizing antibody titer values of seropositive samples for each virus were compared against each other / against serological data of heterologous enteroviruses.

	EV-A71(+) vs EV-A71(-)	***CVA6(+) vs CVA6(-)***
EV-D68(+)	<0.001	<0.001
	EV-D68(+) vs EV-D68(-)	***CVA6(+) vs CVA6(-)***
EV-A71(+)	0.56	0.82

	EV-A71(+) vs EV-A71(-)	***EV-D68(+) vs EV-D68(-)***
CVA6(+)	0.66	0.95

**no adjustment for multiple comparison was needed as the degrees of freedom (=3-1) had been taken into account*.

Neutralizing antibody titers of the 921 seropositive samples were compared between the three enteroviruses in a correlation analysis (Figure 13). EV-A71 and CVA6 titers were significantly correlated ($p < 0.001$) and particularly for samples from 11-20-year-olds ($p < 0.01$). EV-A71 and EV-D68 titers were significantly correlated in the 6-10y age group ($p < 0.05$).

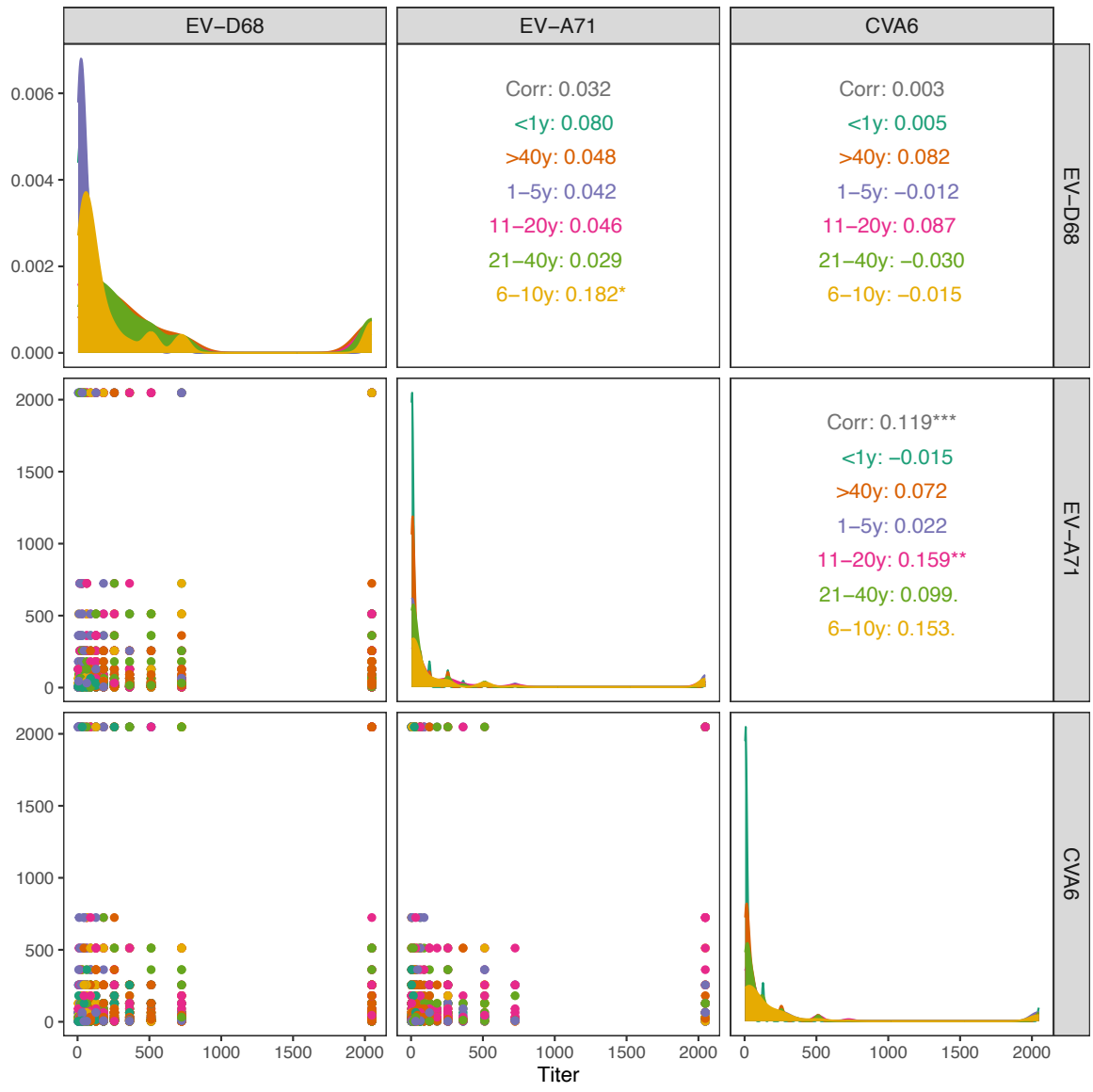


Figure 13 *Correlation matrix plots showing the distribution of neutralizing antibody titers for individuals who were seropositive for all three enteroviruses (n=921) on the left diagonal. Antibody titers are stratified by age groups. (y – years). Scatterplots of each pair of viruses are drawn on the left part of the figure and Pearson correlation is displayed on the right. P values are indicated as '***', '**', and '*' for <0.001, <0.01, and <0.05, respectively.*

2.7 Enterovirus D111 seroepidemiology

Uncommon, rarely detected enteroviruses

Several enteroviruses are only rarely identified clinically and during surveillance, and little data exist on their genetic diversity, prevalence and spectrum of disease associated with infection. This could be due to their physio-chemical characteristics, which makes them susceptible to gastric acid (e.g., respiratory enteroviruses) and are therefore missed by the standard surveillance systems that use stool samples. Alternatively, their detection has classically depended on virus culture, which is quite laborious or uses cell lines that are less sensitive effective in isolating some enteroviruses [342]. Also, rare enteroviruses might seldom cause severe clinical disease and so their clinical spectrum is still unknown [124, 343]. However, advances in molecular biology have increased detection of rare enteroviruses in clinical and environmental samples even in absence of symptomatic infections. Examples include EV-A119, -C104 and -B78, for which no full genome sequences were initially available, and newly reported new types EV-A114, -A119, -A120, -A121, -B86, -B87, -B106, -C019, -C116, -C113 and CVA12, detected in high prevalence in environmental samples indicating widespread, silent circulation [243, 343-346].

Additionally, some of the rare enteroviruses are highly geographically differentiated, e.g., EV-A76, -A119, -B75, -B77, -B97, -C104, -C105, -C109, -C117, CVA20, CVA21, and EV-D94, which are infrequently detected worldwide, therefore presenting significant bias in epidemiological estimates [280, 343, 347]. For example, some new EV-C lineages in sub-Saharan Africa are yet to be detected and described in other regions [348], and a recent analysis showed that EV-B75 lineages circulating in Nigeria [344] appeared to be different from EV-B75 circulating in Gambia, Guinea and Senegal [280] further indicating regional

confinement of circulation. Phylogenetic analysis also showed circulation of two distinct EV-C105 lineages in Europe and Africa, indicating different evolution patterns for the two EV-C105 lineages [349]. EV-D70 emerged in Africa and EV-D94 and EV-D120 have never been isolated outside of Africa. EV-D94 and EV-D120 are seemingly restricted to African habitats that host non-human primates (NHP), consistent with circulation in wild reservoirs in Central Africa and recent emergence from NHPs [112]. However, this could be due to data paucity and not genuinely geographical restriction in virus transmission.

Surveillance of the rare enteroviruses is important since they emerge to cause severe disease and sometimes with atypical or varying manifestations. For instance, EV-A76, -A77, -A81, -A120 CVAB2, CVAB4, EV-B80, -B73, -B97, -B93, -C99, -C105 and EV-D94 strains have been isolated from acute flaccid myelitis cases in African countries and the USA [280, 344, 348, 350]. Novel and rare enteroviruses e.g., EV-C104, -C105, -C109, -C117 and -C118 were detected in mild to severe respiratory illnesses [189, 349-352], and EV-C99 was recently detected in acute gastroenteritis without symptoms of enteroviral syndromes, which is unexpected since enteroviruses are classically not known to be associated with acute diarrhea [353].

Increased human interaction with wildlife, due to urbanization, climate change, changes in land use, the flourishing of live animal markets and farming and trafficking of wild animals, exposes humans to novel pathogens, with population growth and globalization allowing these emerging viruses to spread [354, 355]. Increasing contacts between humans and NHPs, as described in Central Africa, could promote cross-species transmission of new viruses to humans [113, 354]. Zoonotic transmission of HTLV was reported in communities living in close geographic proximity in the Cameroonian forests most likely via exposure occurring at the hunter-primate interface [356]. A recent study

reported genetic identity of enterovirus strains obtained from wild and captive great apes in three national parks in the Congo, and from humans living around and within the parks [357]. Enterovirus infections in NHPs have been reported previously [112, 115, 358] and zoonotic enterovirus transmission was reported in respiratory disease outbreaks occurring simultaneously among gorilla populations and humans residing in nearby research camps in the Central African Republic [359].

EV-A76, -A89 and -A119 have been isolated in humans and animals in Central Africa, in areas deemed to have high contacts between humans and animals, including Cameroon and the Democratic Republic of Congo [113, 360-362]. EV-A76, -A89, -A119 and -B107 strains were determined in previous studies to contain genomic components derived from human and animal parental strains [112, 348] and identification of human EV-B107 and EV-B90 in NHPs in Gabon was attributed to cross-species transfer due to increased contacts between humans and animals [112].

Enterovirus D111 (EV-D111)

EV-D111 is an emerging enterovirus initially recovered from human stool samples collected in the DRC but misidentified as EV-D70 [350] and later detected in a stool sample of a wild chimpanzee in Cameroon [361]. Other EV-D111 strains have been isolated from human stool samples in Central African Republic and Cameroon [241, 348] and more recently, EV-D111 was detected in sewage samples in Nigeria [346]. So far, only eight strains have been reported to date. EV-D111 has enteric tropism, having been detected in feces and found resistant to acid treatment [120]. It is phylogenetically related to EV-D70 and EV-D120 in the capsid and does not have the additional short uORF described for some EV-A, -B and -C [120]. Phylogenetic analyses in the non-capsid genomic regions suggested

that EV-D111 evolved through intertypic recombination and recent zoonotic transmission [120, 346, 348]. EV-D111 induces strong cytopathic effects in many cell lines including L20B, RD, Hep-2c, HEK293T, LLC-MK2 and A104 [120].

Study objective 1:

A probable scarcity of herd immunity to the rare and more recently described enteroviruses could facilitate their emergence and spread in highly susceptible populations, potentially resulting in outbreaks of severe disease. Therefore, it is important to investigate sero-reactivity to such viruses to gain insight into their epidemiology and extent of exposure. For this, human sera (n=627) were tested for anti-EV-D111 neutralizing antibodies to estimate exposure and epidemiology in five countries.

Study objective 2:

Enterovirus circulation in animals has been reported widely and with potential for cross-species transmission. To anticipate zoonotic threats, it's therefore important to screen multiple hosts or reservoir species for enterovirus diversity. Further to objective 1, plasma samples from 32 primates in Cameroon were tested for anti-EV-D111 neutralizing antibodies.

Enterovirus D111 seroprevalence in humans

In this study, the prevalence of serum neutralizing antibodies against EV-D111 in different human populations was assessed using a serum neutralization assay. Serial serum dilutions were incubated with EV-D111, and endpoints determined by visual inspection for virus cytopathic effect (CPE) after three days. Test samples showed a range of neutralization titers (Figure 14) ranging from <1:8 to 1:1024. However, there was no obvious cutoff threshold titer for assigning samples as seropositive or seronegative and we have therefore described seroprevalence at two chosen thresholds, where seropositivity was assigned to samples with neutralizing antibody titers of ≥1:16 and ≥1:32.

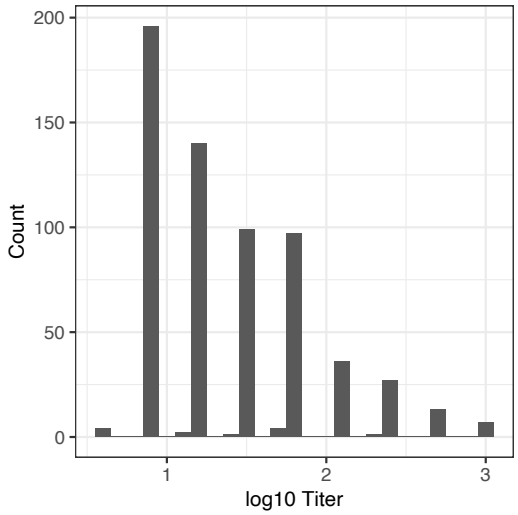


Figure 14 *Distribution of EV-D111 serum neutralizing antibodies in the 227 samples tested in this study*.

Specificity of EV-D111 neutralizing antibody detection

To address the possibility that neutralizing antibodies detected to EV-D111 may not be fully serotype-specific, we compared seropositivity and titers of EV-D111 neutralizing

antibody to those previously determined against EV-D68, EV-A71 and CVA6 for the UK samples. In a pairwise manner, we checked for significant association or independence between EV-D111 serostatus assignments (seropositive or seronegative) and that of the heterologous enterovirus serotypes (Table 12, Fishers Exact tests). Of the 33% (75/227) EV-D111 seronegative samples (titer <1:16), 95% (n=71) were EV-D68 seropositive, 91% (n=68) were CVA6 seropositive and 81% (n=61) were EV-A71 seropositive. There was no statistically significant association between EV-D111 and the other serotypes (*p*-value[a] > 0.05, Table 12).

Table 12 Assessment of EV-D111 neutralizing antibody specificity through pairwise comparison with serology data of heterologous enteroviruses. *p*-value[a] are derived from Fisher's exact tests comparing seroprevalence among the different serotypes, and *p*-value[b] are derived from Kruskal-Wallis tests comparing antibody titer distributions between the different serotypes. Seronegative samples were excluded in the Kruskal-Wallis tests.

	EV-D68 (-)	**EV-D68(+)**	**CVA6(-)**	**CVA6(+)**	**EV-A71(-)**	**EV-A71(+)**
Cutoff (≥1:16)						
EV-D111(-)	4 (5.3%)	71 (94.7%)	7 (9.3%)	68 (90.6%)	14 (18.7%)	61 (81.3%)
EV-D111(+)	8 (5.3%)	144 (94.7%)	28 (18.4%)	124 (81.6%)	30 (19.7%)	122 (80.3%)
***p*-value[a]**	1		0.08		1	
***p*-value[b]**	0.06		0.32		0.22	
Cutoff (≥1:32)						
EV-D111(-)	6 (4.2%)	137 (95.8%)	19 (13.3%)	124 (86.7%)	28 (19.6%)	115 (80.4%)
EV-D111(+)	6 (7.1%)	78 (92.9%)	16 (19%)	68 (81%)	16 (19%)	68 (81%)
***p*-value[a]**	0.36		0.26		1	
***p*-value[b]**	0.067		<0.001		0.044	

At the antibody cutoff of ≥1:32, 143/227 (63%) samples were EV-D111 seronegative, for which 96% were EV-D68 seropositive, 87% were CVA6 seropositive and 80% were EV-A71 seropositive (Table 12). Similarly, there was no statistically significant association

between EV-D111 and the other serotypes at the titer cutoff of ≥1:32 (p-value[a] > 0.05) (Table 12). Antibody titers of EV-D111 seropositive samples (threshold of ≥1:16) were not substantially different from titers of heterologous serotypes (Kruskal-Wallis test, p-value[b] > 0.05) (Table 12). At the titer cutoff of ≥1:32, EV-D111 seropositive samples were different by EV-A71 and CVA6 serostatus (p < 0.05, Table 12). Overall, there was no evidence for neutralizing antibody cross-reactivity between the serotypes tested.

EV-D111 seroprevalence in different human population groups

The proportion of seropositive samples with antibody titers of ≥1:16 ranged from 55% to 83% and was highest in the Democratic Republic of Congo (DRC) and lowest in South Africa. The proportion of seropositive samples with antibody titers of ≥1:32 ranged from 32% in South Africa to 68% in the DRC (Table 13). At the seropositivity threshold of ≥1:16, the geometric mean titer was lowest in the UK samples (24 (95% confidence interval [CI], 20-28)) and highest in the DRC samples (64 (95% CI, 50-82)) (Table 13). At seropositivity threshold of ≥1:32, the GMT was lowest in the UK samples (43 (95% CI, 36-52)) and highest in Burkina Faso (106 (95% CI, 73-152)) (Table 13). Generally, samples from the DRC had higher antibody levels followed by the Cameroonian samples, twice as high as antibody levels in samples from South Africa and the UK (Figure 15).

For the UK samples, seroprevalence at the titer of ≥1:16 ranged from 28% in the 21-40-year-olds to 100% in adults older than 60 years in 2006, compared to 45% in 11-20-year-olds to 80% in over-60-year-olds in 2017 (Table 14, Figure 16). At the titer of ≥1:32, seroprevalence was 0% in the 21-40-year-olds and highest in the 1-5-year-olds (90%) in 2006; and in the range of 18% to 60% in 2017 (Table 14, Figure 16). Consistently, EV-D111 GMTs in the UK samples were highest in children aged 1-5 years (84 (95% CI, 55-128) and

34 (16-76) in 2006 and 2017, respectively) and in adults older than 40 years (37 (27-49)). Overall, antibody levels were lower in the ages between 11 and 40 years, both for samples obtained in 2006 and 2017 (Figure 16).

Table 13 Description of serum samples and the seroprevalence estimates of EV-D111 in human samples at two thresholds. (Abbreviations: CMR – Cameroon, DRC – Democratic Republic of Congo, BFA – Burkina Faso, ZAF – South Africa, UK – United Kingdom).

Country	Mean age	No.	Year	Population	Seroprevalence (≥1:16) (%)	Seroprevalence (≥1:32) (%)	GMT (≥1:16)	GMT (1:32)
CMR	>60y	100	2007	General population	77	60	57	81
DRC	39	100	2007	Military population	83	68	64	87
BFA	25y	100	2007	Blood donors	58	40	59	106
ZAF	25y	100	2009	Blood donors (HIV -ve)	55	32	40	76
UK	30y	227	2006, 2017	General population	61	37	23	43

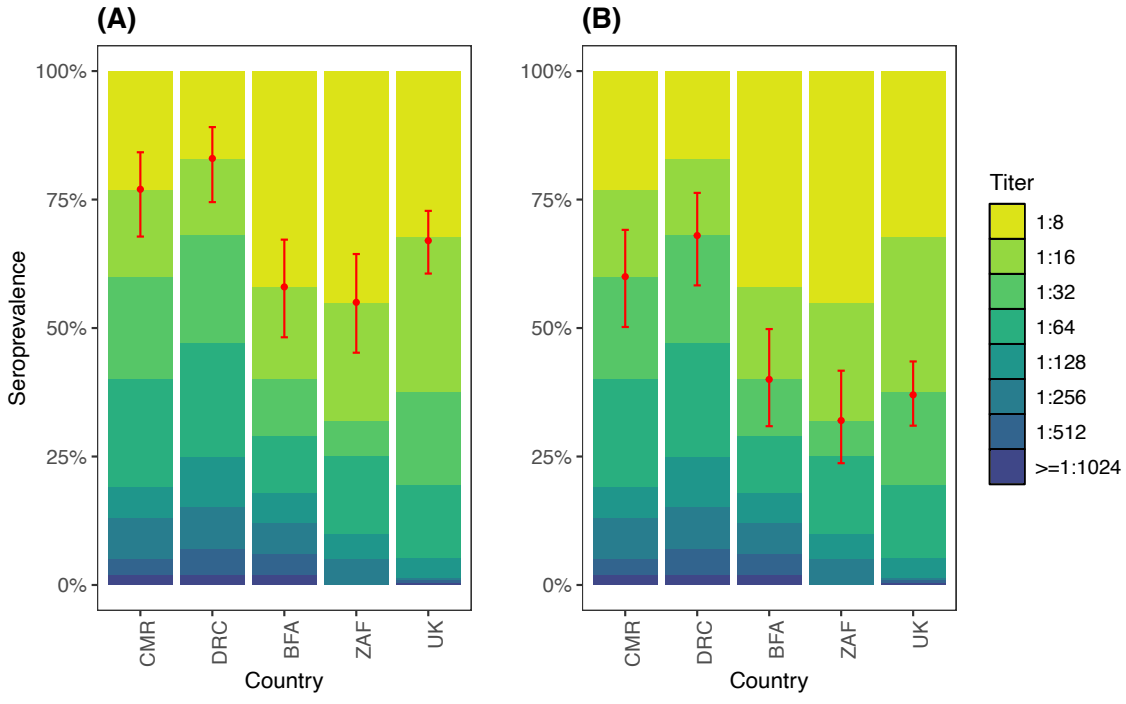


Figure 15 *Seroprevalence of EV-D111 in five countries. The colored bars show the percentage of samples with neutralizing antibody titers 1:8 to ≥1:1024. Point estimates of the percentage that were seropositive at ≥1:16 (A) and ≥1:32 (B) endpoint titers are shown in red and error bars indicate 95% binomial confidence intervals. (Abbreviations: CMR – Cameroon, DRC – Democratic Republic of Congo, BFA – Burkina Faso, ZAF – South Africa, UK – United Kingdom).*

Table 14 Seroprevalence rates of EV-D111 in UK in 2006 and 2017

	2006			2017		
Age group	**n**	**Seroprevalence (≥1:16)**	**Seroprevalence (≥1:32)**	**n**	**Seroprevalence (≥1:16)**	**Seroprevalence (≥1:32)**
1-5y	20	19 (95%)	18 (90%)	15	10 (66.7%)	5 (33.3%)
6-10y	9	6 (66.7%)	3 (33.3%)	9	7 (77.8%)	4 (44.4%)
11-20y	20	8 (40%)	4 (20%)	11	5 (45.5%)	2 (18.2%)
21-40y	64	18 (28%)	0	10	7 (70%)	4 (40%)
41-60y	33	28 (84.8%)	22 (66.7%)	10	7 (70%)	4 (40%)
>60y	11	11 (100%)	9 (81%)	15	12 (80%)	9 (60%)
Total	157	90 (57.3%)	56 (35.7%)	70	48 (70%)	28 (40%)

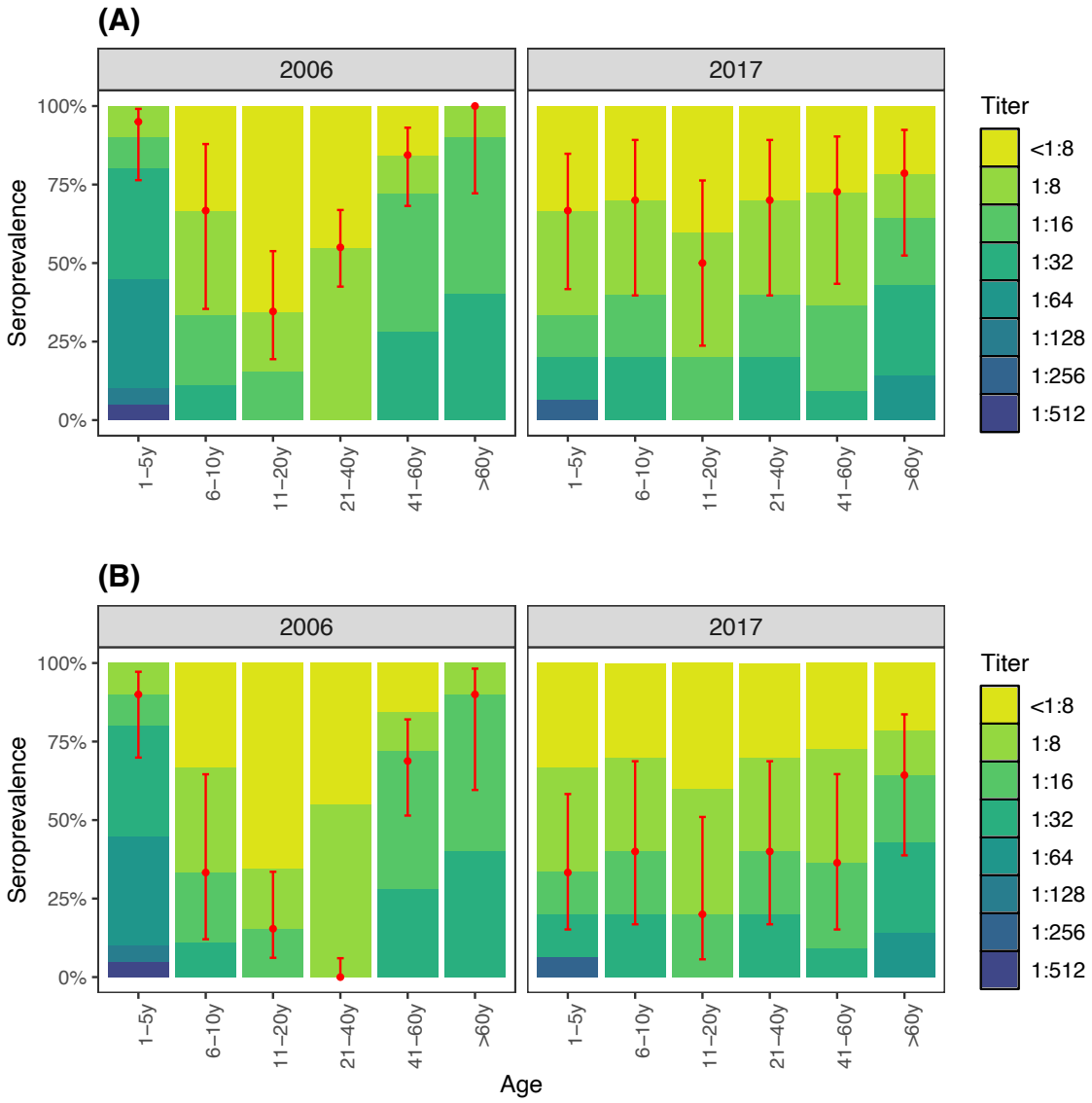


Figure 16 *Seroprevalence of EV-D111 in the UK. The colored bars show the percentage of samples with neutralizing antibody titers 1:8 to ≥1:1024. Point estimates of the percentage that were seropositive at ≥1:16 (A) and ≥1:32 (B) thresholds are shown in red and the error bars indicate 95% binomial confidence intervals.*

EV-D111 seroprevalence in non-human primates

Anti-EV-D111 neutralizing antibodies were also tested in serum from non-human primates (NHP) (n=32) collected in two wildlife sanctuaries in Cameroon. Antibodies were detected in titers ranging from 1:4 to 1:512. Seroprevalence at the titer of ≥1:16 was 62% (8/13) and 42% (8/19) in apes and Old-World Monkeys (OWM), respectively (Table 15). Among the OWMs, the highest levels of antibodies were detected in drills (*M. leucophaeus*) (75%, Table 15). At the titer of ≥1:32, seroprevalence was 40% (13/32) and higher in gorillas compared to other NHP species (Table 15). Generally, gorillas and chimpanzees showed extensive previous exposure to EV-D111 (Figure 17), demonstrating wide circulation among apes. Overall, the geometric mean titers at antibody cutoffs of ≥1:16 and ≥1:32 were 72.0 and 57.5, respectively.

Table 15 Seroprevalence of EV-D111 in apes and Old World Monkeys.

		EV-D111	
Taxonomic name (common name)	**No.**	**Seroprevalence (≥1:16)**	**Seroprevalence (≥1:32)**
Apes			
Pan troglodytes (chimpanzee)	8	4 (50%)	3 (38%)
Gorilla gorilla gorilla (gorilla)	5	4 (80%)	4 (80%)
Total	13	8 (62%)	7 (54%)
Old World Monkeys			
Mandrillus Sphinx (mandrill)	3	1 (33%)	1 (33%)
Papio Anubis (olive baboon)	2	1 (50%)	1 (50%)
Cercopithecus preussi (Preussi's monkey)	2	1 (50%)	1 (50%)
Cercopithecus pogonias (crowned monkey)	2	1 (50%)	0
Cercopithecus mona (mona monkey)	2	1 (50%)	1 (50%)
Cercopithecus nictitans (spot-nosed monkey)	1	0	0
Cercopithecus pogonias (crowned monkey)	1	0	0
Erythrocebus patas (patas monkey)	2	0	0
Mandrillus leucophaeus (drill)	4	3 (75%)	2 (50%)
Total	19	8 (42%)	6 (32%)

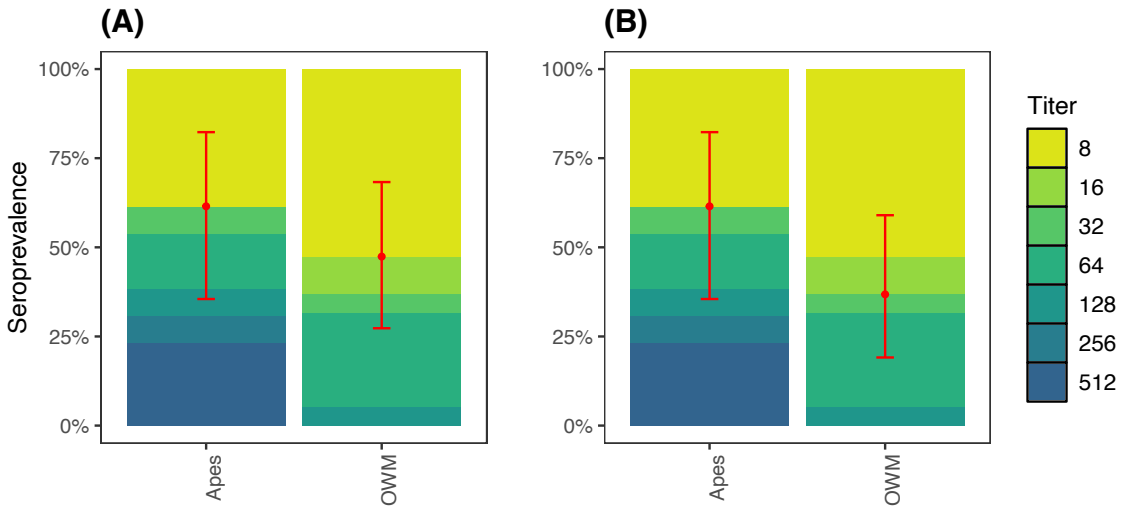


Figure 17 *Seroprevalence of EV-D111 in animal sera. The colored bars show the percentage of samples with neutralizing antibody titers 1:8 to ≥1:1024. Point estimates of the percentage that were seropositive at the ≥1:16 and ≥1:32 endpoint titers are shown in red and error bars indicate 95% confidence intervals of the proportions. (OWM: old world monkeys)*.

Overall, the EV-D111 seroprevalence in NHPs was slightly lower but matching with the seroprevalence in human populations. Although cross-reactivity among different enteroviruses was not evaluated in the animal sera, and even without EV-D111 RNA detection in these animals, these results indicate prior exposure to EV-D111 and support existing knowledge of enterovirus infections in wild and captive animals. Enterovirus infections in animals may have been acquired from human or dietary sources [113]. Animals are thought to play a role in the natural cycle of enterovirus circulation and a large genetic diversity of enteroviruses either distinct to animal populations or resembling human EV species has been documented, comprising members of enterovirus species A, B, C, D, J, and H [112]. High frequencies of active enterovirus infections (95%) were

reported among mandrills and other Old World monkey species inhabiting remote regions of Cameroon without human contact [363].

Enterovirus infections in apes are widespread and active infections have also been reported. For instance, wild chimpanzees in Cameroon excreted EV-A76, -A89 and -A119, which were closely related to human strains including EV-A76 and -A89 strains isolated in patients with AFP [348, 361, 362, 364]. Fatal myocarditis caused by human Coxsackievirus B was reported in a primate in Denmark, and Coxsackievirus B3 was shown to have transmitted between humans and chimpanzees [365, 366]. Among non-human primates at a zoo in Bangladesh, 12.5% of all enteroviruses detected (8/64) were previously detected in humans and the most common was echovirus 24 (E24) [367]. Twenty human enterovirus serotypes from species A, B and C were detected in synanthropic NHPs in Bangladesh [368]. EV-C99, a human enterovirus, was potentially responsible for acute flaccid myelitis illness in a chimpanzee in Congo [369], and recently (2009-2010), human enteroviruses were shown to circulate naturally among chimpanzees and mandrills in Gabon [112].

Novel enterovirus types, including EV-B112 in a chimpanzee, EV-B113 in a mandrill, EV-122 and EV-123 in wild monkeys, were identified more recently suggesting that NHPs could be potential sources of new enterovirus infections in humans [112, 360]. Humoral responses (detection of total immunoglobulins) to human enterovirus species EV-C were recently detected in gorilla samples [370], and serologic testing of NHP samples showed evidence of extensive exposure to Echovirus 11, EV-A76 and EV-D94 [114]. Long-term fecal shedding of enteroviruses, the environmental stability of enteroviruses and contamination of nest sites, could perpetuate infections among NHPs [114]. Higher population connectivity among animal groups and supply of susceptible animals may support cross-species transmission and indigenous enterovirus circulation in the animal population [371].

Though there was no proof of active circulation of EV-D111 in the wildlife centers in Cameroon at the time of sampling, detection of EV-D111 neutralizing antibodies in wild and captive animals provides evidence for broad circulation of EV-D111 among NHPs and could indicate their role as reservoirs and possible sources of virus infection or emergence. Given their close evolutionary relationship with humans it is therefore essential to continue exploring and monitoring EV circulation in these species.

Overall, this study provides broad evidence of EV-D111 circulation in humans and animals and a further indication of the host range of enteroviruses. Still, EV-D111 remains poorly understood as there are no published studies on seroprevalence and data on clinical infection are limited. The correlates of immunity of EV-D111 are unknown and considering the relatively low to moderate seroprevalence reported here, and its detection in AFP cases, EV-D111 may emerge in immunologically naïve human populations and should therefore be considered a potential public health risk.

EV-D111 remains poorly understood as there are limited data on clinical disease and there are no published studies on its seroprevalence. We examined the seroprevalence of EV-D111 in human samples obtained from five countries and in serum from non-human primates. The neutralization assay used here was comparable to those used previously for seroepidemiology investigations of other enteroviruses by us [213, 292] or in other studies. However, we did not have a concrete basis for selecting a threshold or cutoff for estimating seropositivity since there are no previous studies on EV-D111 seroepidemiology that recommend an optimal cutoff or inform on immunological background of the population, or any existing international standards for EV-D111 serological surveys. Also, the titer distribution in Figure 1 did not show a visual inflection point that would be interpreted as a cutoff value. Determining appropriate cut-offs for seropositivity can be challenging and could hinder comparability of epidemiological data between settings and over time.

We identified high EV-D111 seroprevalence in the Central African countries (Democratic Republic of Congo, Burkina Faso and Cameroon), where the virus was previously detected in human and animal fecal samples [348, 361]. Uncontrolled 'bushmeat' hunting and close interaction between humans and NHPs have been reported in the region, creating a risk of exposure to viruses circulating in wildlife and vice versa [354]. Although the sample sizes were small to make conclusive inferences (n=100 per country), the observed seroprevalence could suggest occurrence of asymptomatic or pauci-symptomatic infections, unrecognized outbreaks, or endemic circulation of EV-D111, and hence an epidemiological indicator of virus activity in regions of limited enterovirus surveillance. Recently, EV-D111 strains were detected in sewage in Nigeria and in stool specimens in Angola, further supporting EV-D111 circulation in the wider African region [346, 372].

EV-D111 seroprevalence in the UK was similarly high; this was unexpected as the virus has not been detected there in clinical cases or in more general population-based screening, such as sewage surveillance [243]. There are several potential reasons for these observations. Firstly, the high seroprevalence could be attributed to assay non-specific cross-reactivity with other enteroviruses. However, we demonstrated that EV-D111 neutralizing antibody titers were independent of those against EV-D68, EV-A71 and CVA6 (Table 2). Lack of serological cross-reactivity between EV types has been a general principal underlying past seroepidemiology studies and originates from marked sequence heterogeneity and structural differences between serotypes in their encoded VP1 and VP3 proteins, the target for serum neutralizing antibodies [373].

Nonetheless, some degree of antigenic relationship among enteroviruses cannot be fully ruled out given the sheer number of enteroviruses and potential for shared neutralizing epitopes (currently >100 serotypes assigned). Indeed, most serotypes have been assigned on the basis of >25% genetic divergence in VP1 [374] rather than using the original assignment criterion of a demonstrated lack of serological cross-reactivity with other serotypes, so this possibility has not been systematically evaluated [13]. Indeed, there are reports of cross-neutralization among enteroviruses within species A although infrequent or occurring in only a small proportion of patients [375-377]. It is important to note that although cross-binding of poliovirus to the polyclonal sera obtained from EV-D111 infected mice was proposed, no data on cross-neutralization were shown to support that observation. Particularly for samples with high neutralizing antibody titers (≥1:32) against EV-D111, we therefore do not believe that cross-reactivity accounts for high seroprevalences observed in the current study.

It is possible that the high seroprevalences observed in the study were results of extensive circulation of EV-D111 in the UK and elsewhere, but which would have remained undetected because of its possibly largely mild or asymptomatic nature. Non-polio enterovirus infections are not notifiable in the UK, and even if diagnosed, only samples obtained from more severe cases are forwarded to the national reference laboratory for typing [204, 345]. As discussed elsewhere [378], current monitoring of EV infections in the UK and most developed countries is not systematic. Consequently, EV infections are massively underdiagnosed, making it possible that EV-D111 and perhaps a range of other EVs have circulated undetected for many decades.

However, if EV-D111 was actively circulating in the UK, even if asymptomatically, EV-D111 strains should have been detected in sewage samples collected from the UK. A recent study using a PCR-based metagenomic approach demonstrated extensive diversity of EVs, but not EV-D111 [243], contrasting with the finding of EV-D111 strains Nigeria [346]. Since the virus has consistently been found or detected in stool samples from cases [120], this suggests an enteric tropism and therefore might be represented in sewage if it was circulating in the UK. However, the sewage surveillance may not capture short-term outbreaks, few and far between yet able to infect a substantial proportion of the population during its circulation. Our findings indicate a notable residual population without antibodies to EV-D111 who are potentially susceptible to infection at least in four of the five countries studied. This could facilitate outbreaks and poses a risk of disease to 'naïve' populations. Our findings contrast with the results of seroepidemiology studies for other enteroviruses, particularly in the UK, that typically report nearly 100% seroprevalence in the adult population [213, 292].

EV-D111 and polioviruses share tissue tropism and routes of dissemination [379], which could suggest a specific, but not general, instance of anti-enterovirus antibody cross-reactivity [380]. EV-D111 neutralizing antibody profiles in 2006 in the UK were higher in children aged 1-5 years and in adults over 60 years of age, but lower in the ages between 11-60 years. Geometric mean concentration and seroprevalence of anti-poliovirus antibodies was shown to be highest children between 1-4 years of age due to OPV in 2012 [381, 382] and in adults who we presume to have had natural poliovirus infections before the introduction of the vaccine but also likely received the oral vaccine during its widespread use in the 1960s [383, 384]. To some extent, this observation supports the idea that high anti-poliovirus antibody levels in these two age groups could have contributed to EV-D111 seropositivity via cross-reactivity although this theory still lacks scientific proof.

This is our hypothesis and has no implications on the interpretation of our findings. It could be tested experimentally by measuring poliovirus neutralizing antibody levels across age classes and relating these data to EV-D111 seropositivity. This forms the basis for a future study. The lower EV-D111 seroprevalence in the 11-60-year-olds in the UK might be attributed to mild, or asymptomatic or short-lived infections with low measurable antibody response as suggested for coronavirus infections [385, 386]. Alternatively, it could simply indicate no serological evidence of previous EV-D111 infection due to interrupted virus circulation or low force of infection in these age group.

This is the first seroepidemiology study of EV-D111 where we provide substantial serology-based evidence for extensive EV-D111 circulation in humans and animals, and a further indication of the host range of enteroviruses. Further serology studies are needed to understand the ecology and epidemiology of EV-D111. The correlates of immunity of EV-D111 are unknown and considering the relatively low to moderate seroprevalence

reported here, and its detection in acute flaccid paralysis, EV-D111 may emerge in immunologically naïve human populations and should therefore be considered a potential public health risk.

Discussion

Enteroviruses cause a broad spectrum of clinical illnesses, including acute respiratory infection, febrile rash, hand-foot-and-mouth disease, meningitis, encephalitis, and, rarely, acute flaccid paralysis. Non-polio enteroviruses are emerging threats to global public health. New variants have emerged to cause outbreaks and severe disease manifestations than previously known [13]. EV-D68 has been linked to severe respiratory disease and neurologic disease (acute flaccid myelitis, aseptic meningitis and/or encephalitis) [131, 387]. Brain stem encephalitis and non-cardiogenic pulmonary edema are commonly encountered in EV-A71 outbreaks and were associated with a high mortality rate; and neurologic and neuropsychiatric sequelae appear to be common in children who survive EV-D68 and EV-A71 central nervous system infection [13].

One postulation for emergence of severe enterovirus infections is population-wide changes in disease susceptibility. A good example is the considerable reduction in EV-A71 immunity observed in Taiwan in 1994 to 1997 compared to 1989-1993, which was deemed to be the precursor of a large HFMD epidemic in Taiwan in 1998 [388]. Other hypotheses include changes in virus antigenicity, transmissibility, cellular tropism, and pathogenicity.
Virus neutralization assays provide quantitative data on functional antibody levels that inhibit viral infection, at least in vitro, and are useful to measure virus prevalence. Memory responses to previously encountered pathogens can alter the immune response and the course of subsequent infections [389]. Although antiviral T and B cell memory certainly contribute some degree of protection, strong evidence of a protective role for neutralizing serum antibodies exists. Serological protection against infection and severe disease

conveyed by neutralizing antibodies has been shown in murine models and humans, and neutralizing antibodies are candidates for clinical use as monoclonal antibodies [211].

Here, seroprevalence studies were conducted to (i) assess exposure levels to four enteroviruses (EV-D68, EV-A71, CVA6 and EV-D111) and (ii) discern changes in exposure and population immunity. These studies are the first serological investigations into the prevalence of enteroviruses in England, UK, and differences in seroprevalence reflect their cumulative epidemiology during 2006-2017. The four enteroviruses were selected because of their clinical impact and evidence for emergence of strains with altered pathogenicity [14]. Before the 2014 worldwide outbreaks, EV-D68 was rarely identified as a cause of respiratory disease since testing of respiratory specimens for enteroviruses was uncommon. CSF and stool samples are still far more likely than respiratory samples to be assayed for enteroviruses [21]. The data here show a high seroprevalence and nearly universal exposure to EV-D68 before adulthood, with most children developing antibodies by 2–5 years of age.

Geometric mean titers were highest for EV-D68 compared to EV-A71 and CVA6. Biochemically, EV-D68 shares some common features with rhinoviruses and it was shown that EV-D68 could be neutralized by monotypic antiserum specific for HRV87 indicating that the two viruses represent strains of the same picornavirus [390]. The shared biological characteristics of EV-D68 and rhinoviruses and cross-reactivity make the true prevalence of EV-D68 infection difficult to determine. EV-A71 seroprevalence was lower than that of EV-D68 and antibody titers declined with age. It is not fully known whether different EV-A71 genogroups are uniformly neutralized by polyclonal immune sera.

Antibodies elicited against one genogroup were shown to confer cross-neutralization against other genogroups [287, 391], however, a recent study demonstrated that post-

infection sera and monoclonal antibodies from genogroup B5- or C4-infected patients exhibited reduced titers with genogroup C1 viruses [392]. C2 and C5 strains with mutations in the capsid region were also shown to escape neutralization [287], and these studies indicate that EV-A71-specific antibody response may consist of a fraction of poorly neutralizing antibodies against contemporary viruses among a subset of previously infected individuals. EV-A71 C1 and C2 genogroups commonly circulate in the EU/EEA region, including UK [206, 251, 283]. Potential antigenic variation among EV-A71 genogroups may have resulted in the antibody profiles observed in the UK population.

The interpretation of polyclonal antibodies in sera is complex and challenging due to lack of information on history of enterovirus infections. Nonetheless, the four studies here document greater circulation and force of infection, particularly in infants and young children, over the period in which enteroviruses emerged as important clinical pathogens and potential causes of paralytic disease. Seroprevalence of EV-D68, EV-A71 and CVA6 was >70%, increasing with age and over time, which suggests that enteroviruses may be far more common than indicated by clinical surveillance. Changes in virus activity reflect altered transmissibility and pathogenicity and emphasize the importance of continued surveillance for emerging enteroviruses. Stochastic changes in the biological properties of enteroviruses and population immunity could contribute to an increase in virus transmission and pathogenicity, with unprecedented implications for clinical severity [34].

Conventionally, newborns and young infants acquire indirect protection by maternal antibodies transferred through the placenta and lactation. Neonatal passive immunity can mitigate the risk for severe infections in the early postnatal period [214]. Enteroviral infections affect neonates much differently, often more severe than older children. Severe life-threatening complications of enterovirus infections include hepatic necrosis with

coagulopathy, meningoencephalitis, and myocarditis and these usually present during the first week of life. Factors affecting severity and outcome include presence or absence of passively acquired serotype-specific maternal antibodies [15].

In the samples studied in this thesis, anti-EV neutralizing antibodies were demonstrated at high levels in women of childbearing age (19-45 years) and could reflect maternally transferred antibody levels. Maternal EV-A71 antibodies are efficiently transferred to neonates but decline rapidly, with a half-life of 42 days (95% CI 40–44) [215, 393]. It was shown that the time to loss of protective immunity was longer (5 months) in neonates whose mothers who had titers ≥1:128 [215]. Generally, if this is presumed to be representative of most enteroviruses, then EV-D68 immunity in infants in England as shown here would last longer than that of EV-A71 and CVA6 [215].

Nevertheless, even with the high antibody levels seen in the 19-45y age group, ubiquitous EV infections and significant disease burden are still recorded in young infants <6 months who might be incapable of mounting a robust immune response [106]. For instance, in UK, enterovirus-related meningitis in infants <90 days old was more than twice that for bacterial meningitis and less than 1% of infants developed significant complications or died of their infection [394, 395]. CVA6 (23%) and EV-A71 (6%) were shown to be predominant causes of severe neonatal and pediatric disease in England, in 2012-2013 [396], and this could be attributed to inefficient transplacental transfer, maternal nutrition, differential production and accumulation of maternal of IgG subclasses or lack of persisting antibodies in infants [215, 397]. Paired mother-child samples are required to study maternal enterovirus immunity in the UK population. The antibody levels observed in women of child-bearing age should be considered during further studies of enterovirus infections in England.

Serologic studies are crucial for clarifying disease dynamics, detecting past infections regardless of clinical symptoms, determining correlates of protection and duration of immunity, and for understanding the extent of past transmission [52]. However, the timing (appearance) and magnitude of antibody development should be considered during assay interpretation since detectable antibody responses might only appear 2–3 weeks after infection [398]. Recently infected individuals might be seronegative, which also depends on the infection prevalence, i.e., when infection prevalence is low, the number of false-positive results could be substantial and even outnumber the true-positive results [398]. The latter is particularly critical during an evolving epidemic and may vary given the recency of infection [399].

Other serological variables and immunologic mechanisms to be considered include antibody waning, within-host heterogeneity, antibody isotype, assay characteristics/properties and differences in exposure risk over time [400]. Other constituents of human serum could affect neutralizing ability, which highlights the importance of sample matrix for neutralization antibody measurement. The polyclonal nature of human serum – mix of antibodies to multiple proteins that recognize different neutralizing epitopes – also contains lipids and carbohydrates [401]. Age and underlying clinical conditions can also affect the immunologic outcome during infections [400].

The four seroepidemiology studies described here used residual clinical samples, which reflect people generally more ill than the general population and might come with biases inherent in clinical testing criteria. The samples lacked information beyond basic demographics, such as underlying conditions or pathologies, infection history, or potential risk factors that could affect inferences on epidemiology and transmission. Convenience

samples, such as used here, are also prone to chance variations in sampling between geographic regions.

Although all efforts were made to include equal sample sizes for all ages, the serosurveys were not powered to provide precise seroprevalence estimates for certain age classes. The available specimens for some age classes had insufficient volumes, thus limiting the numbers tested and generalization of the results. Thresholds of immune protection for most enteroviruses are also not fully established, and therefore, concrete inferences on levels of protective immunity in could not be made in this thesis.

In conclusion, EV-D68, -A71, CVA6 and EV-D111 were determined to be commonly circulating viruses in UK and beyond, and sera from infants <6 months neutralized enteroviruses paralleling maternal immunity. Seropositivity rates in 2006, before the periods of virus outbreaks and greater disease severity, were generally high and particularly in the ages most vulnerable to disease (1-10 years). Therefore, changes in susceptibility and presence of immunologically susceptible population appear unlikely to be a cause of the recent enterovirus outbreaks and severe disease emergence.

Chapter 3: Enterovirus clinical virology data in England, UK, 2006-2017

Enterovirus surveillance in the post-polio world

Non-polio enterovirus surveillance is difficult due to the broad range of existing enterovirus types and diseases associated with them. Surveillance for an enterovirus infection is considered mostly when there is a large burden (hospitalization, disability or death), and intervention or treatment is available. In the post-polio eradication era, emerging and re-emerging enteroviruses point to existence of pathogens capable of causing severe disease highlighting the importance of enterovirus surveillance as an early warning system for the detection and response to outbreaks [402]. For instance, in aseptic meningitis, correct and timely identification of enteroviruses as the cause is deemed important to reduce unnecessary use of antibiotics and alleviate psychological strain as viral meningitis has a much more favorable prognosis compared to bacterial meningitis [402]. However, a major challenge to understanding the epidemiology, disease burden, and triggering factors of enteroviruses is the absence of systematic surveillance platforms, which impedes documentation of their true burden and collective impact on human health [402].

WHO's Global Poliovirus Elimination Action Plan provides advice on enterovirus surveillance and parallel data on non-polio enterovirus detection. However, the recent Global Action Plan for polio containment demands poliovirus handling in biosafety level 3A laboratories [403]. This has led to shutdown of reference laboratories without such facilities resulting in interruption of the supplementary non-polio surveillance [23].

Nonetheless, several other surveillance systems provide avenues for monitoring non-polio enteroviruses. In Japan, HFMD syndromic and virologic surveillance data are collected through the National Epidemiological Surveillance for Infectious Diseases, which comprises a national network of 3000 pediatric medical sentinel sites [375, 404] and in China, all probable and laboratory–confirmed HFMD cases are notifiable to the Chinese Centre for Disease Control and Prevention (China CDC) in Beijing [106]. The Asia-Pacific Network for Enterovirus Surveillance (APNES) comprises a multi-country collaboration between Cambodia, Malaysia, Vietnam, and Taiwan [405].

In the EU/EEA region, 11 countries have a reporting or systematic surveillance system for EV-D68 established via sentinel influenza surveillance for respiratory pathogens or AFP surveillance [24, 184]. Most EU/EEA countries have established laboratory-based national enterovirus surveillance systems where diagnostic laboratories send selected samples for further typing at a national reference laboratory and over 5000 samples are typed annually by such systems [378]. Still, HFMD surveillance in the EU/EEA countries has rarely been implemented effectively, with only two countries possessing an established specific HFMD reporting system. Enterovirus surveillance is seen as a complex task given the large number of known types and the variety of diseases they cause [23].

In England and Wales, NHS microbiology laboratories routinely report clinically significant infections, including enteroviruses, electronically on a voluntary basis to UKHSA. For enteroviruses, these reports contain records on demographics (age, gender and geographical region), sample type, diagnostic method, date of detection, serotype, and biological site (blood, CSF, gastrointestinal or respiratory) [204]. Also, as part of the PHE's active polio surveillance commitment to the WHO, NHS laboratories are requested to

submit all positive enterovirus strains to the PHE Virus Reference Department (VRD) for confirmation and molecular typing, and for enhanced national enterovirus surveillance.

This chapter describes clinically or laboratory-confirmed EV-A71- and CVA6-associated infections in England, UK, from 2006 to 2017. The data were used to complement the measurements of enterovirus prevalence estimated using serology methods (previous chapter) and catalytic models (next chapter).

Virology data

Demographic (age and gender) and strain-specific (sample type, date of detection, and serotype) data from 2006 to 2017 for all positive enterovirus strains were obtained from the UKHSA's Enteric Virus Unit. EV-D68 data were only available for the period 2009 to 2017.

Data description and summary

The analyses performed here as part of data description are not aimed at examining long-term changes in virus circulation or incidence, but primarily look at seasonality and annual cycles of enterovirus detections. We assumed that the seasonality or occurrence/periodicity of detections was not affected by changes in clinical practices or laboratory diagnostic methods. During 2006-2017, a total of 20221 enterovirus positive samples were sent for molecular typing. Enterovirus positive samples increased annually and substantially from 190 in 2006 to 2670 in 2017. In the period 2009 to 2017, there were only 156 EV-D68 detections spanning from two in 2010 to 65 in 2014 (Figure 1(A)). The contribution of EV-D68 to the total number of enterovirus detections in 2014, the year that global outbreaks were reported, was only 2% (Figure 1(B)). In 2014, 16 (25%) and 12 (18%) infections were observed in the <1-year-olds and 1-5-year-olds, respectively.

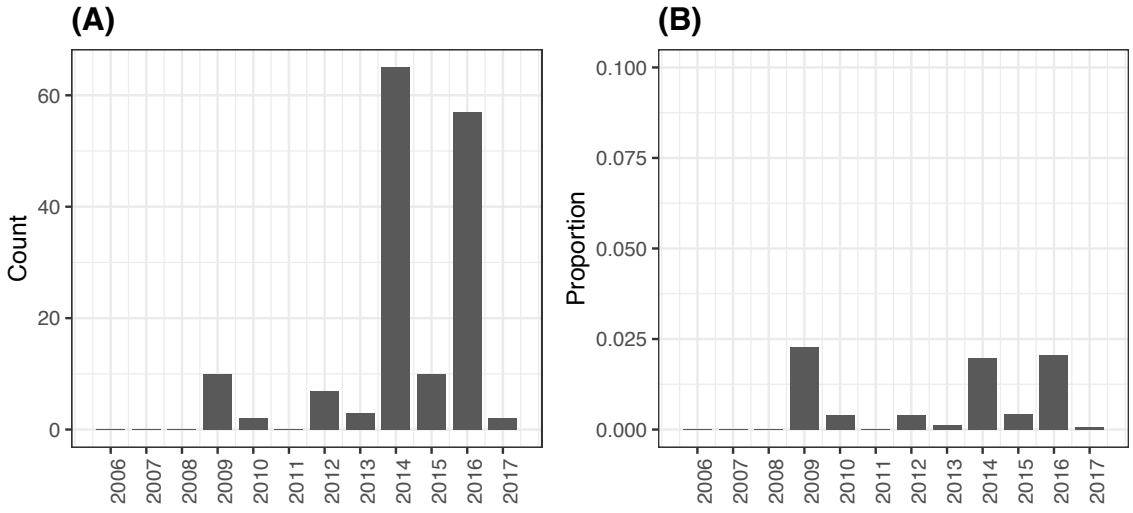


Figure 1 *Number of samples identified as EV-D68 since 2009 to 2017 (A). Panel (B) shows the proportion of EV-D68 samples where the denominator was the total number of enterovirus positive samples by year*. *EV-D68 data were not available before 2009*.

Of the 20221 samples, 402 and 1519 were identified as EV-A71 and CVA6. A sharp increase in CVA6 detections was recorded, from two (1%) in 2007 to 250 (10%) in 2017 (Figure 2(A, left)). EV-A71 detections show a distinct biennial pattern, and a general increasing trend from 2006 to 2013, with the highest years reporting no more than 100 detections (Figure 2(A, right)). CVA6 detections clearly increased over time from 2010, congruent with the emergence of CVA6 globally, with total detections surpassing 150 per year since 2014.

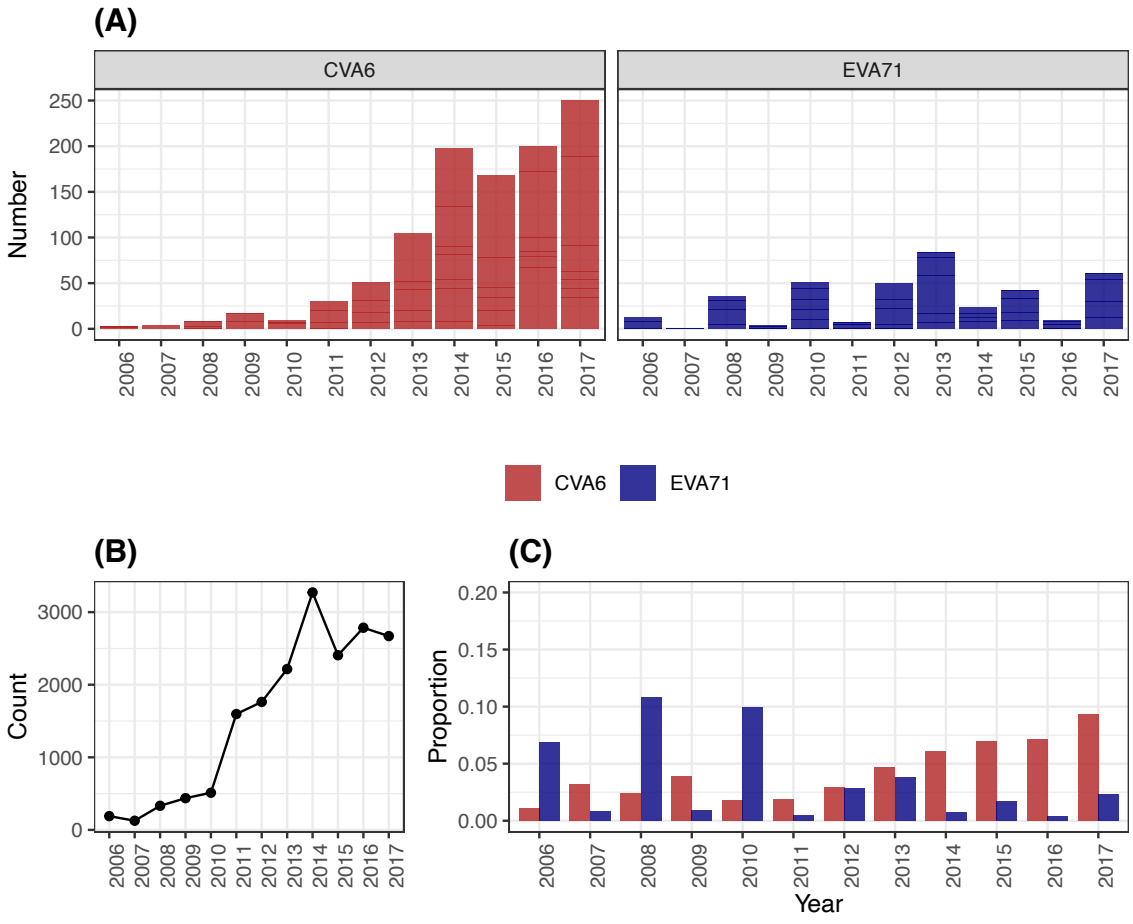


Figure 2 *The number of EV-A71 and CVA6 detections each year from 2006 to 2017 are shown in panel A, while the total number of enterovirus positive samples in the same period*

are depicted in (B). Panel C shows the proportion of samples that were EV-A71 and CVA6 in each year.

Most EV-A71 infections were reported in even years and CVA6 infections reported every year. About 10% of all enterovirus-positive samples in 2006, 2008 and 2010 were identified as EV-A71 but this proportion decreased below 3% since 2012 (Figure 2(C)). The peak months for EV-A71 detections were July and August, whereas most CVA6 detections were detected in October to December. The highest monthly detections were 20 EV-A71 positive samples in July 2013 and 74 CVA6 positive samples in October 2017 (Figure 3).

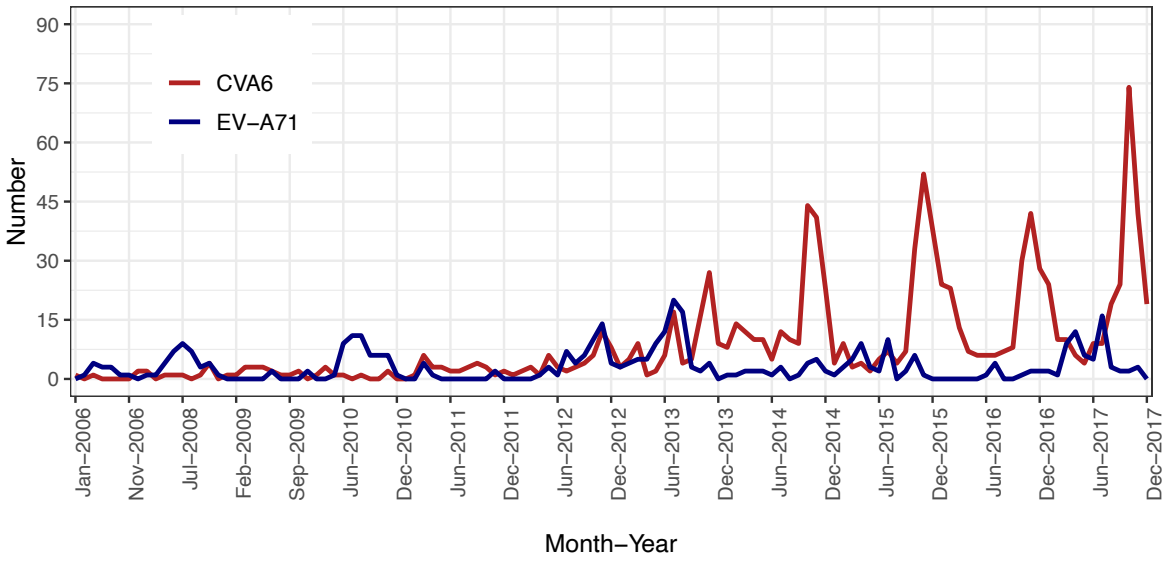


Figure 3 *Monthly distribution of EV-A71 and CVA6 detections in the period 2006-2017. EV-D68 data were too scarce (n=88 in total) to visualize monthly and hence EV-D68 is not shown.*

EV-A71 infections were mostly identified in feces (32%), followed by cerebrospinal fluid (CSF) (26.2%), respiratory specimens (12.1%), vesicle or skin swabs (5.5%) and blood (6.3%)

(Table 1). Data were not available for 21 EV-A71 positive samples. 73.5% CVA6 infections were detected in vesicle or skin swabs reflecting its association with HFMD; 13.2% were detected in respiratory specimens, 8.1% in feces, 4.3% in CSF and 4.1% in blood. Data were not available for 86 CVA6 positive samples. EV-D68 was frequently detected in respiratory swabs (Table 1).

Table 1 EV-D68, EV-A71 and CVA6 positive samples by biological site of detection (no. (%)) submitted to the Public Health England Enteric Virus Reference Department during 2006–2017.

	Blood	**CSF**	**Feces**	**Respiratory**	**Skin**	**Tissue**
EV-D68	0	1 (1.1%)	6 (6.8%)	69 (78.4%)	1 (1.1%)	0
EV-A71	24 (6.3%)	100 (26.2%)	122 (32%)	46 (12.1%)	21 (5.5%)	25 (6.6%)
CVA6	42 (4.1%)	44 (4.3%)	84 (8.1%)	136 (13.2%)	759 (73.4%)	19 (1.8%)

Most enterovirus positive samples were obtained from infants under three months of age (3730/9636, 39%;), and adults 21-40 years (2309/9636, 24%) (Figure 4). Data on age were not available for 10585 samples. EV-A71 detections were highest in infants <3 months old (58%), while 15% and 17% detections were in children 4-12 months and 1-5 years old, respectively (Figure 4(A)). CVA6 infections were mostly diagnosed in older children (>1-5 years, 52%) followed by younger children aged 4-12 months (23%). Unlike EV-A71, only small number of CVA6 infections (5%) were reported in infants <3 months (Figures 4(B)).

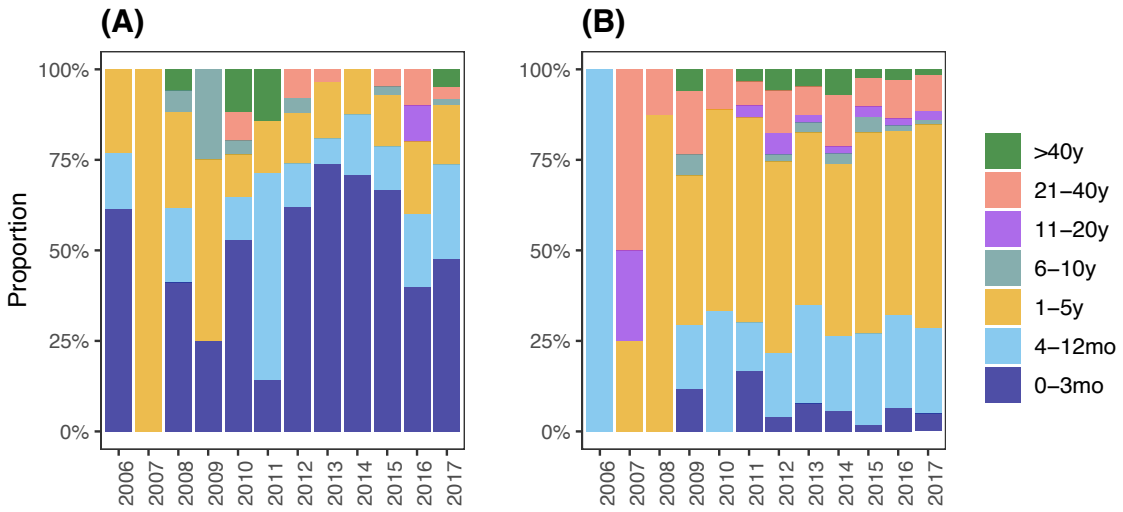


Figure 4 *Proportions of EV-A71 and CVA6 samples by age group for each year from 2006 to 2017. EV-D68 data were scarce (n=88 in total) and therefore only EV-A71 and CVA6 are shown.*

Time series analysis

A time series (TS) is a frequency spectrum depicting occurrence of a phenomena in discrete time. In epidemiology, time series methods are used to explore disease dynamics, including regularity in epidemic cycle periods, seasonal changes and impact of interventions like immunization on seasonal and longer-term epidemic cycles [406]. Time series methods have been applied to study measles and pertussis epidemics [407, 408], Dengue epidemics in Thailand [409], and HFMD and enterovirus epidemics in Japan [34, 375]. Here, spectral and time series methods were used to characterize seasonality, trends, and periodicity of EV-A71 and CVA6 detections. The EV-D68 data were too scarce for time series analysis.

In the first instance, data aggregation by month showed that EV-A71 detections were irregular with no apparent seasonal pattern and varied little from month to month. CVA6 detections were more regular and showed an apparent seasonal pattern, with higher detections in late autumn i.e., October to December, and lower detections between April and July (Figure 5).

Time series decomposition

An additive decomposition model available in the '*statsmodels*' library in Python was used to characterize the enterovirus detections at time *t* by reducing the data into trend (m_t), seasonality (s_t), and noise (e_t) components. Seasonality describes the periodic signal or recurring cycle(s) and trend describes whether the time series is decreasing, constant, or increasing over time (overall rise or fall in the mean). The noise component or random residual describes the variability in the data that cannot be explained by the decomposition model.

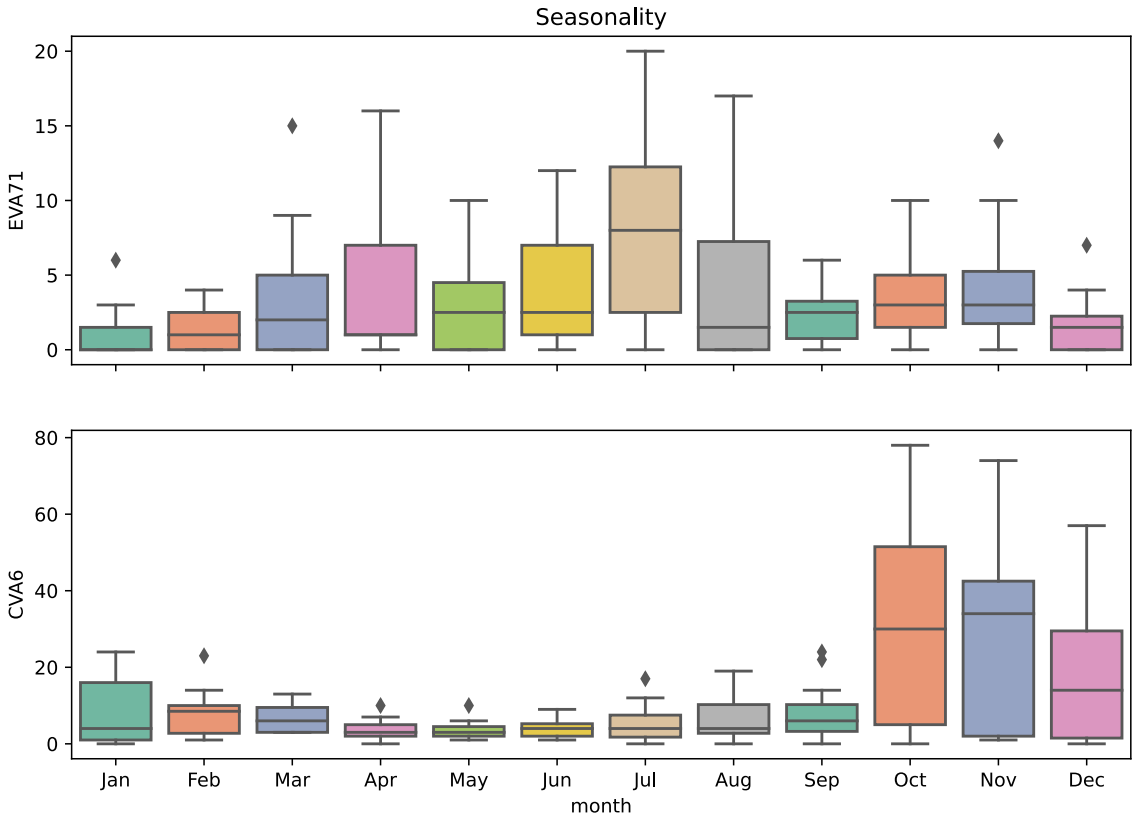


Figure 5 *Seasonality of EV-A71 and CVA6 detections in the period of 2006 to 2017 based on monthly data.*

Time series decomposition showed a strong seasonality of CVA6 with a period of 12 months and an increasing trend of detections over time. EV-A71 had variable seasonality with a period of 24-months and exhibited irregular trend (Figure 7).

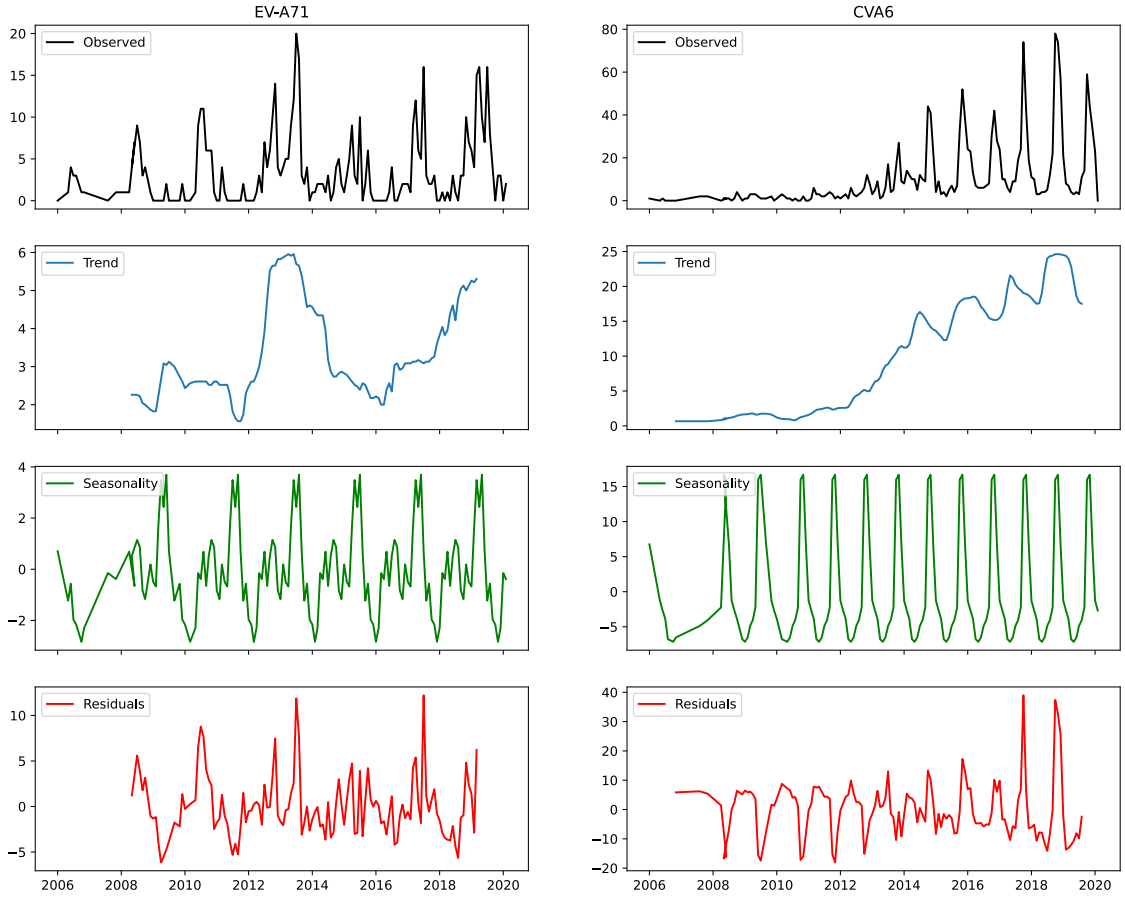


Figure 6 *Time series decomposition of monthly EV-A71 and CVA6 detections. The time series are disaggregated into three components - trend, seasonality and residuals. The trend component captures the overall level of the series, the seasonal component captures patterns that repeat every season, and the residuals are random errors or statistical noise, what is left over after considering trend and seasonality*.

Correlation analysis

An autocorrelation function (ACF), a scaled down version of autocovariance function that evaluates similarity between an original dataset and the lagged version of itself over successive time intervals, was also used to check for seasonality. ACF is based on the construction of autocorrelation coefficients r_k (k = 1, ... ,N, where N is the length of time

series) and reflects the correlation between datapoints at different times within a time series. R_k ($-1 \leq r_k \leq 1$) measures the correlation at a displacement (lag) of k datapoints. For each lag k, the ACF goes through the time series and checks if the observed value covaries positively or negatively with a datapoint k time steps away. A significantly autocorrelated time series means that previous values (lags) may be helpful in predicting the current value.

An autocorrelation plot of CVA6 shows autocorrelation peaks at every 12th month at least for the first 5 years (see months 12, 24, 36, 48 and 60 in Figure 7 (right panel)), depicting that the CVA6 series correlates well with itself at a lag time of 12 months. This suggests a regular incidence cycle of 1-year period or annual seasonality, consistent with the decomposition model. In contrary, the EV-A71 correlogram is hard to interpret, particularly after the 48th month, and the autocorrelation peaks are not consistent indicating irregularity in the incidence cycles. There are multiple low amplitude peaks, and the highest autocorrelation peak is seen at lag 24 and slight peaks are noticeable at lags 48 and 72 suggesting a possible biennial seasonality (Figure 7 (left panel)). For both serotypes, autocorrelation decreases as the lag increases (Figure 7) since the number of datapoints used to estimate the correlation decreases by 1 for every integer increase in lag.

Synchrony (similarity in timing and amplitude of incidence cycles) between the EV-A71 and CVA6 detections was checked using the Spearman correlation coefficient of variance-stabilized (square root transformed) time series. Spearman correlation was 0.087 implying no synchrony in detection of the two enteroviruses.

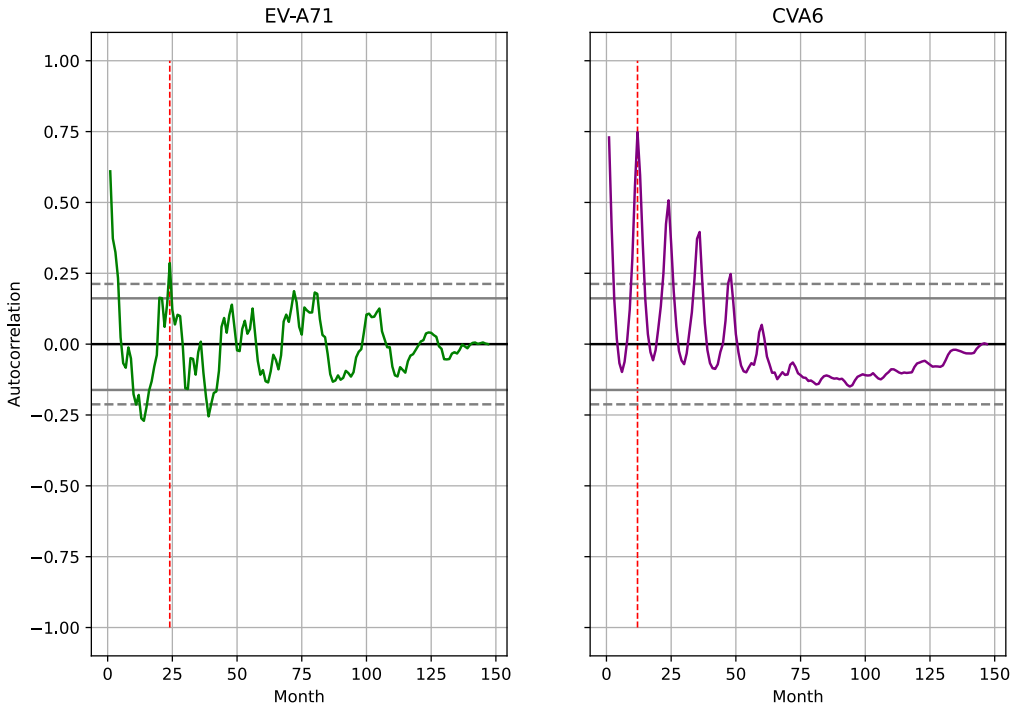


Figure 8 *Correlogram of the reported EV-A71 and CVA6 detections. The x-axis has the month index as the lag (the dataset comprised 147 months). Autocorrelation (y-axis) of +1 represents a perfect positive correlation, -1 represents a perfect negative correlation. The horizontal solid and dashed gray lines above and below 0 are the approximate 99% and 95% confidence limits, respectively, calculated based on randomly distributed data and test for departure from randomness. The red dashed line indicates highest autocorrelation peaks.*

Periodicity of enterovirus occurrence

Local wavelet power spectra of monthly detections were used to evaluate and characterize periodicity of enterovirus occurrence. Wavelet decomposition characterizes a time series as a function of frequency and time [410]. The Morlet continuous wavelet power spectrum of standardized (mean of 0 and unit variance) time series was calculated

to highlight temporal changes in detection patterns and periodicity over time using the *WaveletComp* package in R (R Core, 2020). The EV-A71 and CVA6 data were smoothed and detrended using 'loess'. In a power spectrum, periods of high and low time- and frequency-dependency are color-coded red and blue, respectively, as seen in Figure 8 (red to blue color gradient) and periodicity is displayed on Y axis against time of sampling on X axis.

For EV-A71, peaks of high periodicity are seen at four months and ten months in 2013, and at around 3-4 months in 2015, 2017 and 2019 (Figure 8(A)). There was also an underlying periodicity of 24-30 months (or 2-3 years) seen clearly as a region of high statistically significant power from 2007 to mid-2019 (the continuous red region with a black line between 16 and 32 months (Y axis) in Figure 8(A)). This suggests biennial periodicity of EV-A71, consistent with autocorrelation results above.

For CVA6, two statistically significant regions of high power are seen within the 6 and 12 period bands (the red region between 4 and 8 and 16 months – Y axis in Figure 8(B)), suggesting intermittent semi-annual cycles and a persistent annual mode of periodicity. This is more evident from 2014. Wavelet decomposition of the CVA6 data did not identify significant periodicity before 2014 due to the paucity of reported infections. The average power of statistically significant periods for CVA6 changed or increased with time and the signal became more prominent from 2013 onwards (color gradient shifted from low (blue) to high (red), Figure 8(B)), which corresponds to increased detection rates.

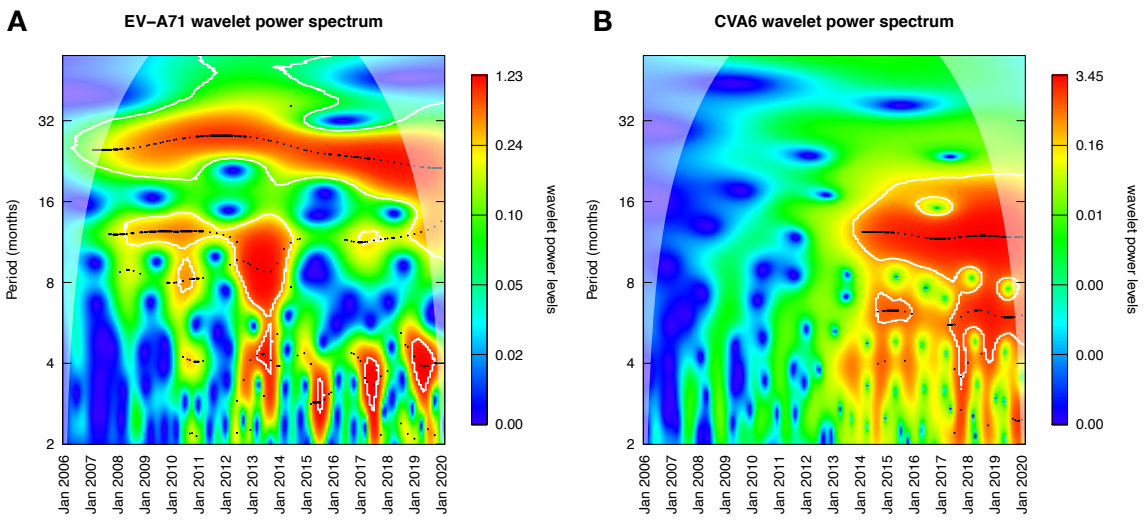


Figure 8 *Wavelet power spectrum of monthly EV-A71 and CVA6 detections from January 2006 to December 2019. Color spectrum depicts strong-weak (red-blue gradient) periodicity. White contour lines surrounding red areas indicate boundaries of statistical significance ($p < 0.1$) and black dotted lines indicate wavelet power ridge. The white shaded region abutting the y-axes mark the cone of influence, an area where the wavelet transforms are potentially affected by edge effects and therefore should be interpreted cautiously. X-axis is time (year) and y-axis is period (in months).*

Discussion

Clinical enterovirus surveillance is important for understanding infection burden by capturing clinically significant infections, identifying emerging strains, linking serotypes with disease phenotype and for developing guidance to manage infections [411]. Surveillance is also useful for characterizing virus epidemiological dynamics and potentially predicting the course of outbreaks or future epidemics. However, this is dependent on coherent surveillance systems with good temporal and spatial reporting and since enterovirus infections are commonly subclinical, virological data from case notification and active sentinel surveillance are generally much less available [412, 413]. This chapter describes EV-A71 and CVA6 detections and seasonality patterns and shows there was an increase in enterovirus positive samples in the period 2006 to 2017. This could reflect improved enterovirus detection through exclusive use of PCR in clinical laboratories [204], and less likely an indication of substantial increase in enterovirus-associated disease.

The measured seroprevalence (previous chapter) shows that EV-A71 and CVA6 circulated widely before 2006, which discounts a substantial increase in enterovirus disease or transmission from 2006. Secondly, diagnostic practices in general, including enterovirus infections, have changed over time in England and Wales. The use of PCR for virus detection increased from 36% in 2000 to 45% in 2011 and likely approached 100% already in 2015, replacing the slow and laborious virus culture entirely [204, 395]. The introduction and almost exclusive use of PCR for enterovirus typing in diagnostic laboratories meant that diagnosis was faster compared to virus isolation in culture, making it more favorable for clinicians/laboratories to do enterovirus screening.

Other factors that have been attributed to increased enterovirus detection include changes in clinical practice comprising collecting different sample types for suspected enterovirus infections and considering or investigating a broad range of clinical syndromes. Changes in clinical practice or diagnostic procedures such as the threshold for investigating and hospitalizing patients with suspected viral infections or performing lumbar puncture [204], may have also influenced the observed increase in positive detections. Previously, typing enterovirus positive samples was mostly done for individuals with neurological infections, while respiratory infections, HFMD, myocarditis, hemorrhagic conjunctivitis and post-mortem investigations were scarcely included in laboratory-based enterovirus surveillance [24].

Now, these clinical syndromes are increasingly investigated for enterovirus infections [395] and different clinical samples are collected for enterovirus testing with clinicians being encouraged to include stool and/or respiratory samples in addition to CSF for neurological infection [24]. A good example is detection of species C enteroviruses (C104, C105, C109 and C117) in respiratory illness only after including respiratory samples in the routine screening panel [347].

Despite the enhanced diagnostic methods and improved clinical practices, the virology data presented here are still sparse given the high seroprevalence that was measured in the UK population. Also, the lack of clinical information including hospitalization, disease severity, enterovirus-related deaths impede accurate estimation of infection burden. This is consistent with sub-clinical nature of most enterovirus infections. Differences in clinical practices might have influenced the number and type of samples obtained from patients and sent to UKHSA for molecular typing. Routinely, laboratory testing is done at the discretion of the clinician, likely biasing results towards

more severe presentations. For instance, CSF samples are more likely to be obtained for testing from individuals with neurological symptoms compared to throat, feces or rectal swabs where the viral loads would be higher, and enterovirus excretion prolonged [21, 414]. Also, delayed lumbar puncture due to atypical and varying clinical presentations, especially in older adults, and the absence of CSF pleocytosis may impede timely diagnosis of enteroviral infections [395], hence reducing the likelihood of a positive pathogen detection.

Moreover, clinical diagnosis of enteroviruses is often confounded by bacterial or viral sepsis. For a long time, enterovirus infections were not considered in the differential diagnosis of febrile neonates [415]. This practice may still be ongoing in some hospitals, therefore confounding the true incidence of infections. Also, infants with fever are often first placed on antibiotic treatment while undergoing diagnostic evaluation for bacterial sepsis, and/or on acyclovir if they are assumed to have disseminated herpes simplex virus (HSV) infection [415]. Even with timely lumbar puncture, testing for enteroviruses may be delayed or not considered given the high prognosis and greater clinical management of febrile illness. This consequently decreases the likelihood of positive enterovirus detection.

A challenge with the molecular assays for clinical enterovirus surveillance is the potential primer mismatches with increasing genomic diversity, which increases the likelihood of missed detection. Also, respiratory enteroviruses e.g., EV-D68 might be reported as rhinoviruses [21, 416]. Before widespread application of PCR, some laboratories primarily relied on virus isolation for enterovirus characterization and most likely used L20B and RD cells recommended for poliovirus surveillance [417]. These cells are less sensitive to some enteroviruses including EV-A71 and CVA6, which increases the likelihood of false negatives [418].

Recent modelling analysis determined that there was a change in viral characteristics of CVA6, including an increase in pathogenicity around 2010, which could explain its global rise in incidence [34]. The low numbers of EV-A71 and CVA6 positive samples obtained from older children and adults despite the high seroprevalence in these age groups (previous chapter) is consistent with benign infections. The high incidence of EV-A71 diagnosis reported in the 0–3-month age group may be underpinned by the low neutralizing antibody levels measured in infants <6 months old, and/or by the >4-fold attrition in mean titers in the 21–40-year age group (the implied maternal immunity) as discussed in the previous chapter. However, it should be noted that infants 0-3 months old are vulnerable and most susceptible to severe infection outcomes and hence a higher propensity for sampling at most emergency care or in-patient settings [394].

Time series analysis methods showed annual periodicity or seasonality of CVA6 and 2–3-year seasonality of EV-A71. A 3-year periodicity for EV-A71 was reported in Japan during 1996 to 2016 [375]. However, these inferences do not indicate accurate disease seasonality in UK and should be interpreted with caution since the data are based on voluntary and passive surveillance, and largely dependent on patient care practices. Sampling date for several samples could not be verified, further impeding inference on virus occurrence. Moreover, the patient could have been admitted for different medical condition(s) and happened to have EV detected in their sample, thus the data are not entirely representative of disease incidence. The paucity of data limited the quantitation of coherence between the two serotypes. Coherence is a measure of correlation and provides information on degree of association, whether two series are linearly correlated or move simultaneously (synchronously) at a particular time and frequency.

Notwithstanding the limitations discussed here, the virology data supplemented our estimation of the epidemiology of enteroviruses in the UK in the absence of systematic surveillance platforms. Other surveillance methods, such as environmental surveillance, might provide an improved picture of the epidemic process of enterovirus infections. Analyses of environmental samples provides supplementary methods to investigate virus circulation, changing epidemiology, importations and reintroductions, and early detection of emerging viruses [345, 419]. Detection of viruses in wastewater could be used as an early warning system for incipient outbreaks before the causative pathogens have been recognized in health care. This has been observed for viruses causing gastroenteritis [420] and for multiple instances of cryptic circulation of SARS-CoV-2 [421, 422]. Environmental surveillance is easily organized and standardized, does not depend on clinical reporting, is more cost-efficient, detects viruses causing sub-clinical infections, is not prone to referral bias and complements clinical data with potentially more accurate representation of viruses in circulation [22, 419].

Chapter 4: Mathematical modelling of Enterovirus A71 and Coxsackievirus A6

Summary

Compared to case-based surveillance, serological data indirectly provide a better estimate of disease and a useful resource for quantifying infection dynamics. Mathematical models attempt to describe the process by which observed data are generated. The widespread use of mathematical models based on serology data for understanding disease epidemiology and guiding policy motivated the work in this chapter. The objective here was to reconstruct historical changes in EV-A71 and CVA6 transmission using seroprevalence data fitted to catalytic models by estimating the force of infection (FOI, per capita rate at which a susceptible person acquires infection). Catalytic models assume an unspecified mechanism that results in a susceptible individual becoming infected, its magnitude defining the FOI.

Here, catalytic models were implemented in a Bayesian framework and assumed either constant or time varying FOI and antibody waning (seroreversion). Overall, the models estimate unchanging FOI over time for both enteroviruses, which suggests that virus transmission has not changed over time and the increase in clinical detection of these two viruses as described in the previous chapter could be explained by increased virus pathogenicity. The models also show evidence of age-dependent risk or infection and / or seroreversion.

Introduction

Force of infection

The force of infection (FOI) describes the rate at which a susceptible person acquires infection. It is a key parameter in mathematical models estimating infection prevalence and effectiveness of preventative measures [423]. FOI measures transmission intensity and depends on the number of infectious individuals and their contact rate with susceptible individuals [423]. At equilibrium (disease-free or endemic), the magnitude of FOI is inversely related to the average age of infection [89]. An association between declining FOI and demographic transitions (changes in birth and death rates) was linked to the increase in the average age of dengue disease [31, 424]. Although the basic reproduction number (R_o), the average number of secondary infections resulting from the introduction of an infectious individual in a completely susceptible population, is key to infectious disease transmission dynamics, the difficulties in obtaining direct estimates of R_o make FOI estimation more relevant [423]. FOI can distinguish age-related changes in rates of infection independent of changes in the proportion of susceptible individuals in each age class [89].

FOI is important for evaluating the impact of vaccination programs and other interventions e.g., vector control in Dengue fever [425, 426]. It can vary over time and by age [424, 427, 428] and may exhibit extensive global and local heterogeneity as seen for Dengue and Chagas disease [31, 429]. FOI estimates have been used to calculate incidence, critical vaccination coverage threshold and R_o [103, 430], for comparison of infection prevalence temporally [431], and for describing endemicity of dengue in multiple countries based on age-stratified seroprevalence data [104].

Catalytic models

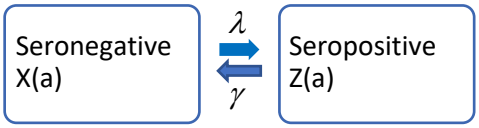
Hugo Muench first proposed the use of a catalytic curve or model for derivation of constant FOI from incidence and serological data. The name 'catalytic' was inspired by the similarity to the equations used to study the processes that drive chemical reactions [432]. Muench's catalytic curve was written as

$$\pi(a) = k(l - e^{-\lambda a})$$

where *k* is the proportion susceptible, π is the proportion previously infected prior to age a, and l is the proportion which may show evidence of exposure. The model follows individuals from birth and assumes a life-long constant FOI (λ) independent of age and year [423].

After Muench's seminal work, models with linear, non-linear (exponentially damped linear model) and polynomial description of changes in FOI with age were later developed (see [423] for detailed overview). Catalytic models have been used extensively with seroprevalence data to estimate FOI or probability of seroconversion [429, 433-436]. In these models, antibody titer is assumed to represent the cumulative exposure during an individual's lifespan, and an individual's age at the time of sample collection is used as a proxy for historical time. Since seroprevalence tends to increase with age because of increased exposure (i.e., augmented immunity), stochastic catalytic models can be constructed where individual seroconversion and seroreversion dynamics are described by a Markov chain with two states S^- and S^+.

Generally, for age *a,* catalytic models describe the rate of conversion (λ) from seronegative (X) to seropositive (Z) and the rate reversion to seronegative (γ) if there is decline in transmission and waning immunity [437]:



The seroconversion rate (λ) is the FOI and reflects disease transmission intensity or relative decline in the susceptible population and is related to (host) factors affecting antibody production; while the seroreversion rate reflects host factors (e.g., genetics or age) affecting antibody decay after infection [437].

For infections with life-long immunity, the proportion seronegative for age *a*, *X(a)*, declines with increasing age in dynamics represented by the differential equation

$$x'(a) = -\lambda(a)x(a)$$

where X(a) is defined by a negative exponential distribution in a solution

$$\mathrm{X(a)} = \mathrm{e}^{-\lambda \mathrm{a}}$$

The proportion seropositive, Z(a), or the rate of change in the proportion infected with age is then defined as follows:

$$Z(a) = 1 - X(a) = 1 - e^{-\lambda \mathrm{a}}$$

The reversible catalytic model assumes that antibody detection or seropositivity declines at a rate γ and individuals return to the seronegative status. $1/\gamma$ is the duration of antibody persistence in years. Here, the dynamics of seropositive individuals are expressed by the differential equation

$$\frac{dZ(a)}{da} = \lambda - (\lambda + \gamma)\mathrm{Z(a)}$$

which is integrated to give the solution,

$$\mathrm{Z}(a) = \left[1 - e^{-(\lambda+\gamma)a}\right]\left[\frac{\lambda}{\lambda+\gamma}\right]$$

However, the simplistic assumption of a constant FOI model has been questionable and disputed in studies considering multiple cross-sectional or longitudinal surveys [438]. Infection frequency and distribution of disease may be shaped by outbreaks or changes in human behavior that affect the risk of exposure or might be dependent on the epidemiological setting or age structure, with more intense transmission resulting in a younger median age of infection [433, 439]. In such cases, age- and time-dependent catalytic models were deemed more suitable and applied where the constant FOI assumption did not hold [89, 440].

Bayesian inference

In classical inference methods, model parameters are regarded as fixed quantities. A likelihood approach to statistical inference uses only the likelihood or data to calculate the Maximum Likelihood Estimate (MLE) of parameters, θ, defined as the value of θ which maximizes the likelihood of the observed data. In Bayesian inferencing, parameters are regarded as random variables with appropriate prior distributions, and the main object of interest is the posterior distribution, i.e., the distribution of the parameters given the data [441]. The Bayesian approach to parameter estimation uses formal probability distributions to represent uncertainty about a parameter's value and properties of the mechanisms that produced the parameter [441]. In contrast to frequentist methods, the subjective-Bayesian methods interpret models as nothing more than summaries of tentative personal bets about how the data and the parameters would appear, rather than models of a real random mechanism [441].

A prior distribution or assumption, $p(\theta)$, represents our knowledge about the likely values of unknown parameters independently of data. The Bayes' theorem updates the prior assumptions using the likelihood (observed data whose probability of occurrence depends on θ) to obtain a posterior distribution [442]. The posterior distribution $p(\theta \mid Data)$ is a probability distribution that summarizes the state of knowledge about an unknown parameter conditional on the prior and observed data, and provides information concerning parameter uncertainty that might be very hard to obtain using the classical inference methods [443]. The posterior distribution is proportional ($\propto$) to the normalized product of the prior distribution and the likelihood function, the conditional probability of data given θ, $p(Data|\theta)$:

$$p(\theta|\, Data) \propto p(Data \mid \theta) * p(\theta)$$

The choice of prior distribution contributes to the posterior and therefore to inference. A vague or non-informative prior (one that assigns equal or close to equal probability to a wide range of plausible values) yields results quite like maximum likelihood-based inferences [442]. Non-informative priors lessen the influence of the prior distribution on the posterior distribution. Information from previous studies can be incorporated through an informative prior distribution by placing prior restrictions on the possible values or by assigning a prior probability distribution based on available data or summary statistics [442]. Informative priors can improve efficiency and limit bias in estimating the parameters of interest. As the sample size increases, the estimated Bayesian point and interval estimates for the odds ratio will be driven more and more by the observed data and less by the prior [442]. The choice of prior is often encountered during sensitivity analysis, where different priors are evaluated to examine how the conclusions

depend on the choice of prior (i.e., evaluate the robustness of the model to the choice of prior) [442].

Modern Bayesian inference methods use Monte Carlo methods to estimate posterior distributions. A commonly used method is the Markov chain Monte Carlo (MCMC) algorithm which iteratively draws random samples from target (posterior) distributions using a sequential 'memoryless' ('Markovian') process, where the current sample is used as a steppingstone to generate the next random sample [444].

With Bayesian inference, the summaries of parameters posterior distributions include posterior means or median and the 95% credible intervals, which is the interval containing the true parameter with 95% probability. These summaries are Bayesian alternatives to the maximum likelihood estimates and 95% confidence intervals, respectively [444]. Unlike confidence intervals, which are typically calculated by assuming large sample approximations, the Bayesian credible intervals are appropriate for small samples [443].

Study objective

Increased virus transmissibility reflecting possible changes in tissue tropism, receptor use or persistence of virus shedding and longer periods of infectivity, might have led to enterovirus emergence in the recent decades. This study aims to fit catalytic models to the EV-A71 and CVA6 seroprevalence data from England, UK, to estimate the force of infection and reconstruct historical changes in virus transmission.

EV-A71 and CVA6 circulation dynamics in England, UK

Data

The EV-A71 and CVA6 age-stratified seroprevalence data for the years 2006, 2011 and 2017 as reported in *Chapter 2* were used in model fitting.

Models

Catalytic models were used to estimate the force of infection or attack rate and describe how the proportion of seropositive individuals changed with time. Two model categories were implemented – constant FOI and piece-wise constant FOI. The piece-wise models assumed time dependency, i.e., time-varying FOI. For mathematical simplicity, several general assumptions were made: (i) infections result in detectable serological markers (antibodies), (ii) homogenous mixing i.e., every individual is equally at risk of infection, (iii) disease-related mortality is negligible compared to all-cause mortality, (iv) negligible migration or stable age distribution, (v) no portion of the population is free from exposure, and (vi) a perfect laboratory test was used – that serology assays uncovered seropositivity with 100% accuracy. The status of individuals was described by a Markov Chain with two states, seronegative and seropositive.

General theory used: the age-specific seroprevalence $\pi(a)$ represents the expected proportion of immune individuals at age a and is the complement of the susceptible proportion $x(a)$, i.e., $\pi(a) = 1-x(a)$. Therefore, given the serostatus of an individual born in year τ during a serosurvey conducted in year t, $X^{\tau}(t|c)$, where $t \geq \tau$ and using a seropositivity cut-off c, the probability of being seropositive was given by:

$$Pr(X^{\tau}(t|c) = 1) = 1 - S^{\tau}(t)$$

where S^{τ} is the proportion of seronegative or susceptible individuals born in year τ.

At a seropositivity cut-off c, the number of seropositive individuals born in year τ in a serosurvey in year t, $z^{\tau}(t|c)$, was modelled using a binomial distribution:

$$z^{\tau}(t|c) \sim \text{Binomial}(n^{\tau}(t|c), \Pr(X^{\tau}(t|c) = 1)),$$

where $n^{\tau}(t|c)$ is the sample size in serosurvey year t for individuals born in year τ.

For the piece-wise constant FOI models, the proportion of seropositive individuals at age *a* in a serosurvey in year *S* is a function of the cumulative exposure from birth (i.e., the cumulative FOI or cumulative hazard). Age is used as an indicator of the cumulative time of potential infection risk. The probability of being seropositive is calculated as cumulative infection risk experienced from age one, when excluding the year of birth due to maternal antibodies, to the year of serosurvey, i.e., $\int_{Y_{B+1}}^{Y_S} \lambda(t)d(t)$. See the illustration diagram below. With λ_t as the FOI in year *t*, the probability to being seropositive in cross-sectional year *S* for an individual of age *a* (age in years) is equal to:

$$1 - \exp\left(-\sum_{t=S-a+1}^{S} \lambda_t\right)$$

Effectively, everyone contributes to the annual probability of infection (FOI) as demonstrated below:

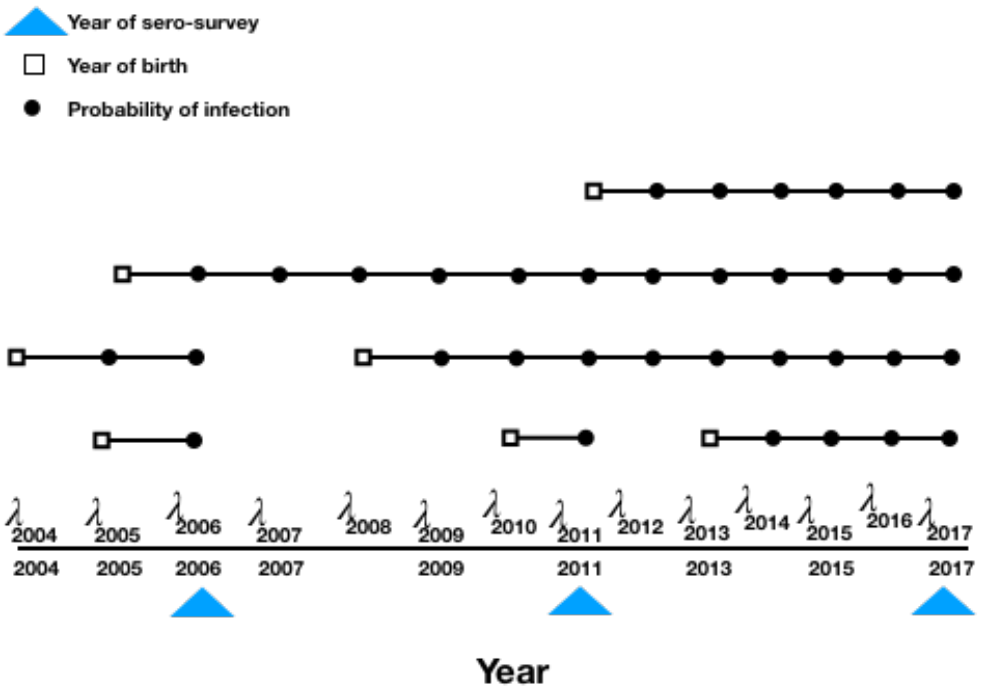


Illustration of the cumulative infection risk experienced by individuals. Consider, (i) an individual born in 2011 whose sample was seropositive in the 2017 serosurvey. Their probability of being seropositive is calculated as the cumulative FOI from 2012 to 2017, and the data from this individual contributed to six annual FOI estimates, i.e., for years from 2012 (when they are one years old) to 2017 (the year of the survey). In this model (and model 4, see below), it is assumed that the risk of infection starts from age one after waning of maternal immunity. (ii) A second example, consider a seropositive individual in the 2011 serosurvey who was born in 2008. This individual has a cumulative hazard of infection of three years (2009, 2010, 2011) and their data contributed to the annual FOI estimates for those three years.

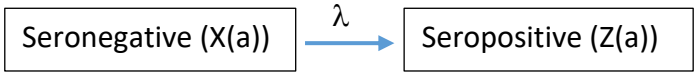
Here, we fit six catalytic models to three cross-sectional EV-A71 and CVA6 serosurveys to explore whether the seroprevalence data support changes in transmission over time and whether there was evidence of loss of antibody detectability and age-

dependence in the risk of infection. Model 1 is the simplest model, which assumes that individuals seroconvert at a constant rate λ and remain seropositive throughout their lives. Under this model, the proportion of individuals who have seroconverted at age *a* is given by [433]:

$$z(a) = 1 - e^{-\lambda a}$$

Model 1 assumes a constant force of infection (λ) over time $\lambda(t, a) = \lambda, \forall t, a$ and no seroreversion. The dynamics are described by the differential equation:

$$z'(a) = \lambda - \lambda z(a)$$



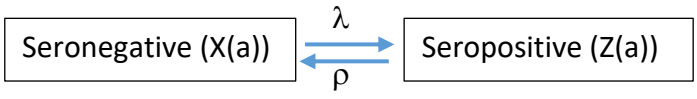
Model 2 is a reversible catalytic model, i.e., it assumes that individuals who have seroconverted can serorevert (become seronegative) at a rate ρ, where the duration of antibody detection follows an exponential distribution with a mean of $1/\rho$. In this model, the proportion of individuals seropositive at age *a* is given by [433]:

$$z(a) = \frac{\lambda}{\lambda + \rho}\left(1 - e^{-a(\lambda+\rho)}\right),$$

with seropositivity plateauing at $z(\infty) = \frac{\lambda}{\lambda+\rho}$.

Model 2 assumes a constant force of infection (λ) over time and seroreversion, with seroreversion rate ρ. The model's dynamics are described by the differential equation:

$$z'(a) = \lambda - (\lambda + \rho)z(a)$$



We extend Models 1 and 2 by allowing the FOI to change over time following a random walk and time step of 1 year (Models 3 and 4).

Model 3 assumes a time-varying FOI i.e., risk of infection for seronegative individuals was homogenous among age groups in any year but varied annually. We assumed time-heterogeneity in FOI on the basis that drastic improvements in sanitary conditions have occurred over the last decades. This assumption has been made for other fecal-orally transmitted pathogens (e.g., hepatitis A virus and cholera, [445, 446]). Also, the occurrence of sporadic outbreaks and large epidemics of EV-A71 and CVA6 in the last two decades motivated the time heterogeneity assumption. The time-varying or piece-wise constant FOI models were used to estimate annual probability of infection:

$$\lambda(t, a) = \lambda_t.$$

Let's denote the sampling year by *S*. For an individual of age a_j in year *S*, the probability of being seropositive will depend on the FOI during the years between when they were born, $S-a_j$, and the sampling year, *S*, which we denote by $\lambda_{S-aj}, \ldots, \lambda_{S-1}, \lambda_S$. We assume individuals cannot be infected the year they are born. The probability for a 1-year-old being seronegative in year *S* is given by:

$$P_{-(a_{1,S})} = e^{-(\lambda_S)}$$

For an individual of age a_j, the probability to be seronegative in year *S* is calculated from the cumulative risk of infection from the year the individual was age one $S-a_j+1$ to the year *S:*

$$P_{-(a_{j,S})} = \exp\left(-\sum_{t=S-a_j+1}^{S} \lambda_t\right)$$

Model 4 assumes a time-varying FOI with seroreversion denoted by parameter (ρ). However, the seroprevalence data were insufficient to accurately estimate both the time-dependent FOI (i.e., λ_t) and seroreversion parameters, and the FOI and the rate of sero-reversion seemed to be highly correlated. Hence, we fixed the value of ρ and and compared the model fits. We present the results for three different durations of seropositivity: 6 months, 5 years and 20 years. Let's denote by *S* the sampling year. For an individual of age a_j in year *S*, the probability of being seropositive will depend on the FOI during the years between when they were born, $S-a_j$, and the sampling year, *S*, which we denote by λ_{S-aj}, ..., λ_{S-1}, λ_S.

For this model, we assume individuals cannot be infected the year they are born. Here, the probability for a 1-year-old being seronegative in year S is given by:

$$P_{-(a_{1,S})} = e^{-(\lambda_S+\rho)} + \frac{\rho}{\lambda_S+\rho}\left(1 - e^{-(\lambda_S+\rho)}\right)$$

where the first term is the probability of not getting infected and the second one is the probability of getting infected and seroreverting.

The probability for a 2-year-old in year *S* to be seronegative depends on the probability to be seronegative at age 1 (in year *S*−1) and the probability of not getting infected or getting infected and seroreverting at year *S*,

$$P_{-(a_{2,S})} = P_{-(a_{1,S-1})} \times [e^{-(\lambda_S+\rho)} + \frac{\rho}{\lambda_S+\rho}\left(1 - e^{-(\lambda_S+\rho)}\right)]$$

$$P_{-(a_{2,S})} = P_{-(a_{1,S-1})} \times P_{-(a_{1,S})}$$

By recursion, the probability to be seronegative for an individual aged a_j in year *S* is:

$$P_{-(a_{j,S})} = P_{-(a_{j-1,S-1})} \times [e^{-(\lambda_S+\rho)} + \frac{\rho}{\lambda_S+\rho}\left(1 - e^{-(\lambda_S+\rho)}\right)]$$

As the probability of seroconverting during a year is independent of age, we can write:

$$P_{-(a_{j,S})} = \prod_{i=0}^{j-1} P\,(\text{not seroconverting in year } S-i)$$

$$P_{-(a_{j,S})} = \prod_{i=0}^{j-1}\left(e^{-(\lambda_{S-i}+\rho)} + \frac{\rho}{\lambda_{S-i}+\rho}(1-e^{-(\lambda_{S-i}+\rho)})\right)$$

Age-dependent FOI models

The model fitting results indicated that historical FOIs were persistent over time for both enteroviruses. Since enterovirus infections occur mainly in children under 5 years, we additionally explored whether there was evidence of FOI declining with age by extending the time-constant FOI models above. We modified Model 1 and 2 to assume that the force of infection follows an exponential relationship with age, i.e., FOI decays exponentially by age:

$$\lambda(a) = \lambda_0 e^{-\beta a}.$$

where $\lambda_0 > 0$ and $\beta > 0$.

Model 5 denotes the time-constant model with an age-dependent FOI and no seroreversion and Model 6 denotes the same model but with seroreversion.

For Model 5, the seropositivity at age a is given by [433]:

$$z(a) = 1 - \exp\left(\int_0^a \lambda(a')da'\right).$$

This expression implies that, if $\lambda_0 > 0$ and $\beta > 0$, seropositivity increases with age and plateaus at: $z(\infty) = 1 - \exp\left(\frac{\lambda_0}{\beta}\right)$.

Model 6 included age and seroreversion and the corresponding quantity is given by [433]:

$$z(a) = 1 - \exp\left(\int_0^a \lambda(a')da'\right) - \rho \int_0^a \exp\left[-\int_{a'}^a (\lambda(a) + \rho)\, da''\right] da' ,$$

This model implies that, if $\lambda_0 > 0$, $\beta > 0$ and $\rho > 0$, that seropositivity reaches a peak before declining towards $z(\infty) = 0$. Intuitively, this is because older age groups lose antibody detectability without it being replenished by infection.

The table below shows the summary of the models and assumptions made.

Table 1 Summary of models used. The listing of the models is based on quality of fit.

	Time-varying FOI?	**Age-dependent FOI?**	**Seroreversion?**
Model			
5	No	Yes	No
6	No	Yes	Yes
2	No	No	Yes
3	Yes	No	No
4a 4b 4c	Yes Yes Yes	No No No	Yes Yes Yes
1	No	No	No

We fitted the models to the seroprevalence data using a binomial likelihood:

$$p(a) \sim \text{Binomial}\big(n(a), \text{z(a)}\big)$$

where p(a) is the number of seropositive individuals at age a, n(a) is the number of individuals of age a who were tested, and z(a) is the proportion seropositive at age a under the Models 1-6. The models were fit to the first 80 age classes (ages 1 to 80 years). To avoid the effect of maternal antibodies, we excluded seroprevalence data for individuals <1 year of age. However, the models allowed individuals to be infected at the year of birth or age 0.

We report the estimated annual probability of infection or attack rate for year t, pt, which is the proportion of the susceptible population that becomes seropositive each year and can be derived from the FOI estimate as follows:

$$p_t = 1 - e^{-\lambda_t}$$

The models were fit under a Bayesian paradigm, and the priors specified for the parameters are given in Table 2. Across the model types, we set priors for λ which were uninformative with respect to the annual probability of infection. For the constant FOI models (Models: 1, 2, 5, 6), we chose a prior on λ which corresponded to a uniform prior over [0, 1] for the annual probability of infection. We used the same prior for our initial FOI value for the time-varying FOI models (Models 3 and 4) and a random walk prior on the FOIs from then on.

Table 2 Description of the different catalytic models and priors used.

Model	**Prior**
1 – Constant FOI (λ)	$\lambda \sim \text{exponential}(1)$
2 – Constant FOI (λ) with seroreversion (ρ)	$\lambda \sim \text{exponential}(1)$ $\rho \sim \text{exponential}(20)$
3 – Time-varying FOI (λ_t)	$\lambda_{t>t_1} \mid \lambda_{t-1} \sim \text{Normal}(\lambda_{t-1}, \sigma)$ $\lambda_{t=t_1} \sim \text{exponential}(1)$ $\sigma \sim \text{exponential}(1)$
4 – Time-varying FOI (λ_t) with seroreversion (ρ)	$\lambda_{t>t_1} \mid \lambda_{t-1} \sim \text{Normal}(\lambda_{t-1}, \sigma)$ $\lambda_{t=t_1} \sim \text{exponential}(1)$ $\sigma \sim \text{exponential}(1)$
4a: duration of seropositivity = 6 months	$\rho = 2$
4b: duration of seropositivity = 5 years	$\rho = 0.2$
4c: duration of seropositivity = 20 years	$\rho = 0.05$
5 – Age-dependent constant FOI (λ_0)	$\lambda_0 \sim \text{exponential}(1)$ $\beta \sim \text{exponential}(20)$
6 – Age-dependent constant FOI (λ_0) with seroreversion (ρ)	$\lambda_0 \sim \text{exponential}(1)$ $\beta \sim \text{exponential}(20)$ $\rho \sim \text{exponential}(20)$

For Model 2, which included seroreversion, we assumed that the typical duration of immunity was 20 years, and in Figures 1 and 2, we show the prior predictive simulations of the age-profile of seropositivity for Models 1, 2, 5 and 6. We do not show prior predictive simulations for Models 3 and 4 since these models only allow extra variation in λ over and above the constant FOI models. The plots in Figures 1 and 2 illustrate that a wide range of seropositivity profiles were possible given our choice of priors.

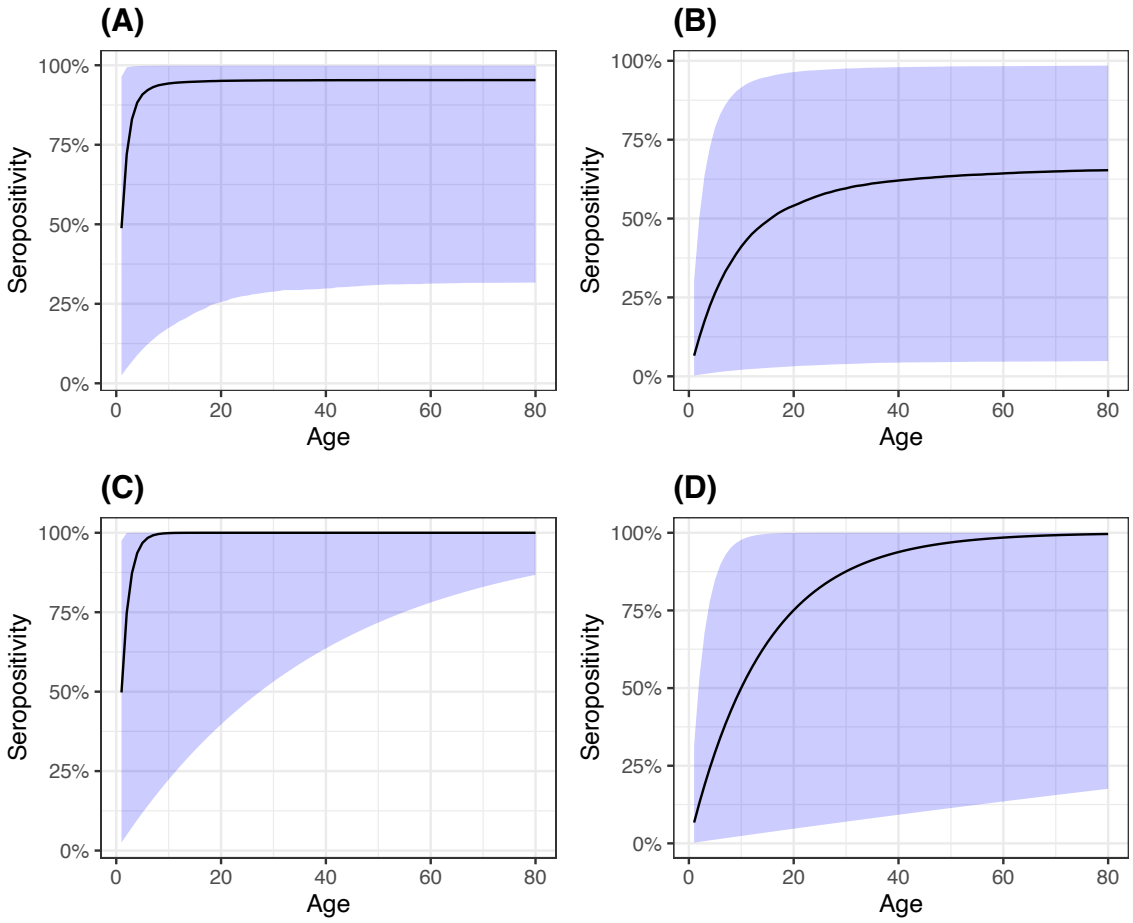


Figure 1 *Prior predictive simulations of the age-profile of seropositivity to assess the appropriateness of priors of parameters ρ and λ used in the constant FOI models (Model 1 - panels A and B, and Model 2 - panels C and D). That is, the figures show the implications of a prior in terms of what it says the data are going to look like. Panels A and B shows the*

simulated seropositivity using exponential(1) and exponential(10) on λ, respectively. Panel C shows simulations using exponential(1) on λ and exponential(20) on ρ, while panel D shows the simulated seropositivity using exponential(10) on λ and exponential(20) on ρ. The shaded area is the 95% interval, and the solid line is the mean estimate of seropositivity.

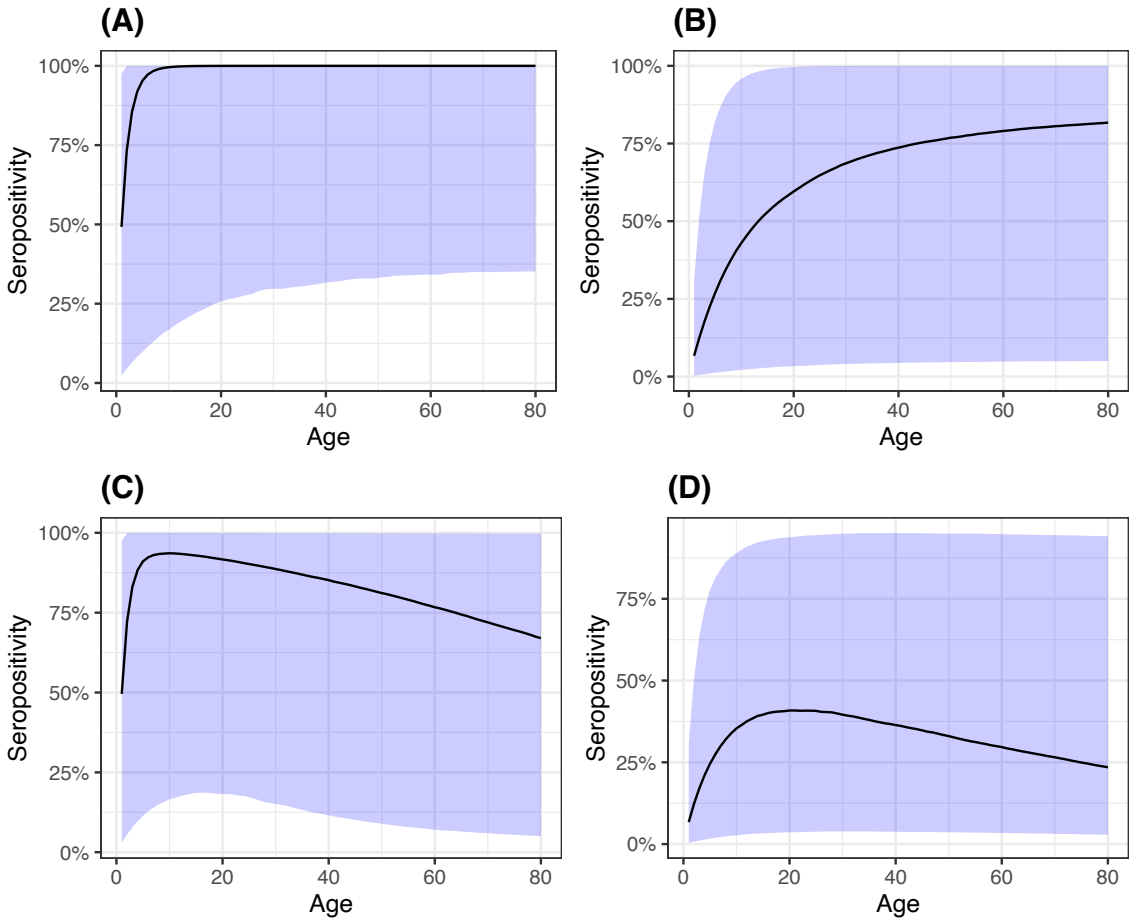


Figure 2 *Prior predictive simulations of the age-profile of seropositivity to assess the appropriateness of priors of parameters β, ρ and λ used in the age-dependent FOI models (Models 5 - panels A and B, and Model 6 - panels C and D). That is, the figures show the implications of a prior in terms of what it says the data are going to look like. Panels A shows the simulated seropositivity using exponential(1) on λ and exponential(20) on β; while panel B shows the simulated seropositivity using exponential(10) on λ and*

exponential(20) on β. Panel C shows simulations using exponential(1) on λ, exponential(20) on β and exponential(20) on ρ; while panel D shows simulations using exponential(10) on λ, exponential(20) on β and exponential(20) on ρ. The shaded area is the 95% interval, and the solid line is the mean estimate of seropositivity.

Sensitive analyses

We also conducted prior sensitivity analyses where we explored how model fitting and parameter estimation varied for differing priors on λ. We explored how FOI estimates from Models 1 and 2 change as we assume a different prior distribution ($\lambda \sim exponential(10)$) that would allow for lower seropositive values. Using the alternative prior in Model 1, the mean annual probability of infection was estimated at 0.062 (95% Credible Interval, 0.058– 0.067) and 0.079 (0.073-0.084), for EV-A71 and CVA6 respectively. With Model 2, the mean annual probability of infection was estimated at 0.25 (0.2–0.3) and 0.4 (0.33-0.49) for EV- A71 and CVA6, respectively; and a mean duration of seropositivity of 16 (12-23) and 11 (8- 16) years for EV-A71 and CVA6, respectively. The results of sensitivity analyses showed that model fitting was insensitive to the choice of priors on λ.

Posterior predictive analysis

Posterior predictive checks were used to assess model fit to observed data. Posterior predictions of seroprevalence were generated with the binomial random number generator, '*binomial_rng*' and posterior draws of FOI. Agreement between empirical and predicted seroprevalence profiles by age were visually inspected.

Model implementation

The models were fitted with the RStan package in R, which implements the Hamiltonian Monte Carlo No-U-Turn-Sampler (NUTS) sampling algorithm to explore the joint posterior parameter distributions [447]. Four chains were run in parallel with 10000 iterations each, and warmup of 3000 iterations. Convergence (when the chains have moved to an equilibrium or a stationary point and are sampling from the target distribution) was assessed visually using MCMC trace plots, and by checking the effective sample size (ESS, >200) and Gelman-Rubin's R-hat (<1.1) and potential scale reduction factor statistics. R-hat checks whether the chains reached a stable posterior distribution and ESS is the estimated number of independent samples accounting for autocorrelations generated by MCMC. The posterior distributions were summarized using means and 2.5th and 97.5th percentiles reported as 95% credible interval (CrI).

Model comparison

Models selection and comparison were based on the Pareto-smoothed importance sampling leave-one-out information criterion (PSIS-LOOIC), a Bayesian information criterion adapted to hierarchical models, which balances the goodness of fit of the model with model complexity, and therefore balances the risks of overfitting and underfitting [447]. PSIS-LOOIC was calculated using the *loo* package in R (http://mc-stan.org/loo/reference/loo_compare) and evaluates out-of-sample prediction accuracy using the LOO-CV (leave-one-out cross validation) method. Model performance was ranked based on overall expected log pointwise predictive density (ELPD) by summing the individual *elpd* estimates [448].

Results

We fit models allowing either a historically constant FOI or those with time-varying FOIs to three cross-sectional EV-A71 and CVA6 serosurveys. There were four time-constant models: a model with an age-constant FOI; a model with FOI which declines with age; another incorporating only seroreversion; and another with both age-dependent FOIs and seroreversion (see Methods above). Consistently across both enteroviruses, the time-constant models incorporating either an FOI varying with age and/or seroreversion fit the data better than the models allowing time-varying FOI (Table 3); which, in turn, provided a better fit than the simplest model with a constant FOI and no seroreversion. Collectively, these results indicate that there is no evidence of substantial variation in the force of infection over the past few decades in either enterovirus. Because of this, here we focus only on the three best performing models (Models 2, 5 and 6; Table 3), which all had time-constant FOIs and either age-dependent FOIs and/or seroreversion.

Table 3 Model comparison and ranking.

	EV-A71		CVA6	
Model	**elpd_diff**	**se_diff**	**elpd_diff**	**se_diff**
5	0	0	0	0
6	-0.4	0.006	-3.1	0.27
2	-2.7	0.02	-2.1	0.06
3	-45	2.87	-82.2	3.5
4a	-45.4	2.89	-83	3.67
4b	-45.1	2.86	-82.2	3.5
4c	-45	2.87	-82.1	3.5
1	-256.2	42.1	-258.7	48.3

Model comparison and ranking of the fitted catalytic models was done using the approximate leave-one-out cross-validation (LOO-CV) method. LOO-CV calculates the expected log pointwise predictive density (ELPD) for each model, which is a measure of the

overall model fit accounting for model uncertainty. Model ranking is based on the differences in the ELPD and standard error estimates ('elpd_diff', and 'se_diff', respectively), where the differences are calculated relative to the model with the largest ELPD, which in this study was Model 2. The ordering in which the models are displayed corresponds to the quality of fit of EV-A71.

Model comparison indicated that either age-dependence or seroreversion were necessary to explain the patterns of variation in seroprevalence, although the data characteristics meant we could not differentiate between these two hypotheses, and a model including both seroreversion and age-dependent FOIs fared no better (Table 3). Figure 3 shows the raw seroprevalence data (colored points) and the modelled seropositivity (solid line and shading) as a function of age across the three serosurveys for the two enteroviruses.

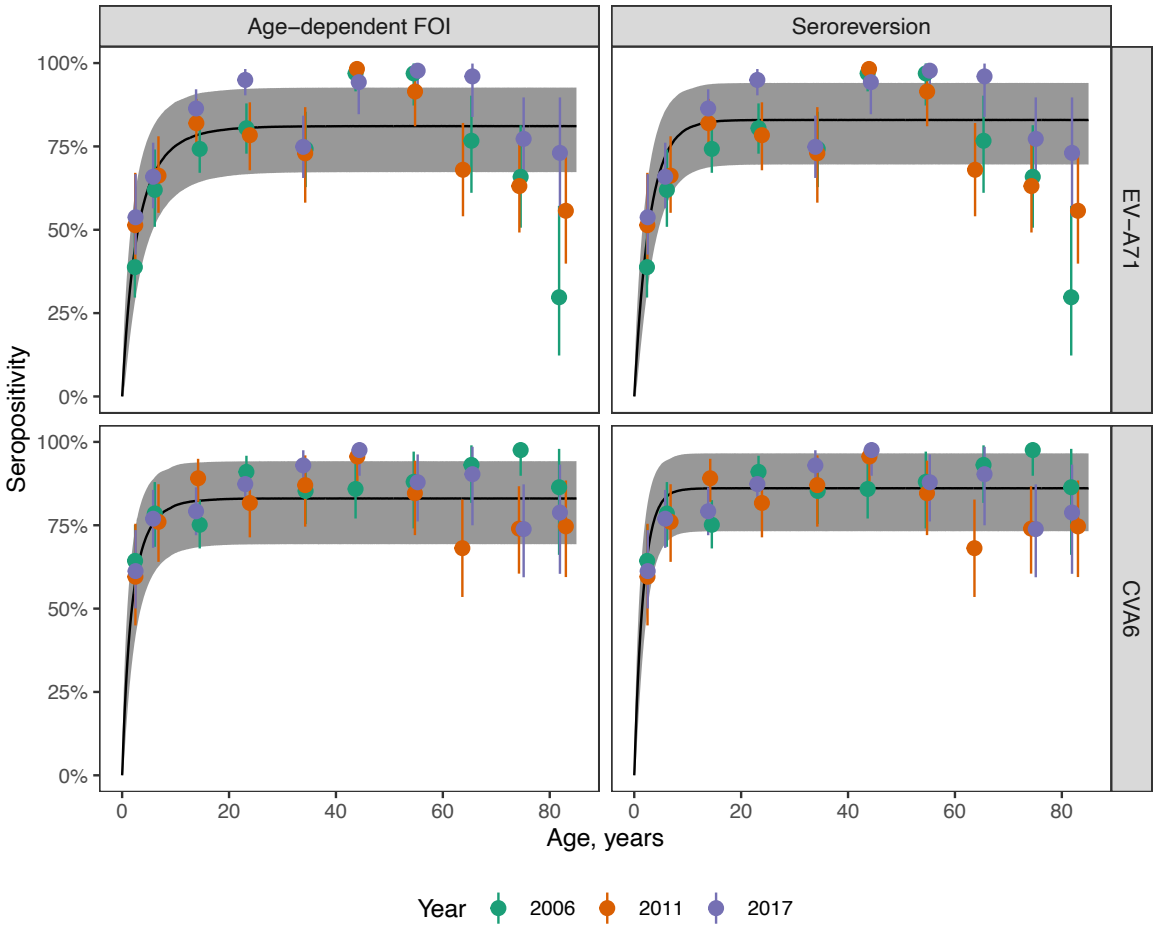


Figure 3 *Model fit to data. The model fits for Model 2 (constant FOI with seroreversion) and Model 5 (age-dependent FOI) are shown for EV-A71 and CVA6. The observed seropositivity values across age are shown as filled circles colored by year of sample collection. The shaded region indicates the binomial sampling errors.*

The columns in the Figure 3 indicate model fits for the Models 2 and 5, which included either an FOI which declines with seroreversion or age. For each virus (i.e., each row), the two models provided very similar fits and mirrored the patterns of variation in the data: particularly, the rapid increase in seropositivity in the first years of life owing to a high FOI; and the plateauing of seropositivity after the teenage years. For EV-A71, these two models could not explain the decline in seropositivity older ages, but Model 6 which

incorporated both an FOI that declines with age and seroreversion replicated the pattern of declining seropositivity (see Figure 4).

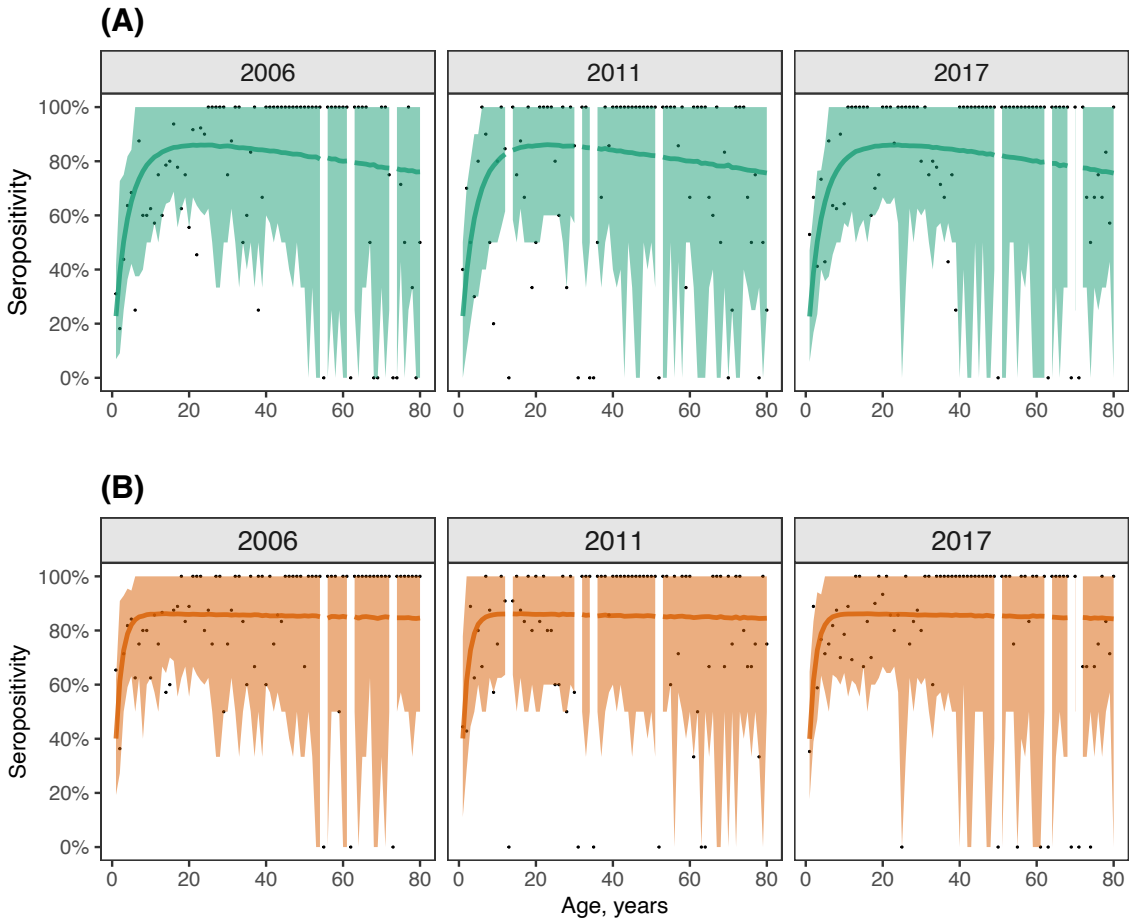


Figure 4 *Model fit to data. Model 6 (age-dependent force of infection and seroreversion) fit to data for EV-A71 (A) and CVA6(B). The observed proportion of samples that were seropositive are shown as black circles. The solid lines and shaded area represent the model's mean predicted seropositivity estimates and 95% Bayesian Credible Intervals. The gaps in the plots indicate absence of data in the corresponding age(s).*

We do not discuss Model 6 further, however, because (i) it provided a similar fit to the data but was more complex than models with either age-dependent FOIs or

seroreversion (Table 3) and (ii) the inferred FOI from this model was infeasibly high (see Table 4), likely reflecting the difficulty in separating the influence of an age-dependent FOI from seroreversion from our data.

Table 4 Parameter estimates of the catalytic models.

Model	**EV-A71: mean (95% Credible Interval)**	**CVA6: mean (95% Credible Interval)**
1 – Constant FOI (λ)	λ = 0.064 (0.06 – 0.07)	λ = 0.082 (0.076 – 0.088)
2 – Constant FOI (λ) with seroreversion (ρ)	λ = 0.3 (0.24 – 0.37) ρ = 0.062 (0.04 – 0.086)	λ = 0.58 (0.43 – 0.78) ρ = 0.1 (0.067 – 0.14)
5 – Age-dependent constant FOI (λ_0)	λ_0 = 0.31 (0.25 – 0.38) β = 0.19 (0.15 – 0.25)	λ_0 = 0.52 (0.41 – 0.65) β = 0.3 (0.22 – 0.41)
6 – Age-dependent constant FOI (λ_0) with seroreversion (ρ)	λ_0 = 0.27 (0.21 – 0.35) β = 0.08 (0.003 – 0.17) ρ = 0.015 (0.0009 – 0.06)	λ_0 = 0.51 (0.39 – 0.65) β = 0.001 (0.00004 – 0.005) ρ = 0.12 (0.072 – 0.19)

Parameter estimates resulting from the different catalytic models using priors defined in Table 2.

The model fits indicated a more rapid increase in seroprevalence with age in CVA6, which is explained through higher estimated FOIs for this virus (Table 4). To illustrate these differences, in Figure 5, we show the modelled probability that an individual has been infected by the two enteroviruses at a given age, which illustrates the greater chance of having been infected by CVA6 than EV-A71, particularly during younger ages. Model 5 with an FOI that declines with age predicts that some individuals never become infected during their lifetimes (20%, 95% Credible Interval [CrI], 17% - 23% for EV-A71; 18%, 95% CrI, 15% - 21% for CVA6), whereas the model with seroreversion indicates that, if an individual lives long enough, they will have been infected by both enteroviruses (Figure 5).

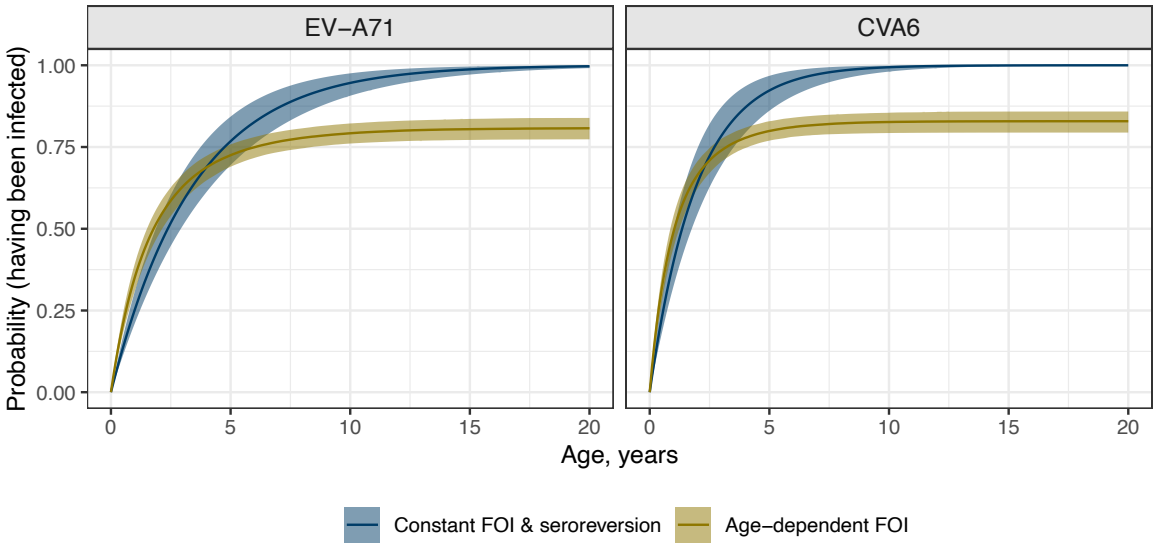


Figure 5 *Estimates of the probability of having been infected. This figure shows the estimated probabilities of infection for EV-A71 and CVA6 by age for the models allowing seroreversion (Model 2) and age-dependent force of infection (Model 5). The solid line indicates the mean posterior estimate, and the shaded region represents the 95% credible interval*.

In the age-dependent models, FOIs fell sharply with age, and the rate of decline in FOI was highest for CVA6 (Figure 6A), with our estimates suggesting that an overwhelming proportion of all first infections occur in individuals under 5 years (CVA6: 96.8% (95% CrI, 96.2% - 97.5%); EV-A71: 90% (89.6% - 92%)). The corresponding results from Model 2 with seroreversion, whilst lower, also indicate that the bulk of infections occur in those under 5 (CVA6: 92% (95% CrI, 86.8% - 97%); EV-A71: 77% (69.5% - 84.8%)). In the models including seroreversion, the rate of decline of antibody detectability was faster for CVA6 than EV-A71 (Figure 6B). For both enteroviruses, the probability of detecting antibodies 12 years after infection was estimated at less than half.

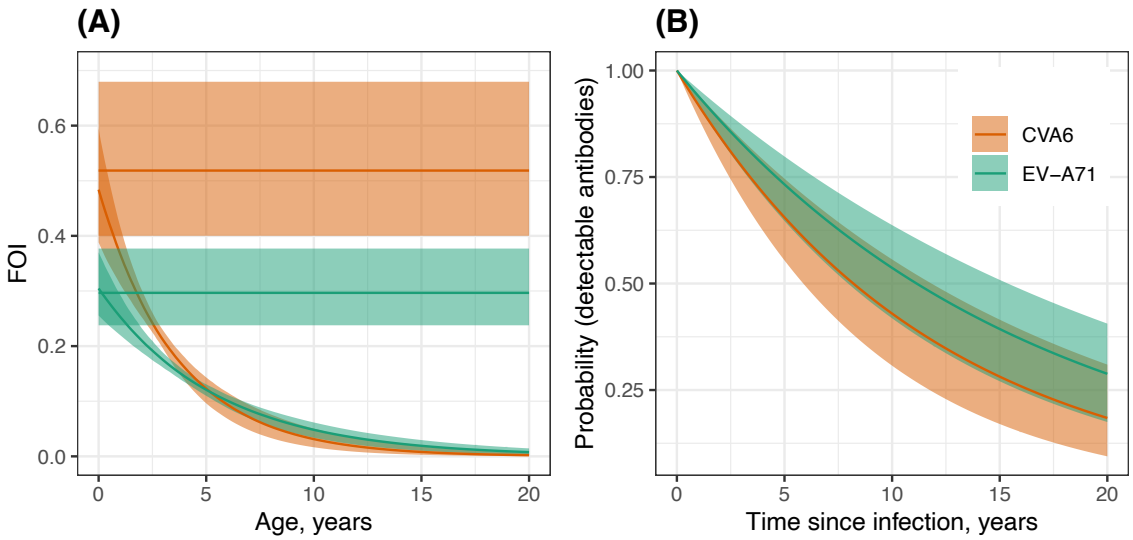


Figure 6 *Force of infection and probability of antibody detectability by age. Panel (A) shows the age-dependent force of infection estimated using Model 5 and the constant force of infection estimated using Model 2 (straight line); panel (B) shows the probability of detecting serum neutralizing antibodies by age for EV-A71 and CVA6.*

We also performed a range of prior sensitivity analyses across the models (see Methods), and the estimates were largely unchanged (Table 5) meaning our conclusions above remained the same.

Table 5. Parameter estimates resulting from sensitivity analyses.

Model	**EV-A71: mean (95% Credible Interval)**	**CVA6: mean (95% Credible Interval)**
1 – Constant FOI (λ)	λ = 0.064 (0.06 – 0.07)	λ = 0.082 (0.076 – 0.088)
2 – Constant FOI (λ) with seroreversion (ρ)	λ = 0.28 (0.23 – 0.35) ρ = 0.06 (0.04 – 0.08)	λ = 0.52 (0.4 – 0.67) ρ = 0.086 (0.06 – 0.12)
5 – Age-dependent constant FOI (λ_0)	λ_0 = 0.3 (0.25 – 0.37) β = 0.18 (0.14 – 0.24)	λ_0 = 0.49 (0.38 – 0.61) β = 0.28 (0.2 – 0.37)
6 – Age-dependent constant FOI (λ_0) with seroreversion (ρ)	λ_0 = 0.26 (0.2 – 0.33) β = 0.075 (0.004 – 0.16) ρ = 0.015 (0.0012 – 0.06)	λ_0 = 0.47 (0.37 – 0.6) β = 0.0017 (0.000057 – 0.0054) ρ = 0.11 (0.065 – 0.16)

These are parameter estimates from sensitivity analysis on the prior for λ for the time-constant FOI models. See Methods for a detailed description of sensitivity analyses.

The simplest model, Model 1, which assumes a constant FOI over time, estimated a mean annual probability of infection (API) of 0.064 (95% Credible Interval (CrI), 0.06-0.069) for EV-A71 and a slightly higher estimate for CVA6 of 0.082 (0.076-0.088). For both serotypes, model I estimated increasing seroprevalence with age, which is expected and consistent with the model assumption (Figure 1). The model did not capture the high seroprevalence in the younger age groups (<10 years) and its estimated seroprevalence does not match the observed/empirical seroprevalence profiles for older age classes (>50 years).

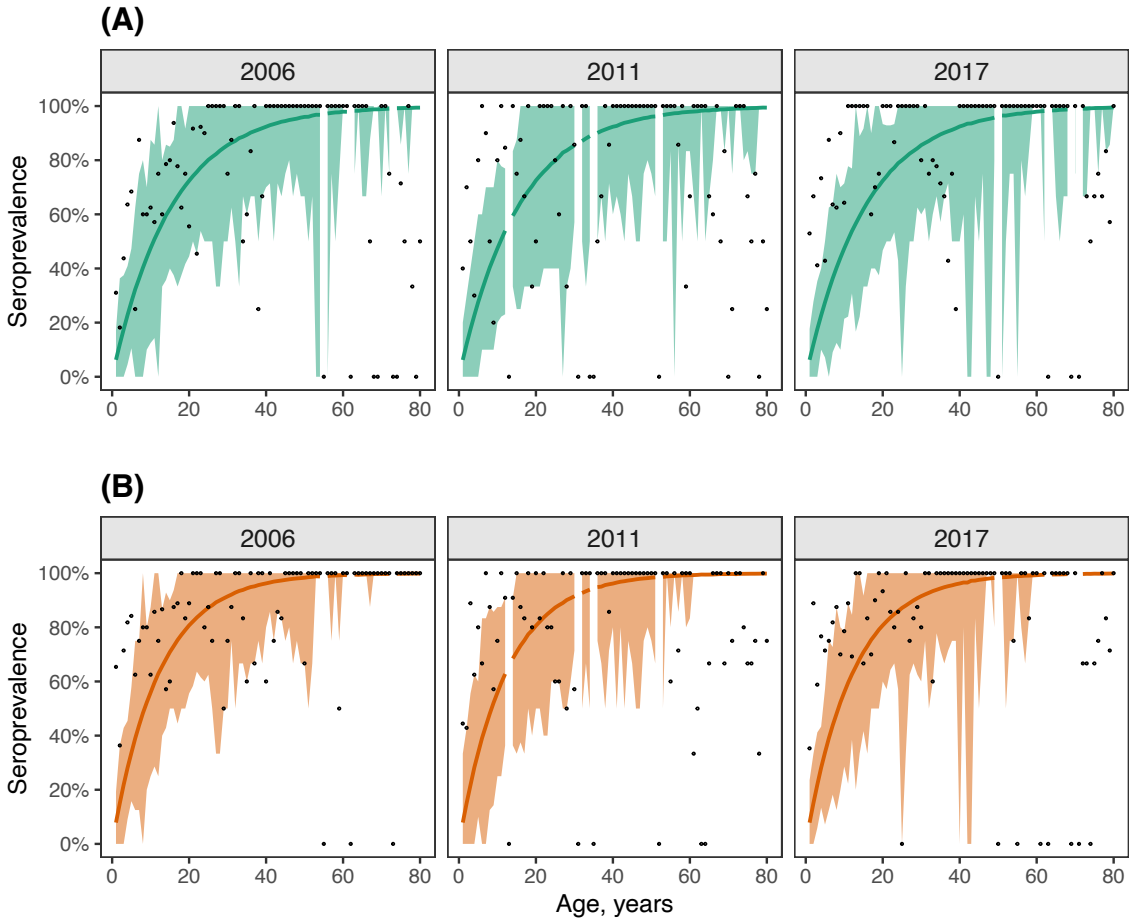


Figure 7 *Model fit to data. Model 1 (constant force of infection) fit to data for EV-A71 (A) and CVA6(B). The observed proportion of samples that were seropositive are shown as*

black circles. The solid lines and shaded area represent the model's mean predicted seropositivity estimates and 95% Bayesian Credible Intervals. The gaps in the plots indicate absence of data in the corresponding age(s).

Discussion

Compared to case-based surveillance, serological data could be more powerful for defining plausible epidemic trajectories by allowing inclusion of susceptible and recovered individuals into mathematical inferencing methods [99]. The objective of this chapter was to use seroprevalence data to estimate the force of infection (FOI) of EV-A71 and CVA6 and infer changes in virus transmission dynamics in England, UK. The best performing model assumed a constant FOI over time and seroreversion, suggesting that seroreversion or waning immunity was important in explaining the observed seroprevalence, and that virus transmission did not vary over time. This model estimated an average seroconversion rate of 25% and 46% for EV-A71 and CVA6, respectively. The time-varying FOI models (Models 3 and 4) estimated a small general increase in FOI, and nearly similar values for both enterovirus serotypes for the period 2006 to 2017. In all models, the FOI estimates for CVA6 were slightly higher than EV-A71's.

Detection of CVA6 in clinical specimens increased over time from 2006 to 2017 as described in *Chapter 3*. For both serotypes, our data and models do not support significant changes in transmission intensity over this period. The alternative explanation for the observed increase in CVA6 detections in England in 2006-2016 is a change in virus pathogenicity (i.e., a higher probability of an infection to result in disease). These results agree with a modelling study of sentinel surveillance data from Japan [34]. CVA6 started to cause large outbreaks of HFMD in Japan in 2011, after years of smaller outbreaks, mostly associated with herpangina rather than HFMD. The modelling study mentioned above tested different hypotheses that could explain the sudden occurrence of large CVA6-related HFMD outbreaks from 2011, and found that an increase in pathogenicity, but not

changes in transmission, explained the Japanese data best [34]. To illustrate a change in CVA6 pathogenicity, a new genetically distinct CVA6 variant was found in atypical HFMD cases in Edinburgh, UK, around 2013, and contained amino acid substitutions that potentially represented host adaptations and may have functionally affected virulence, pathogenicity and transmissibility [301, 330].

Although not recorded in the UK, acute EV-A71 infection waves occurred in Austria, France, Germany, and the Netherlands in the 2000-2010 decade [251], and EV-A71 outbreaks of severe neurological diseases occurred in neighboring European countries in the late 2010's, including Spain in 2016 [12] and Germany in 2019 [13], and the USA (Colorado) in 2018 [14]. Our results do not support the hypothesis that these outbreaks were due to increased transmission of EV-A71, but the analysis of seroprevalence data from the countries affected would allow to corroborate this.

The catalytic models generally captured the observed age-stratified seroprevalence, which lies within the 95% credible interval of the posterior predictive distribution. However, the wide intervals in the predicted seroprevalence (Figures 4 and 6) could be driven by the priors or it indicates that model parameter values are consistent with the data. The posterior predictive distribution is also conditional on the observed data, and in our case, the small sample size per age class. This suggests that some constraints or additional data are necessary.

Models which included seroreversion (i.e., loss of detectable antibodies) were favored (along with those including age-dependent FOIs) in the model comparison and estimated a duration of antibody detection of 15 (11-22) years for EV-A71 and 9 (6-14) years for CVA6, suggesting that reinfections occur for both serotypes but not frequently. There are no substantial data or empirical estimates of duration of neutralizing antibodies

for EV-A71 or CVA6, and the only data that come close are from a recent study which showed that neutralizing antibody titers for EV-A71 and CVA16 remain high, years after illness onset [450]. However, CVA6 was not tested in that study and the longest sampling time was 26 months after illness onset, hence not a firm picture of the kinetics of antibody responses in enterovirus infections. Despite the different pattern on the number of EV-A71 and CVA6 detections reported over time in England, the analysis of seroprevalence data suggest strong similarities in the transmission dynamics of these two serotypes during the study period. This probably reflects shared transmission routes.

More precise age-specific seroprevalence estimates, for the younger age classes, would allow inference of possible transmission changes at a finer timescale for the most recent years. As such, prioritizing sampling younger ages over older ones, may be a strategy to maximize the information that will support the estimates of the force of infection. Similarly, sampling more often, may allow to infer transmission changes at a finer timescale. This could be particularly important for EV-A71, which exhibited a 2–3-year cycle as seen in Chapter 3. However, with data at only three time points, we were only able to estimate general trends in the force of infection over several years.

Other limitations are inherent to our study. We assumed complete single-serotype neutralizing antibody reactivity, but we cannot discount potential non-specific serologic cross-reactivity arising from varied exposure histories with other enteroviruses. Presence of overlapping viral receptor repertoires [451, 452] and cross-serotype immunological interactions between enteroviruses has been documented [453]. Ecological forcing and interactions between closely related EV serotypes such as those causing HFMD were also illustrated in recent studies [375, 454]. However, these factors are unlikely to bias our results since virus neutralization tests, as used in our study, are more sensitive and specific

for detecting virus-specific neutralizing antibodies, and any cross-neutralization is very limited/absent. Although classifying individuals either as seropositive or seronegative based on a cut-off provides a straightforward estimation of seroprevalence, it leads to a loss of information. The reconstruction of historical transmission patterns could be improved by using a model based on antibody levels. A future direction for this work is to develop a model framework that uses antibody titers.

The analyses here provide an improved picture of EV-A71 and CVA6 transmission in England and may be useful for estimation of disease burden and severity of infections caused by these viruses. It further underscores the value of serological data to unravel transmission patterns, particularly for pathogens that cause many asymptomatic infections and disease for which surveillance is passive. The interpretation of the number of the reported infections from the passive surveillance systems may lead to erroneous hypothesis about the levels of pathogen circulation, as has been demonstrated here.

This work contributes to our understanding of the causes underlying the emergence of enterovirus related disease outbreaks and supports the hypothesis of phenotypic changes as a driver of enterovirus emergence. There is a need for further virology studies of EV-A71 and CVA6 to investigate the potential mechanisms of evolution of phenotype and pathogenicity including the genetic determinants of replication, tropism and receptor usage that have contributed to their emergence. Such investigations would include identification of clones of contemporary strains based on evolutionary trees, followed by detailed mapping or characterization of phenotypic effects associated with key genetic mutations.

The modelling framework here could be adapted to integrate uncertainty from the laboratory assays such as sensitivity and specificity; population demographics and

contemporary data from case-based surveillance or syndrome surveillance for improving parameter estimation [455][456]. A key challenge when modelling serological data is parameter identifiability, where the data may not provide sufficient statistical power to allow precise parameter estimation [457]. The rate of seroreversion (ρ) could not be reliably estimated with the current seroprevalence data when assuming time-varying FOI. Instead, we fixed ρ in the parameterization of model 4 suggesting that the data were insufficient. Since there are no empirical data on enterovirus seroreversion rates, there was no information to define a proper prior distribution of ρ. Previous studies found that seroreversion rates vary by age [437, 457], and that the population-level estimation of seroreversion can be challenging when the force of infection varies geographically and by time [458].

Generally, the measured antibody responses in cross-sectional studies reflect cumulative exposure and immunological memory. This could have implications in the estimated seroprevalence and without substantial contemporary surveillance data of enterovirus infections describing virus prevalence in England, the proportional contributions of the serology data to the estimated change in FOI could not be fully assessed. Future modelling studies should account for reinfections or antibody boosting for accurate estimation of virus transmission.

The results here contribute to our knowledge on enterovirus transmission and present an initial estimation of infection burden of EV-A71 and CVA6. The analyses here show that insights on transmission dynamics can be obtained using simple models when there is no strong evidence that basic epidemiological assumptions are violated.

Chapter 5: Assessment of reference materials for virus neutralizing assay standardization and harmonization

Summary

Regular participation in external quality control programs helps laboratories identify gaps in their procedures. Biological standards and reference materials are important in assay standardization for evaluation of vaccine efficacy and quality control, and in seroprevalence studies relying on neutralizing antibody quantification. Calibration to a common reference material improves assay standardization globally, reducing variability across laboratories. This work was part of a collaborative study aiming to establish the 1st WHO International Standard (IS) for antiserum to EV-D68, to enable harmonization and comparison of results across multiple assay formats. Additionally, neutralization assay protocols for Echoviruses were compared among laboratories of the ENPEN network. Inter-laboratory concordance was better for the EV-D68 assays, but virus neutralization assay formats differed between laboratories for the Echoviruses, which limited comparability of results. The discordance in antibody measurements between laboratories emphasizes a further need for assay standardization and calibration, which is crucial for continued monitoring of infections.

Introduction

Measuring serum neutralizing antibodies is important for evaluating vaccine immunogenicity and efficacy, and in seroepidemiological studies, and therefore having reagents to support standardization would be beneficial. There are increasing demands to standardize laboratory methods for measuring past or current infections and to establish relevant biological reference standards to ensure that methods are accurate, sensitive, and reproducible [449]. Serum neutralization assays come in multiple formats and this challenges accurate comparison of neutralization titers from multiple clinical trials in vaccine research. Assay formats vary widely by viral strains, cell lines, use of complement, and results reporting formats, leading to potential variation in antibody measurement against vaccine candidates [450].

A biological reference standard serves as a calibrator against which the concentration or potency of an analyte present in a biological sample is measured and defines an internationally agreed, arbitrary unit that allows comparison of biological measurements [451]. Using a candidate International Standard (IS) as a reference and expressing the results in International Units significantly reduces the variation between laboratories. Inclusion of an appropriate calibrated antibody standard for adjustment of original titers substantially increases reproducibility of serological results from different laboratories and significantly improves regulatory evaluation of vaccine efficacy [452]. This was demonstrated for Influenza, SARS-CoV-2 and HPV16 immunoassays where absolute titers were adjusted relative to a calibrated standard and inter-lab variation was drastically reduced and acceptable agreement of results achieved [450, 452, 453].

International Standards (IS) established by the World Health Organization (WHO) are the highest order biological references that enable laboratories globally to calibrate their bioassays and report measurements that are comparable [450]. There is a wide repertoire of WHO standards available, including 22 biological reference preparations in the "immunoglobulins and human sera" category and 83 biological reference preparations in the "vaccines/toxoids/toxins" category (https://www.who.int/bloodproducts/catalogue/en, accessed on 20 April 2022). For enteroviruses, an IS for EV-A71 was established in 2018 [454], but none exists for EV-D68. Development of an international standard for EV-D68 would have important implications for seroepidemiology studies as it would contribute to the harmonization of assay read-outs, hence facilitating the comparison of assays conducted in different laboratories and increasing confidence in results.

Given the importance of qualitatively and quantitatively detecting antibody responses in the current research settings, and likely in future prophylactic scenarios against enteroviruses, the National Institute of Biological Standards and Controls (NIBSC) initiated a collaborative project to investigate the possibility of developing an international standard serum to measure EV-D68 neutralizing antibodies, which would be suitable for the evaluation of the immunogenicity of EV-D68 vaccines. On the same note, the European Non-Polio Enterovirus Network (ENPEN) initiated a study to identify the capacity for echovirus serosurveillance studies in Europe by comparing and evaluating virus neutralization assay protocols used across different laboratories. Harmonization of the echovirus serology protocols will be useful for surveillance seeing as echovirus infections are common and clinically relevant as they are associated with meningitis and meningoencephalitis outbreaks [455, 456].

The first aim of this chapter was to characterize eight candidate anti-EV-D68 sera in neutralization assays to assess their suitability for use as the 1st International Standard for anti-EV-D68 serum and for harmonization of EV-D68 neutralization assays. The second objective was to evaluate variability between virus neutralization assay protocols for Echoviruses across different laboratories in the ENPEN network.

Materials and Methods

NIBSC EV-D68 panel

Eight candidate samples, labelled S1 to S8, produced from plasma donated by healthy individuals for use as reference standards for anti-EV-D68 serum were sent by NIBSC to 15 laboratories. S1-S4 were freeze-dried and reconstituted in 0.4ml of sterile water before use; S5 and S6 were liquid presentations; while S7 and S8 were freeze-dried and reconstituted in 0.5ml of sterile water before use. S1/S3 and S2/S4 were duplicates of the same sample. An EV-A71 international standard was also supplied, in freeze-dried medium, and reconstituted in 0.5ml of sterile water before use. All samples were stored in 0.1ml aliquots at $\leq$-20°C.

Two EV-D68 isolates, (i) the 1962 prototype Fermon strain (labelled '*virus 1*') and (ii) a 2016 subclade B3 strain (labelled '*virus 2*') were provided to evaluate the difference in neutralization capacity of the serum candidates to new and old strains. The choice of subclade B3 was to reflect the most commonly circulating EV-D68 strain at the time of the study. Both viruses had been grown in RD cells and were supplied with known titers: virus 1 had 1 x 10^8 $TCID_{50}$/ml and virus 2 had 3.2 x 10^7 $TCID_{50}$/ml and stored in 0.1ml aliquots at $\leq$-70°C. Virus titers were confirmed prior to the neutralization assays. Ten serum samples used in the EV-D68 seroepidemiology study (Chapter 2) were added here to check for seroreactivity to the two challenge viruses mentioned above. Also, our 2016 EV-D68 strain from Finland (EV-D68/FIN/2016) (used in *Chapter 2*) was also used here as an additional challenge virus against the eight candidate samples. Positive and negative EV-D68 controls used in Chapter 2 were added to the testing panel.

ENPEN Echovirus panel

Five echovirus strains of type E1, E18, E30-G1, E30-G6 and E6 with unknown viral titers were sent alongside four dilutions of an IVIg (intravenous immunoglobulin) (1:1, 1:4, 1:16 and 1:64) to 12 laboratories across Europe. The IVIg samples were labelled as S1-S4 and echoviruses labelled as V1-V5. In our lab, virus concentration and neutralizing antibody titers were determined using the protocols described in *Chapter 2*. Neutralizing antibody titers were determined as the end point dilution based on the last serum dilution showing development of cytopathic effect (CPE). Assay protocols and results were compared among nine out of 12 laboratories which reported back.

Data analysis

Geometric mean titer (GMT) for each sample was calculated. The geometric coefficient of variation (GCV), which gives the measure of percentage of relative variability, was also calculated as '1 – geometric standard deviation * 100' to assess between-assay variability. GMT and GCV were calculated using the PKNCA package in R (R Core, 2020).

Results

NIBSC EV-D68 panel

A blinded panel of eight serum samples were tested for neutralizing antibodies against two EV-D68 strains in three independent assays for each virus. Independent titer determinations of the challenge viruses were performed prior to the neutralization antibody measurements.

Our data

All the sera showed neutralization activity against both strains tested with S1-S3 having higher neutralization titers against the Fermon strain and S4 having high neutralization titers against both Fermon (virus 1) and subclade B3 (virus 2) strains (Table 1). However, neutralization titers against virus 2 were lower than those against virus 1 for all samples except S4 and S8, which showed small differences. Samples S7 and S8 had lower neutralizing titers against virus 1 compared to S1-S6. There was no intra-assay variability, i.e., variability between duplicates for individual samples within an independent assay. Inter-assay variability (measured for replicate assays) as illustrated in Table 1 was minimal and ranged from 0% (no difference in replicates) to 49% (2-fold difference between replicate assays). The ratios of GMTs against the two viruses were calculated for each sample (Table 1) and used to compare data between laboratories.

Table 1 End point neutralization titer results against the NIBSC EV-D68 challenge viruses

	Assay	**Sample**								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**EV-A71 IS**
Virus 1	**1**	5793	5793	5793	2896	2896	2896	724	724	724
	2	5793	5793	5793	2896	2896	2896	724	1448	724

	3	5793	5793	5793	2896	2896	2896	724	724	724
	GMT	5793	5793	5793	2896	2896	2896	724	912	724
	GCV	0	0	0	0	0	0	0	49	0
Virus 2	**1**	724	724	1448	2896	362	362	362	724	181
	2	724	724	1448	1448	362	724	362	724	181
	3	724	1448	724	2896	362	362	362	724	181
	GMT	724	912	1149	2299	362	456	362	724	181
	GCV	0	49	49	49	0	49	0	0	0
Virus 3		4096	5793	5793	2048	2896	2896	1024	724	362
GMT ratio between viruses (%)		800%	635%	635%	125%	800%	635%	200%	125%	400%

GMT: geometric mean titer, GCV: geometric coefficient of variation.

Ratio calculated as: GMT of virus 1/GMT of virus 2

The NIBSC candidate sera and the EV-A71 IS were also tested in duplicate against another EV-D68 strain circulating in 2016 (virus 3). In this test, the candidate sera showed equally higher levels of neutralizing reactivity to virus 3, compared to titers observed with virus 2 (Table 1). Viruses 1 and 2 were also tested against 10 UK serum samples used in the EV-D68 study in *Chapter 2*. Generally, the UK samples had similar titers against virus 1 and 2, and these titers were comparable to titers against virus 3 as observed in *Chapter 2* (Table 2).

Table 2 Neutralizing antibody titers of the PHE sera against the three EV-D68 strains

Sample	Age (years)	Gender	Titer – Virus 1	Titer – Virus 2	Titer – Virus 3
PH01	1	F	32	32	32
PH02	3	F	128	128	128
PH03	8	M	256	256	256
PH04	19	M	256	387	256
PH05	3	F	64	64	64

PH06	6	F	256	256	256
PH07	11	M	256	256	362
PH08	5	F	256	256	256
PH09	36	M	>1024	>1024	>1024
PH10	23	M	>1024	>1024	512

Data comparison between laboratories

Data from other laboratories were shared among study participants for review and self-evaluation. S5 and S4 showed the largest differences in neutralization titers against virus 1 and 2, respectively. S7 and S8 had the smallest differences in titers against virus 1, while S1, S3, S5, S7 and S8 had the smallest variation in titers against virus 2 (Figure 1). Neutralization titers for S1 to S5 against virus 1 were symmetrical, while titers for S2, S4 and S6 against virus 2 were right skewed. Our own results against virus 1 for S5 and S7, and against virus 2 for S2, S4, S5 and S7, were within the 75th percentile (Figure 1). However, our titers for S1 to S4 and S8 against virus 1, and for S1, S3 and S8 against virus 2 were extreme and distant/outliers from other laboratories.

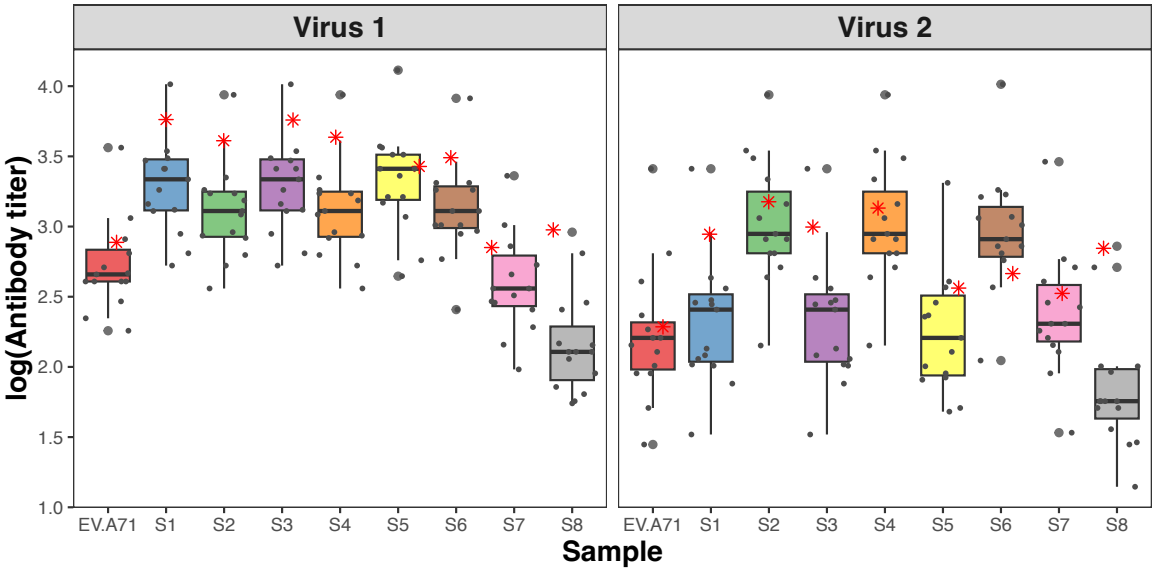


Figure 1. *Distribution of laboratory mean end point titer estimates against virus 1 and 2 for various serum sample. Our data are indicated by '*'. Data from other labs are indicated by grey circles. Note: S1 and S3, S2 and S4 were duplicates of the same sample.*

Intra-assay precision was good among the participating laboratories at the rates of 92% and 94% for virus 1 and 2, respectively. Inter-assay variability, as illustrated by the between-assay geometric coefficient of variation (GCV) in Figure 2, ranged from 0% (no difference in results obtained for a sample in all three assays) to 139% (for sample 5 with virus 2) with only few instances of high inter-assay variability observed. The median %GCV ranged from 20% to 42% for virus 1 and from 30% to 50% for virus 2 (Figure 2). Between-laboratory %GCVs were below 100% for virus 1 (panel A in Figure 2) and variability in %GCV values was higher for titers against virus 2, and notably for sample 5 (panel B in Figure 2). Our own %GCV values compared poorly with other laboratories for virus 1 but aligned well with other labs for virus 2 for five serum samples (Figure 2). Overall, our %GCV values were mostly lower than other labs indicating we had better inter-assay agreement. All between-laboratory %GCVs were >100% indicating high variability between laboratories (Table 4).

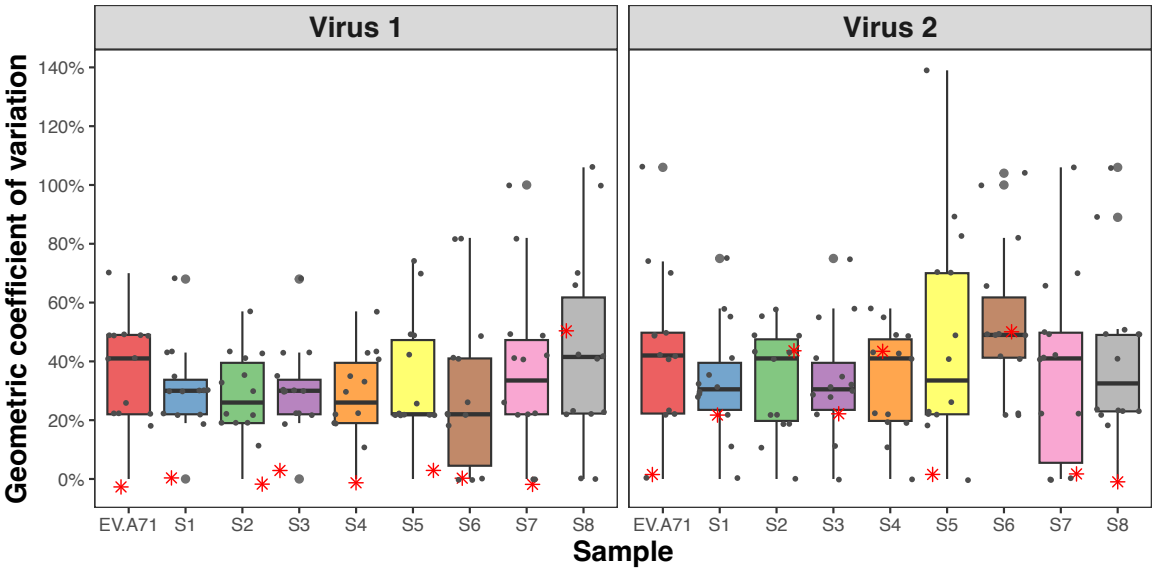


***Figure 2**. Inter-laboratory variability in geometric coefficient of variation (%GCV) for the two EV-D68 viruses. Our data are indicated by '*'. Grey circles indicate outlier values. Note: S1 and S3, S2 and S4 were duplicates of the same sample.*

Summary calculations of mean, %GCV and median end point titers across laboratories are shown in table below.

Table 4 Summary Table for reported end point titers for all labs combined.

Virus	Sample	Mean	GCV	Median
1	EV-A71	520	105%	456
1	1	2014	121%	2170
1	2	1340	123%	1290
1	3	2014	121%	2170
1	4	1340	123%	1290
1	5	2149	125%	2580
1	6	1331	117%	1290
1	7	390	121%	362
1	8	146	130%	128
2	EV-A71	170	191%	161
2	1	217	191%	256
2	2	1092	169%	887
2	3	217	191%	256

2	4	1092	169%	887
2	5	177	166%	161
2	6	901	166%	813
2	7	244	170%	203
2	8	69	180%	57

A comparison was performed by calculating the ratio between the reported end point titers against virus 1 relative to titers against virus 2 for each study sample in each laboratory. The ratio of titers ranged from 0.3 to 51, with the largest titer variations between the two EV-D68 viruses being observed in S5. Overall, the results show consistency across all labs for S2, S4, S6-S8, and the EV-A71 standard. S1 and S3 show large variation in titers between laboratories for virus 1 and 2 (Figure 3).

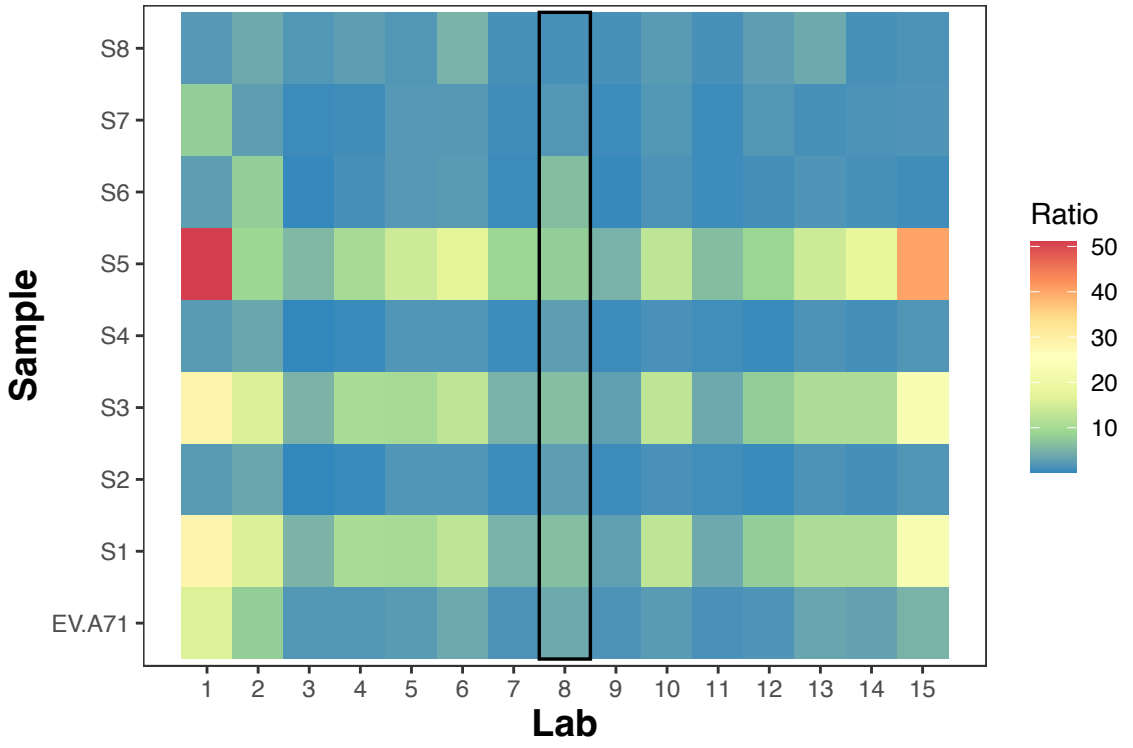


***Figure 3**. Ratio of reported geometric mean titers using virus 1 relative to virus 2. Our lab is indicated by the black border. Note: S1 and S3, S2 and S4 were duplicates of the same sample.*

ENPEN Echovirus testing panel

Neutralising antibody titres were determined against five echovirus types V1-V5 (E1, E18, E30-G1, E30-G6 and E6, respectively) in four samples S1-S4 (IVIG 1:1, 1:4, 1:16, 1:64 dilutions). Different laboratories used their own protocols.

Method or assay comparison

Incubation lengths, cell type, whether use of cell suspension or monolayer, the number of cells used and the methods of titre calculation, all varied between laboratories (Table 5). Nine laboratories used RD cells, and one lab using three cell lines. The number of cells per well varied among labs and ranged from $1x10^4$ to $5x10^5$. Half of the participating laboratories used cells in suspension and inactivated serum complement before setting the assay. The virus-sample incubation period was between one to three hours and the incubation period after adding cells varied from three to seven days. All participating labs used an equal volume of 100TCID50 of virus and sample except for one which added 200 TCID50 of virus. All labs used CPE or crystal violet staining for assay read-out.

Table 5 Characteristics of the virus neutralization assay protocols. The blank/white spaces indicate unreported data. Our lab results are shown in red. (M) = monolayer, (S) = suspension.

Lab	Cell type	Cells/well	(M)/(S)	Virus/sample volume (μL)	Virus:Sample incubation	Titer calculation	Incubation period
1	HT29, RD, A549	Not counted	S	50/50	1	Reed Muench	7

2	RD	18000	M	50/50	3	Reed Muench	6
3	RD	15000			2		3
4	RD	20000		25/25			5
5	**RD**	**15000**	**S**	**50/50**	**1**	**Reed Muench**	**3**
6	RD	25000	M	25/25	3		7
7	RD	25000	M	50/50	3		3
8	RD	500000	M	25/25	3	Reed Muench	6
9	CaCo		S	50/50	3		5
10	RD	14400					6
11	RD	Not counted	M	30/30	3		4
12	RD, LLC-MK2	RD: 15000 LL: 10000	S	25/25	1	Reed Muench	RD = 3, LLC-MK2 = 4

Up to 3log10 TCID50/mL differences in virus concentration were observed between labs (Figure 4). Our virus concentrations were within the interquartile ranges, and the concentrations for E30-G1, E30-G6 and E6 were close to the median values. Half of the participating labs conducted virus backtitration and the reported virus titers were between 10^1.5 and 10^2.5 TCID50/well.

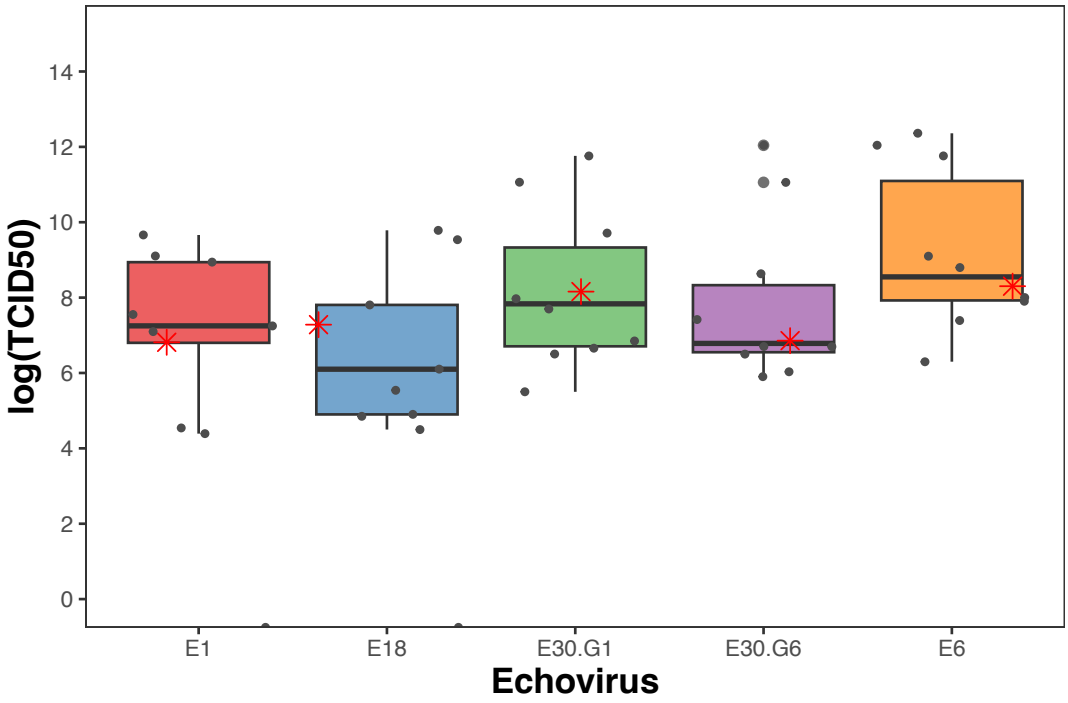


Figure 4*. Virus titers as obtained in different lab conditions. Gray dots indicate data from other labs, '*' indicates data from our lab.*

Neutralizing antibody titers

Antibody titers of S1 (IVIG 1:1 dilution) were compared across labs (Figure 5). A wide range of titers were observed among laboratories especially against E1 and E18. Titers ranged from 1:4 to 1:1024 for E1 and E18, from 1:8 to 1:1024 for E30-G1, from 1:8 to 1:1024 for E30-G6 and from 1:64 to 1:1024 for E6. The geometric coefficients of variation for E1, E18, E30 G1, E30 G6 and E6 were 44%, 53%, 28%, 24% and 16%, respectively. Our own antibody titer measurements against E1 and E18 were quite high compared to other laboratories, while those against E30 and E6 were comparable to other labs.

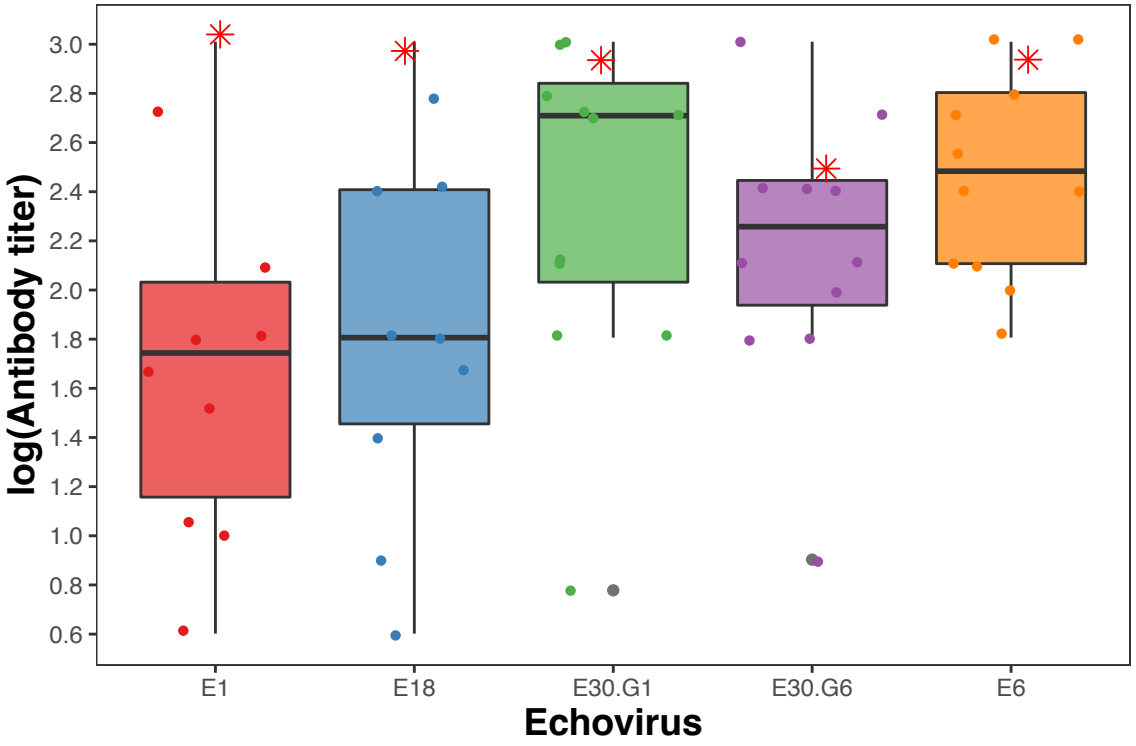


***Figure 5**. Neutralizing titres for sample 1 (IVIG 1:1, neat concentration) against different Echovirus types. Colored dots indicate data from other labs, '*' indicate data from our lab.*

Discussion

Different assays are used for antibody detection and employ different cells and virus strains, resulting in difficulties comparing and interpreting results on virus antigenic and immunogenic properties. Here, two studies were carried out to (i) establish the 1st WHO international standard for human anti-EV-D68 serum and (ii) evaluate variability in enterovirus neutralizing assays across geographically dispersed laboratories. The studies were conducted in a multi-laboratory survey using common specimens to assess feasibility and the degree of agreement across diverse neutralization assay formats.

All eight serum preparations in the EV-D68 panel had good levels of neutralizing activity against two viruses and there was good concordance within and between assays using the same challenge virus, indicating that the assay format used across laboratories was suitable to assess the study materials. However, there was high interlaboratory variation across laboratories (seen as high GCVs), likely due to methodology or local technical variations in assessing neutralizing antibodies in a cell-based system. Technical variations include (i) variability in treatment of sera to remove non-specific inhibitors, (ii) or using cells lines with different receptor specificities, (iii) or variation in other parameters such as size of wells in a plate and serum volume.

S1 and S3, and S2 and S4 which were duplicates from the same sample had similar titers between laboratories. For all testing material, the within-laboratory variation was low and therefore, generally good. Serotype specificity is an important consideration in enterovirus immunoassays and the candidate sera tested showed high cross-reactivity to EV-D68 strains belonging to clades B3 and D, which have been circulating globally in recent years [230, 457]. This indicates that the candidate specimens are suitable as reference

standards to measure the neutralization activity in human sera against a wide range of EV-D68 strains from different genotypes.

Overall, the neutralizing antibody assays used by various laboratories seemed accurate, sensitive, reproducible, and suitable for assessing the study materials, and would be generally fit for evaluation of immunogenicity of EV-D68 vaccines and assessment of the seroprevalence against EV-D68 in future studies. Based on the higher level of cross-reactivity of S2/S4, the original serum pool from which S2 and S4 were derived was proposed as the 1st International Standard for anti-EV-D68 serum (Human) with an assigned potency of 1,000 IU of anti-EV-D68 neutralizing antibodies/ampoule. It was suggested that the serum pool for S1/S3 should be used as a control for neutralization assays.

For the ENPEN Echovirus panel, variability in antibody titers was higher for E30-G1 and E6, but lower for E1. Differences in assay protocols were observed across laboratories including cell type, virus concentration ($TCID_{50}$) and incubation length, which might explain the variability observed in antibody titers. An extended serum-virus incubation time has been associated with a more specific, sensitive and reproducible assay for vaccinia neutralizing antibodies [458], and maybe the reason why some laboratories opted to have a longer incubation period. Majority of the participating laboratories used RD cell lines, but some used different cell lines and so it is likely the differences in titers were also influenced by virus susceptibility in different cell lines.

Cells or tissues are different in their permissibility for primary infection and replication. For instance, a gradient of sensitivity to primary infection across eight cell lines was observed for Influenza viruses resulting in underestimation of neutralization titers [459]. The Echovirus ENPEN panel showed varying virus yields between suspension and

adherent cell culture system, which should be something to be considered during assay harmonization. The Influenza study described in [459] observed no differences in viral yield between suspension and adherent cells but showed that the peak yields were reached earlier with suspension cells.

Given that the four specimens tested against the different Echovirus types were dilutions of IVIg material, the differences in neutralizing antibodies against each echovirus type could indicate antigenic variation and variability in the frequencies of type-specific antibodies in the population. Echoviruses seem to be experiencing antigenic drift from year to year according to a recent study [460]. This could pose a practical difficulty in developing a long-term standard serum for widespread use in echovirus serosurveillance and quite possibly affect the future use of IVIg for prophylaxis of infections with antigenically distant strains, despite their clinical benefit.

Reference antisera or controls were not included with the ENPEN panel to calibrate the results. Reference materials are essential for optimization and validation, evaluation of specificity, sensitivity, precision, and accuracy and for establishing assay parameters e.g., dilution range or calibration curves [461]. Reference standards are also useful for monitoring assay stability, reproducibility, and consistency within and between laboratories [462].

In both studies, each participating lab was responsible for their own protocols and reagents. However, inter-laboratory results are thought to be more homogenous and less variable when virus stocks and cell lines are provided from a common source [463]. Still, the practicality of introducing harmonized procedures and reagents across laboratories is doubtful, particularly where laboratories are required to validate their own assays. It may also not be possible to adopt a consensus protocol, plus the capacity for all laboratories to

use the same reagents may be limited by availability and costs of some commercially available items. Various approaches could be implemented to facilitate use of a consensus protocol, e.g., use of training videos as demonstrated for Influenza serology [464].

In conclusion, comparison of assays is not always straightforward, and the discordance in antibody measurements between laboratories emphasizes a need for greater effort in assay standardization and calibration using multiple samples covering a broad range of neutralization potency. Enteroviruses are predicted to remain in circulation for years to come due to emergence of new strains and lack of vaccines. Monitoring of acute and convalescent infections and the broad spectrum of immunity against enteroviruses will remain a routine task for clinical microbiology and virology facilities. Therefore, harmonization of serology assays and the ability to compare results between laboratories remains crucial.

SUMMARY AND CONCLUSION

Seroepidemiology studies use established methods for assessing population immunity and provide information on pathogen circulation. This thesis maps the landscape of circulation and exposure to three commonly circulating enteroviruses (EV-D68, EV-A71 and CVA6) and one rarely detected enterovirus type (EV-D111). These viruses have emerged recently as causes of outbreaks, including more severe, atypical disease manifestations than previously described.

Virus neutralizing antibody titers were measured in sera and used to estimate age-stratified seroprevalence and force of infection (FOI). High seropositivity was observed for all four viruses indicating extensive transmission. The seroprevalence of EV-D68, EV-A71 and CVA6 did not vary significantly between 2006 and 2017 despite their clinical emergence during this period, implying changes in pathogenicity rather than changes in population immunity. EV-A71 antibody titers declined with age, which could be attributed to the duration of protection related to the population dynamics and homeostatic regulation of memory B and T cells. Differential age-specific seroreactivity was observed for EV-D111, which could signify the neutralization potency of cross-reactive polyclonal responses and requires further studies.

It was hard to compare the measured seroprevalence with other studies, where such data were available, because of differences in seropositivity cutoffs, sampled populations, heterogeneous sampling strategies, time of sample collection, data aggregation, laboratory methods and differences in attack rates. It was also noted that some age classes had inadequate sample size and a greater focus on sampling design is needed for precise and unbiased seroprevalence estimates.

Enterovirus surveillance focused on clinical disease is limited as only a subset of infections are clinically apparent and are detected through virological testing. This was evident based on the virological data presented in *Chapter 3*. Since enterovirus reporting in UK is passive and voluntary, the available data are sparse and insufficient to fully characterize virus circulation dynamics. Enhanced or improved enterovirus surveillance is warranted.

In *Chapter 4*, serocatalytic models fitted to the seroprevalence data showed a small increase in the force of infection of EV-A71 and CVA6 over time. We determine that there has been persistent historical FOI for both enteroviruses and there is evidence of age-dependent risk or infection and / or seroreversion in each case. We found that transmission intensity for both enterovirus serotypes has remained relatively unchanged in a 12-year period and the change in enterovirus-related disease across England during this period could be attributed to increased pathogenicity (probability of disease after infection).

Standardization and harmonization of virus neutralization assays is challenging and poor reproducibility of seroprevalence results is widely cited, limiting comparisons in clinical trials and between seroprevalence studies. Two studies described in *Chapter 5* showed the difficulty of assay comparison and reproducibility, which could be due to varying physical resources and capacity or expertise. Since neutralization assays will continue to play a role in enterovirus surveillance, harmonization and standardization of assay parameters and formats, e.g., use of common protocols and reagents, and international standards to calibrate assays, will be effective in reducing interlaboratory variability and for improved comparison of seroprevalence studies.

In summary, the work presented in this thesis demonstrates the application of serology and a mathematical framework to estimate pathogen prevalence and

epidemiological parameters. This thesis generated novel seroepidemiological data and used innovative methods to conduct an in-depth investigation of non-polio enterovirus epidemiology.

Future studies

The continuing emergence and widespread circulation of pathogenic enteroviruses poses a big public health threat. Efforts to understand enterovirus transmission and pathogenicity require continued input of resources and further investigations. Suggested priorities and studies are listed below:

1) Expand the serological dataset to include nationally representative samples from other years, for example mid to late 1990s and early 2000s. This will enable robust quantification of changes in seroprevalence and virus transmissibility over time. More serology data would also support more accurate estimation of duration of detectable neutralizing antibodies.

2) Further modelling studies could include fitting models that incorporate reinfection and immune boosting, seeing as the seroprevalence data reported here show increase in antibody titer with age. Statistical models used here could be extended to explore the effect of age-specific mixing on the primary estimates of transmissibility - R_o and FOI, and to allow for infection with two or more antigenically divergent strains. This approach has been used to study transmission dynamics for Dengue virus, Influenza and SARS-CoV-2.

3) Increase support for clinical enterovirus surveillance and promote virus genomics to monitor genetic diversity. Thus far, the national (UK) approach to enterovirus monitoring

has focused on voluntary reporting. Given the vast diversity and complexity of non-polio enteroviruses, there is a need for more systematic surveillance and sequencing of circulating strains. This will enable detailed studies on seasonality and periodicity, and estimation of changes in pathogenicity over time. Clinical data would also be useful to study variation of disease by age, and whole genome sequences would be useful for associating coding and non-coding genomic changes with pathogenicity or clinical manifestation. For instance, the sentinel influenza-like illness surveillance platform in the UK could be targeted for EV-D68 monitoring by testing influenza negative respiratory samples.

4) Experimental studies to investigate antigenic evolution and its potential contribution to enterovirus emergence and pathogenesis. Mutations that change virus antigenic phenotype enable strains to circumvent immunity conferred by natural infection and are particularly important. Selection for amino acid changes has been reported in putative neutralizing antibody epitopes for enteroviruses and could consequently affect immune recognition. The change or increase in seroprevalence in UK as observed with our data could suggest antigenic divergence and variability in cross-neutralization such that antigenically different strains cause reinfections leading to a boost in neutralizing antibodies.

To evaluate antigenic evolution, homotypic and cross-neutralization activity to different/alternative enterovirus genotypes/strains could be tested. Seroprevalence data against different viral genotypes could be fitted to a multi-strain model to explore the implications of genotype specific immunity to the overall virus transmission dynamics.

Similar approaches have been developed for the analysis of Influenza and Dengue viruses, where immune boosting play an important role.

The observations and inferences made during this research provide a better understanding of the extent of enterovirus exposure, population susceptibility and immunity, and dynamics of virus transmission. Several constraints and issues were highlighted and need to be addressed to facilitate better enterovirus surveillance. The findings reported here are beneficial for guiding future policies and public health directives focused on enterovirus epidemiology and disease burden. Certainly, better documentation of enterovirus true prevalence and collective impact on human health is still warranted.

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Publications and Contributions

Kamau E, Nguyen D, Celma C, Blomqvist S, Horby P, Simmonds P, Harvala H. *Seroprevalence and Virologic Surveillance of Enterovirus 71 and Coxsackievirus A6, United Kingdom, 2006-2017*. Emerg Infect Dis. 2021 PMID: 34423767; PMCID: PMC8386771.

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Data and code availability

The code for the analyses performed in this thesis are available on GitHub: https://github.com/ekamau/enterovirus_seroepidemiology/