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MOLECULAR EPIDEMIOLOGY OF ACUTE RESPIRATORY VIRUS INFECTIONS

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Molecular Epidemiology of Acute Respiratory Virus Infections

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By

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Populärvetenskaplig sammanfattning

Munskydd, social distansering, dagliga uppdateringar om antalet insjuknande i nyheterna – ingen har undgått den pandemi som drabbade världen i början av 2020 och alla har vi vår egen erfarenhet av när världen stannade upp och med oro höll andan. På bästa sändningstid tolkade och förklarade virologer och epidemiologer nya vetenskapliga rön.

Mitt doktorandprojekt inleddes för att kartlägga enterovirus D68. Viruset fick uppmärksamhet när det 2014 orsakade ett globalt utbrott med luftvägsinfektioner hos barn i förskoleåldern. En del infektioner var så svåra att barn behövde intensivvård och några patienter drabbades också av polio-liknande förlamningar. Enterovirus D68 hade upptäckts redan på 1960-talet och antikroppstester visade att nästan alla någon gång haft infektionen men viruset verkade nu orsaka allvarligare sjukdom. Vi beslöt oss för att undersöka viruset med molekylärepidemiologi, det vill säga kartläggning av arvsmassan.

Stockholm drabbades 2016 av ett stort utbrott av enterovirus D68, vilket vi snabbt kunde upptäcka genom PCR-testning. Eftersom det endast fanns ett begränsat antal beskrivningar av utbrott med enterovirus D68 kartlade vi det närmare, vilket vi beskriver i **Artikel I**. I den och i tidigare kartläggningar av enterovirus D68 hade man huvudsakligen bara läst av en mindre del av virusets arvsmassa. För att kunna göra en mer noggrann kartläggning utvecklade vi en metod för att läsa av nästan hela arvsmassan, vilket vi beskriver i **Artikel II**. Med denna nya metod kunde vi bland annat visa att utbrottet i Stockholm 2016 orsakades av flera parallella introduktioner kopplade till Nordamerika.

Då utbrotten av enterovirus 2014 och 2016 hade drabbat flera länder beslöt vi 2018 att samarbeta med andra forskare i Europa för att få en bättre helhetsbild av epidemiologin. Vi använde också alla tillgängliga virussekvenser i databaser. I **Artikel III** visade vi så att enterovirus D68 hade spritts snabbt geografiskt och att särskilt de yttre delarna av viruspartikeln som utgör bra mål för skyddande antikroppar hade förändrats snabbt.

Genom att förändra ytproteinerna skulle viruset kunna undkomma antikropparna. Infektioner med enterovirus D68 hade dock påvisats främst hos barn, och barn som har sin första infektion har inte antikroppar, så det borde inte finnas någon fördel för viruset att just ytproteinerna muterade. Att det ändå fanns många mutationer i dessa ytprotein talade för att viruset även smittar personer som redan har haft infektioner. Vår tolkning av dessa resultat var att enterovirus D68 troligen orsakar återinfektioner hos vuxna. Att viruset däremot främst påvisas hos barn skulle dels kunna förklaras av att luftvägarna hos vuxna anatomiskt och fysiologiskt är mindre känsliga för infektioner, dels att vuxna har skydd efter tidigare infektioner. De vuxna som smittas därför söker inte sjukvård och blir då inte heller provtagna och enterovirus D68 skulle då också påvisas i mindre utsträckning hos vuxna än hos barn. Då vuxna också reser betydligt mer än barn skulle det också kunna förklara den snabba geografiska spridningen.

Sedan inträffade pandemin med SARS-CoV-2.

SARS-CoV-2 var ett nytt virus, fyra andra coronavirus hade dock sedan länge funnits spridda över världen men fått begränsad uppmärksamhet eftersom de oftast bara ger förkylning. Diagnostiken för luftvägsvirus på Karolinska Universitetssjukhuset hade under flera år utförts i ett fast paket i vilket bland annat dessa fyra coronavirus ingick. I **Artikel IV** analyserade vi dessa data från tio års klinisk rutindiagnostik och fann en uttalad säsongsvariation, där coronavirusen främst påvisades vintertid. Dessutom fann vi ett vartannat-årsmönster, med virusen OC43 och NL63 som förekom samma år, följt av HKU1 och 229E nästa år. Vi såg dessutom att det hos äldre patienter hade beställts få analyser för coronavirus trots att positiva fynd inte var ovanliga, vilket talade för att infektioner hos äldre var underdiagnostiserade.

Slutligen undersökte vi med molekylära metoder hur SARS-CoV-2, som orsakar sjukdomen covid-19, fördes in och spreds i Sverige, vilket vi beskriver i manuskriptet till **Artikel V**. Dels analyserade vi sparade luftvägsprov som under början av 2020 hade undersökts för andra luftvägsvirus, men inte SARS-CoV-2, dels sekvenserade vi prov från kända SARS-CoV-2-fall och analyserade dessa i relation till alla tillgängliga sekvenser i internationella databaser. Analysen av de sparade proven visade att det pågick samhällsspridning av SARS-CoV-2 minst en vecka tidigare än man förut antagit. Analysen av sekvenserna med kopplade data om smittland visade att de flesta utlandssmittade patienter hade smittats i Italien eller Österrike och det var skilda virusvarianter från dessa två länder. Den variant som huvudsakligen förekom i Sverige under första halvåret 2020 var den som påvisades hos patienterna med smittland Österrike.

Då norra Italien tidigt blev bedömt som ett riskområde vidtogs förebyggande åtgärder för de resenärer som återvände därifrån. I de österriska alperna däremot pågick under en tid en okänd smittspridning. Det visade sig vidare att ju större andel av sekvenserna i en svensk region som utgjordes av den österrikiska varianten, desto fler var inläggningarna på intensivvårdsavdelning och dödsfallen orsakade av covid-19. Vi tolkade det som att de förebyggande åtgärderna som vidtogs hade viss effekt för att minska smittspridningen av införseln från norra Italien, medan avsaknaden av tidiga förebyggande åtgärder mot införseln från Österrike bidrog till att just den varianten fick en större spridning.

Med dessa arbeten har jag undersökt virus som orsakar akuta luftvägsinfektioner med molekylärepidemiologiska metoder och visat att dessa metoder är viktiga och värdefulla verktyg för att både påvisa virus och förstå dess spridningsmönster.

Streszczenie popularnonaukowe

Maski, dystans społeczny, codzienne raporty o liczbie zakażonych i zmarłych - nikt nie uniknął pandemii koronawirusa, która wybuchła w 2020 roku. Świat się zatrzymał i wstrzymał oddech.

Punktem wyjścia mojej pracy doktorskej było jednak zbadanie enterowirusa D68. Wirus ten zwrócił na siebie uwagę w 2014 roku, kiedy spowodował światową epidemię infekcji dróg oddechowych u dzieci. Niektóre z nich wymagały intensywnej opieki, a u niektórych wystąpił paraliż, podobny do objawów polio. Chociaż enterowirus D68 został odkryty już w latach 60. XX wieku a testy na przeciwciała wykazywały, że prawie każdy miał kiedyś infekcję, wirus zdawał się teraz powodować groźniejszą chorobę. Postanowiliśmy zatem zbadać enterowirusa D68 za pomocą epidemiologii molekularnej, czyli badania materiału genetycznego wirusa.

W 2016 r. Sztokholm nawiedziła duża epidemia enterowirusa D68, którą udało nam się szybko wykryć za pomocą testów PCR. Ponieważ istniała tylko ograniczona liczba opisów ognisk z enterowirusem D68, zbadaliśmy tą epidemię w Sztokholmie, co opisujemy w **Artykule I**. W tym i w poprzednich badaniach odczytano głównie tylko mniejszą część genomu wirusa. W celu wykonania dokładniejszych badań, opracowaliśmy metodę odczytu prawie całego materiału genetycznego, którą opisujemy w **Artykule II**. Dzięki tej nowej metodzie byliśmy w stanie wykazać między innymi, że epidemia w Sztokholmie w 2016 r. była spowodowana kilkoma równoległymi introdukcjami związanymi z Ameryką Północną.

W 2014 i 2016 roku ogniska enterowirusa D68 pojawiły się w kilku krajach, dlatego w 2018 roku nawiązaliśmy współpracę z innymi naukowcami w Europie. W naszych badaniach wykorzystaliśmy też wszystkie sekwencje wirusa dostępne w bazach danych. W **Artykule III** wykazaliśmy, że enterowirus D68 szybko rozprzestrzenił się geograficznie. Ponadto wykryliśmy, że zewnętrzne części cząsteczki wirusa, które są celami dla ochronnych przeciwciał, szybko się zmieniły.

Zmieniając białka powierzchniowe, wirus może uniknąć przeciwciał. Jednak infekcje enterowirusem D68 wykryto głównie u dzieci, jednak dzieci które przechodzą pierwsze zakażenie nie mają przeciwciał, więc zmiany białek powierzchniowych nie powinno mieć żadnej korzyści dla wirusa. Fakt, że akurat w białkach powierzchniowych było jednak wiele mutacji, wskazywał na to, że wirus zakażał również osoby, które już miały przeciwciała. Nasza interpretacja tych wyników była taka, że enterowirus D68 prawdopodobnie powoduje ponowne infekcje u dorosłych. Z kolei fakt, że wirus jest wykrywany głównie u dzieci, można częściowo wytłumaczyć tym, że drogi oddechowe u dorosłych są mniej wrażliwe na infekcje oraz, że tym ich odporność po wcześniejszych zakażeniach daje łagodniejsze objawy. Osoby te nie zwracają się o pomoc medyczną, nie są więc testowe,

a zatem enterowirus D68 jest u nich wykrywany w mniejszym stopniu niż u dzieci. Dorośli podróżują też więcej niż dzieci, czym możnaby też wytłumaczyć szybkie geograficzne rozprzestrzenianie się wirusa.

Potem nastąpiła pandemia SARS-CoV-2. Był to nowy wirus, ale cztery inne koronawirusy od dawna krążyły, choć poświęcano im niewiele uwagi, ponieważ zwykle powodują tylko przeziębienia. Diagnostyka wirusów układu oddechowego w Szpitalu Uniwersyteckim Karolinska w Sztokholmie od kilku lat była prowadzona w pakiecie obejmującym również te cztery koronawirusy. W **Artykule IV** przeanalizowaliśmy te dane z ponad dziesięciu lat rutynowej diagnostyki klinicznej i stwierdziliśmy, że koronawirusy były wykrywane głównie zimą. Ponadto znaleźliśmy, że poszczególne rodzaje koronawirusów występują co dwa lata. Zauważyliśmy też, że u pacjentów w podeszłym wieku zlecono niewiele testów na obecność tych koronawirusów, mimo że pozytywne wyniki nie były rzadkością, co sugerowało, że spora część infekcji u osób starszych nie była zdiagnozowana.

W manuskrypcie do **Artykułu V** opisaliśmy nasze badania w jaki sposób SARS-CoV-2, który powoduje chorobę covid-19, dotarł do i rozprzestrzenił się w Szwecji. Zbadaliśmy przechowywane próbki z dróg oddechowych, które na początku 2020 r. zostały skierowane pod kątem zbadania zakażeń układu oddechowego innych niż SARS-CoV-2. Analiza ta wykazała, że SARS-CoV-2 rozprzestrzeniał się w społeczności już tydzień wcześniej niż poprzednio zakładano. Ponadto, zsekwencjonowaliśmy wirusy z próbek ze znanych przypadków SARS-CoV-2 i przeanalizowaliśmy je w odniesieniu do wszystkich dostępnych sekwencji w międzynarodowych bazach danych. Analiza tych sekwencji wraz z danymi dotyczącymi kraju zakażenia wykazała, że większość pacjentów zakażonych za granicą została zarażona we Włoszech lub Austrii i że w tych dwóch krajach istniały różne warianty wirusa. Wariant, który występował głównie w Szwecji w pierwszej połowie 2020 r. był tym, który wykryto u pacjentów z Austrii.

Kiedy północne Włochy zostały wcześnie ocenione jako obszar ryzyka, podjęto środki zapobiegawcze dla powracających stamtąd podróżnych. Natomiatst w austriackich Alpach przez pewien czas trwało niewykryte rozprzestrzenianie się zakażeń. Stwierdziliśmy, że im wyższy odsetek wirusów w regionach Szwecji był wariantem austriackiem, tym więcej było przyjęć na oddziałach intensywnej terapii i zgonów spowodowanych przez covid-19. Tłumaczymy to w ten sposób, że podjęte środki zapobiegawcze w pewnym stopniu ograniczyły rozprzestrzenianie się infekcji pochodzących z Włoch. Brak wczesnych środków zapobiegawczych wobec napływu zarażonych podróżnych z Austrii przyczynił się do rozprzestrzeniania się tego wariantu.

Dzięki tym pracom zbadałem wirusy wywołujące ostre zakażenia dróg oddechowych przy użyciu molekularnych metod epidemiologicznych. Wykazałem, że metody te są ważnymi i cennymi narzędziami zarówno do wykrywania wirusów, jak i zrozumienia wzorców ich rozprzestrzeniania się.

Abstract

Acute respiratory virus infections are very common but can also cause severe disease. In my thesis, I have analysed the molecular epidemiology of acute respiratory virus infections caused by enterovirus D68 and coronaviruses.

In **Paper I**, we used real-time PCR and Sanger sequencing to analyse the outbreak of enterovirus D68 in Stockholm in 2016. We found that the outbreak was caused by the subclade B3, and we also described three patients with neurological manifestations. The virus sequences were closely related to concurrent sequences from North America.

In **Paper II**, we developed an assay for whole-genome sequencing of enterovirus D68 a next-generation platform. By using the assay on the samples from the 2016 outbreak, we found that the outbreak was caused by multiple independent introductions of the virus. We also estimated the time to the most common recent ancestor for the subclades B1 and B3 to 2009.

In **Paper III**, we used the whole-genome sequencing assay in a European multicentre study of enterovirus D68 circulation in the 2018 season. We also included sequences in public repositories. We found that the viruses in 2018 belonged to subclades A2 and B3 and that sequences in subclade B3 originated from the circulation in 2016. We also found that enterovirus D68 had a rapid geographic mixing and that residues on the surface of the virus particle had an elevated substitution rate of amino acids. Hence, we proposed asymptomatic reinfections of adults to explain both rapid geographical dispersal and selective pressure on the surface residues.

In **Paper IV**, we analysed stored results from routine clinical diagnostics for the four common cold coronaviruses. The data contained the results from September 2009 to April 2020. At the species level, we found a pattern of alternating biennial circulation, and we also found the circulation of *Betacoronaviruses* to peak earlier than that of *Alphacoronaviruses*.

In **Paper V**, we investigated Sweden's first SARS-CoV-2 pandemic wave in 2020. We analysed stored respiratory samples with real-time PCR for SARS-CoV-2 and found that community transmissions started earlier than previously appreciated. We also sequenced stored SARS-CoV-2-positive samples. To these sequences, we added information from contact tracing records and combined them with data from public repositories. Among cases exposed abroad, we mainly found clades 20B and 20A, whereas clade 20C dominated domestic infections. Furthermore, we found the proportion of clade 20C to be correlated with the cumulative number of deaths due to COVID-19. We interpreted this as early undetected introductions of clade 20C having had a significant impact on the further course of the pandemic in Sweden.

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List of abbreviations

MERS-CoV	Middle East respiratory syndrome coronavirus
PCR	Polymerase chain reaction
SARS-CoV	Severe acute respiratory syndrome coronavirus
VP	Virus protein

1 INTRODUCTION

1.1 Acute respiratory virus infections

Infections of the upper respiratory tract are very common. In 2019, the incidence was estimated at 17,000,000,000 incident cases [2]. These infections are often caused by viruses. Although acute respiratory viral infections are usually mild and self-limiting, they are still a significant cause of death and illness. Worldwide, the influenza virus annually causes an estimated 400,000 deaths [3]. Bronchiolitis caused by the respiratory syncytial virus is estimated to cause 3.4 million hospital admissions annually [4]. Furthermore, the loss of productivity within Sweden due to these infections is estimated at billions of euros per year [5].

Viruses commonly causing acute respiratory infections include the influenza virus, respiratory syncytial virus, parainfluenza viruses, human metapneumovirus, coronaviruses, rhinoviruses, some enteroviruses, some adenoviruses, and bocavirus. In addition, viruses transmitted to humans from animals, such as MERS-CoV and avian influenza, , can cause severe illness and pose a pandemic threat. My thesis focuses on enterovirus D68 and human coronaviruses.

1.1.1 Enterovirus D68

Enterovirus D68 is a genotype in the species *Enterovirus D* and one of over a hundred genotypes in the genus *Enterovirus*. The most well-known genotypes in the genus are the polioviruses. Polioviruses cause the paralytic disease poliomyelitis and belong to the species *Enterovirus C*. The genus *Enterovirus* also includes rhinoviruses, which cause the common cold. All enteroviruses have a non-enveloped capsid and an RNA genome of circa 7.5 kilobases. The viral capsid consists of the four viral proteins 1-4 (VP1-4), of which all but VP4 are exposed on the surface of the virus particle [6]. The structures of VP1-VP3 each contain eight strands (B-I) connected by loops (e.g., the BC loop). VP1 is the main target of neutralising antibodies [7].

1.1.2 Coronaviruses

Coronaviruses have an envelope and an RNA genome of around 27.5 to 30 kilobases. Among the structural proteins, the spike glycoprotein on the surface of the virus particle is the target of neutralising antibodies [8-12]. Before the SARS-CoV-2 pandemic, there were four known species of human coronaviruses: 229E, HKU1, NL63, and OC43. These four viruses are often referred to collectively as the “common cold”, “endemic”, or “seasonal” coronaviruses. Additionally, human infections with the highly pathogenic SARS-CoV-1 and MERS-CoV have also been reported.

1.2 Seasonality

Most respiratory viruses do not occur uniformly over the year, as reflected by the term "flu season". Thus, seasonal circulation of influenza viruses is common knowledge. As reviewed by Moriyama *et al.* [13], this kind of seasonality may be due to the transmission of the viruses being affected by climate factors and variation of people's social behaviours over the year. Climate factors can influence the viability and droplet dynamics of virus particles and host defence mechanisms, such as the efficiency of mucociliary clearance.

As reviewed by Pica and Bouvier [14], the influenza virus is more efficiently transmitted at low temperatures than at high temperatures, and low absolute humidity correlates strongly with the onset of the influenza season. In fact, aerosolised influenza virus particles have the longest viability at the combination of low temperature and low relative humidity, and the viability decreases as either temperature or relative humidity increases [15]. For poliovirus, aerosolised virus particles at high temperatures remain viable longest at high relative humidity and shortest at intermediate relative humidity [15]. For the common cold coronavirus 229E, aerosolised virus particles remain viable longer at low temperatures than at high temperatures [16]. Furthermore, in low temperatures, more virus particles remain viable at high relative humidity, whereas at high temperatures an intermediate relative humidity is most favourable for the virus viability. Similarly, the stability of SARS-CoV-2 virus particles is around five times longer at low than at high temperatures and shorter at intermediate than at low or high relative humidity [17].

1.3 Diagnostic methods

Clinical diagnostics of acute respiratory virus infections are currently based on methods directly detecting the virus (Table 1). Among these, molecular diagnostics using nucleic acid amplification tests are preferred because they provide rapid results with high sensitivity and high specificity [18]. Furthermore, molecular diagnostics often consist of syndromic panels that simultaneously analyse a specimen for several pathogens. Results from such multiplex panels provide a valuable resource for epidemiological studies as patients are consistently tested for all the pathogens in the panel [19].

Table 1. Methods to detect respiratory viruses

Method	Target	Features
Direct detection methods		
NAAT, e.g., qPCR	Viral nucleic acid	Short to moderate turn-around time. Moderate cost. High sensitivity and high specificity. Risk for primer or probe mismatch. Biased: only detects queried sequences. Detected nucleic acid may not be infectious virus particles.
Antigen detection	Viral protein	Very short turn-around time. Can be done at point-of-care. Low cost. Low sensitivity. Can have good specificity. Biased: only detects queried antigens.
(Isolation of virus)	Infective virus	Not in routine clinical use. Moderate to long turn-around time. Relatively unbiased, but some viruses are not culturable. Useful to determine infectivity. Biohazards need to be considered due to the enrichment of infectious virus.
(IFM)	Viral protein	No longer in routine clinical use. Short turn-around time. Low cost. Moderate sensitivity. High specificity. Biased: only detects queried antigens.
Indirect detection method		
(Serology)	Host antibodies	Not in routine clinical use. Useful to determine past infection. Not useful for detecting acute infection.

Methods in parenthesis = not in routine clinical use for diagnostics of respiratory virus infections. IFM = immunofluorescence microscopy; NAAT = nucleic acid amplification test; qPCR = real-time polymerase chain reaction.

1.4 Molecular epidemiology

The best way to study an organism's evolutionary history and relationship to other organisms is to analyse its nucleic acid [20]. Such analyses are instrumental in the study of the epidemiology of infectious diseases. Modern bioinformatic tools allow for user-friendly constructions of phylogenetic trees and integration of temporal, spatial, and other metadata [21]. The topology of such phylogenetic trees is informative, for instance, a continuous immune selection yields a ladder-like structure of the tree [22]. A few examples of knowledge provided by molecular epidemiology are the bottleneck size and serial interval of influenza A virus transmission [23], the transmission structure of rabies virus in endemic circulation [24], and the uncovering of a hidden Zika outbreak [25].

1.4.1 Virus evolution

Molecular epidemiology is possible because of genetic variation. This variation stems from point mutations, insertions, deletions, and recombination. These causes of variation arise during the replication of the genetic material in all organisms. However, in viruses, the substitution rate is high enough to observe in real-time [26]. For enterovirus D68, the substitution rate in the VP1 gene is circa 5×10^{-3} substitutions per site per year [27-30]. Although recombination commonly occurs in other enteroviruses [31], it is rare in enterovirus D68 [29]. The coronaviruses have a primitive proofreading mechanism for genome replication [32]. Their substitution rate is circa 2×10^{-4} substitutions per site per year for the entire genome [33, 34] but higher in the spike gene (around 6 to 8 $\times10^{-4}$ substitutions per site per year) [34-37]. For comparison, the average substitution rate in mammals is around 2×10^{-9} substitutions per site per year [38].

Due to the high error rate of the RNA-dependent RNA-polymerase, the genomes of new viral particles are not identical but instead consist of a so-called "swarm" or "quasispecies" of closely related variants [39]. In a mouse model using poliovirus, this intrahost variability of the virus is a virulence factor [40], by providing a source for selection that allows the virus to adapt to tissue-specific innate immunity responses [41].

1.5 Immunity and immune escape

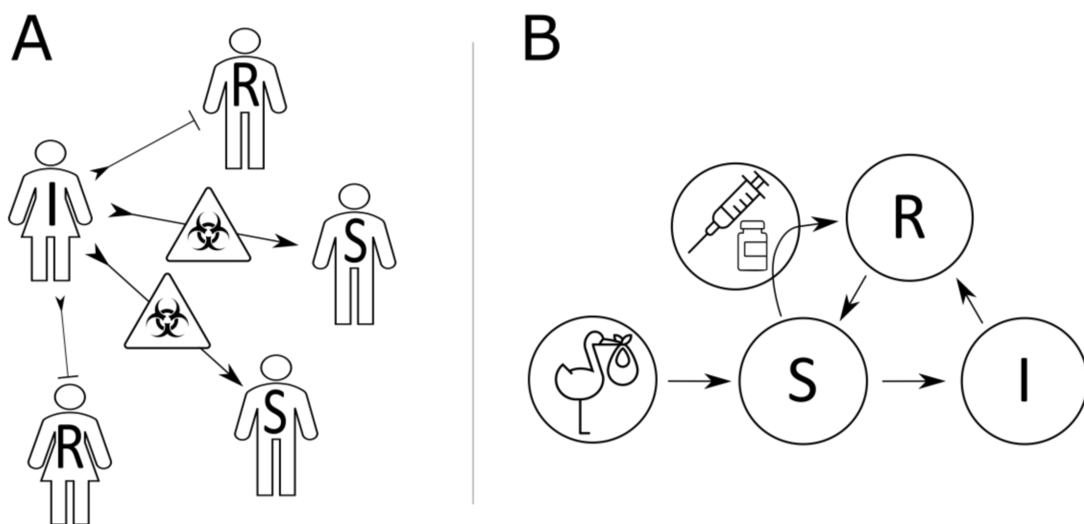
An absolute requirement for viruses is that they must be able to transmit, usually within the same host species but sometimes to other species (i.e., zoonosis). Thus, infectious individuals can transmit the virus and the disease to susceptible individuals, who then become infectious. According to the simple susceptible-infectious-removed (SIR) model, individuals who recover from the infection are no longer contagious and cannot be infected due to acquired immunity (Figure 1A). According to more complex SIR models, this protection can decline with time due to the waning immunity of the individual and immune escape of the pathogen, leading to the individual becomes susceptible again. This process can be viewed as individuals transitioning between three pools in the population: the susceptible, the infectious, and the removed (Figure 1B).

A typical example of immune escape is the continuous antigenic drift of the influenza A virus. Due to random mutations, an influenza virus gains a transmission advantage if it acquires substitutions that decrease the host's protection from prior immunity [42-44]. This continuous immunologic selection pressure results in the abovementioned ladder-like structure of the phylogenetic tree. A similar ladder-like tree structure is seen in viral sequences obtained over time from individuals chronically infected with viruses like hepatitis C virus and human immunodeficiency virus (HIV) [22].

A widespread outbreak may deplete the pool of susceptible individuals, causing the virus to die out in that population; however, the disappearance of the virus causes an

accumulation of susceptible individuals over time, and a subsequent reintroduction of this virus could then trigger an outbreak [45, 46]. In modelling the incidence of enteroviruses, outbreaks occur when the size of the susceptible population reaches a threshold [47]. In the model, a serotype-specific immunity is assumed to be life-long. As the pool of susceptible individuals is then only replenished by births, a declining birth rate causes the time interval between outbreaks to increase. There are sudden deviations from the predicted incidence for the enterovirus genotypes coxsackievirus A6 and echovirus 18, thought to be caused by immune escape due to an antigenic change.

Figure 1. Transmission and immunity, SIR models



Panel A: Infected (I) individuals can transmit the virus to susceptible (S) but not removed (R) individuals who have recovered from the infection.
Panel B: Individuals in the susceptible (S) pool can become infected (I) and, after recovery from the infection, acquire immunity and transit to the removed (R) pool. Removed individuals may return to the susceptible pool due to waning immunity or immune escape of the pathogen. New births also replenish the pool of susceptible individuals. Vaccination allows susceptible individuals to acquire immunity and move into the removed pool without becoming infected.

2 LITERATURE REVIEW

2.1 Enterovirus D68

Enterovirus D68 is the enterovirus genotype that is among the most commonly detected in the upper respiratory tract [48-66]. Primary infections with enterovirus D68 usually occur at a young age. More than half of preschool children are seropositive [67-72], including reports with a 100% seroprevalence rate in 2-year-olds [73-75]. During an outbreak of enterovirus D68 in one kindergarten, more than 70% of children seroconverted, having mainly mild respiratory symptoms [76].

2.1.1 The re-emergence of enterovirus D68

Reports of enterovirus D68 were uncommon before molecular diagnostics, despite seroprevalence studies showing infections with enterovirus D68 to be very common. Enterovirus D68 was first isolated by Schieble *et al.* [77] in the 1960s from four paediatric patients in California, USA. The prototypic strains Fermon and Rhyne originate from two of these patients. Subsequently, enterovirus D68 accounts for only 26 cases (0.05% of reported cases) in the US National Enterovirus Surveillance System in 1970-2005, with the first case reported in 1987 [78]. Similarly, in enterovirus surveillance in the UK during 2004-2011, there are only 14 cases of enterovirus D68 (0.5% of analysed strains), of which 10 in 2009 [79]. Furthermore, in enterovirus surveillance in Belgium 2007-2018 [80] and Ireland 2005-2014 [81], there are no cases of enterovirus D68 until 2014.

In contrast to the sparse detection of the enterovirus D68, seroprevalence studies show that almost all adults worldwide have antibodies to the virus (Table 2), indicating that infections are widespread. The discrepancy between the ubiquity of infections indicated by seroprevalence studies and the paucity of identified cases could be due to mild infections not being detected by hospital-based testing. However, diagnostic challenges are also likely to be critical. For instance, enterovirus D68 mainly causes respiratory symptoms, whereas characterisation of enteroviruses within polio surveillance is performed on stool rather than respiratory samples [82].

The challenge of identifying enterovirus D68 before molecular diagnostics and genotyping is illustrated by viral cultures mostly yielding negative results for samples tested positive for enterovirus D68 by PCR [60, 83, 84]. Indeed, in European enterovirus surveillance, enterovirus D68 is not detected in any country that does enterovirus typing by virus isolation and neutralisation assays [85]. Unlike other enteroviruses, the capsid of enterovirus D68 is acid-sensitive [1, 86-88]. The acid-sensitivity may have hindered detection by serotyping assays, as it was uncommon to do serotyping of acid-sensitive viruses [86]. In addition, an identification of enterovirus D68 by serotyping is hampered by a lack of antisera and immunofluorescence reagents [89].

Table 2. Seroprevalence studies of enterovirus D68.

Authors	**Location**	**Collection year(s)**	**Seroprevalence**
Smura *et al.* [90]	Finland	1983, 1993, 2002	100%
Kamau *et al.* [68]	United Kingdom	2006, 2016	0 to ≈90% of children ≈90 to ≈100% of adults
Karelehto *et al.* [69]	The Netherlands	2006 - 2007, 2015 – 2016	44 to 100% of children 85 to 100% of adults
Sun *et al.* [71]	China	2010	20 to 83% of children 100% of adults
Sun *et al.* [91]	China	2012	51 to 64% of children
Harrison *et al.* [73]	Missouri, USA	2012 -2013	100% of children 100% of adults
Liu *et al.* [74]	China	2012 – 2017	62 to 100% of children 96 to 100% of adults
Chan *et al.* [67]	Malaysia	2013, 2015	32 to 84% of children 82 to 90% of adults
Livingston *et al.* [75]	Missouri, USA	2017	100% of children
Lee *et al.* [70]	Taiwan	2017	18 to 98% of children 100% of adults

However, even with molecular diagnostics, enterovirus D68 can still be missed due to the low sensitivity of some PCR assays [84, 92] or misclassification due to cross-reactivity in the assays with rhinovirus [93]. Nevertheless, with the introduction of molecular diagnostics, the presence of enterovirus D68 was noticed worldwide (Table 3). A significant shift in epidemiology and awareness occurred in 2014, starting with a cluster of severe respiratory illnesses in paediatric patients in the Midwestern US [94], and concurrent cases of acute flaccid paralysis [95]. In Europe, a surveillance study of the 2014 season detects enterovirus D68 in 14 of the 17 participating countries [92]. However, in contrast to the USA, in Europe, the infections cause a limited number of admissions to intensive care units and only three cases of acute flaccid paralysis. Nevertheless, the 2014 outbreak raised awareness about the virus and led to improved surveillance as well as prospective and retrospective studies.

Table 3. Early studies of enterovirus D68

Authors	**Publication year**	**Location**	**Collection year(s)**	**Context***
Schieble *et al.* [77]	1967	California, USA	1962	Paediatric patients with respiratory disease
Savolainen-Kopra *et al.* [96]	2009	Finland	2004-2005	Surveillance of military recruits
Wang *et al.* [97]	2010	California, USA	2003-2005	Surveillance of military recruits
Centers for Disease Control and Prevention [98]	2011	Georgia, USA	2009-2010	Implementation of multiplex PCR-testing
Imamura *et al.* [99]	2011	The Philippines	2008-2009	Paediatric pneumonia study
Kreuter *et al.* [100]	2011	New Hampshire, USA	2008	Case report, fatal meningoencephalitis
Petitjean-Lecherbonnier *et al.* [60]	2011	France	2008	Genotyping of clinical samples
Piralla *et al.* [101]	2011	Italy	2008	Rhinovirus surveillance in hospitalised patients
Centers for Disease Control and Prevention [98]	2011	Pennsylvania, USA	2009	Outbreak, paediatric patients
Tokarz *et al.* [102]	2011	New York, USA	2009	Outbreak, multiplex testing in ILI surveillance
Centers for Disease Control and Prevention [98]	2011	Arizona, USA	2010	Outbreak, paediatric patients
Hasegawa *et al.* [103]	2011	Japan	2010	Paediatric patients hospitalised due to asthma
Kaida *et al.* [83]	2011	Japan	2010	Surveillance of respiratory tract infections
Rahamat-Langendoen *et al.* [104]	2011	The Netherlands	2010	Outbreak, multiplex PCR-testing of hospitalised children

* ILI: influenza-like illness; PCR: polymerase chain reaction

2.1.2 Clinical manifestations of enterovirus D68 infection

2.1.2.1 Respiratory disease

The most common clinical presentation of enterovirus D68 infections are respiratory symptoms, which can range in severity from mild to severe disease [105]. Outbreaks result in increased hospital admissions due to respiratory illness, including admission to intensive care units for respiratory support such as supplementary oxygen or ventilation [94, 98, 103, 104, 106-112]. Some reports suggest that enterovirus D68 causes a more severe respiratory illness than other enteroviruses or rhinoviruses [113, 114], whereas others indicate that enterovirus D68 infections are not more severe [50, 110]. In the latter case, the increased admissions would be the tip of an iceberg during large outbreaks. Patients with severe respiratory disease caused by enterovirus D68 infection often have underlying asthma [29, 48, 52, 60, 103, 106, 107, 109, 113-128].

The receptors for enterovirus D68 are found in the respiratory tract. Enterovirus D68 can use both α2,3-linked and α2,6-linked sialic acids as receptors, though with a preference for the latter [129-131]. Of note, human influenza A viruses also use α2,6-linked sialic acids as receptors, whereas avian influenza A viruses prefer α2,3-linked sialic acids [132]. The α2,3-linked sialic acids are found on alveolar cells in the lower respiratory tract and only at low levels in the upper respiratory tract. In comparison, the α2,6-linked sialic acids are present both in the upper and lower respiratory tract but not in alveolar cells [133]. In conjunction with enterovirus D68 having an optimal growth temperature of 33°C [87, 88, 134], this may predispose the virus to cause upper respiratory tract infections.

2.1.2.2 Neurologic disease

Evidence has accumulated for enterovirus D68 being the causative agent of a form of acute flaccid paralysis termed acute flaccid myelitis [135, 136]. The earliest clusters of patients with this presentation are noted in the US during the 2014 outbreak. By November 2014, the US CDC had received reports of 88 cases, with a peak of cases in mid-September [137]. With the increased awareness after the outbreak, retrospective studies identify additional patients with acute flaccid myelitis and concurrent enterovirus D68 infection also before 2014 [49, 119, 138, 139]. In the scientific literature, the earliest reported case is a young adult with acute flaccid paralysis and enterovirus D68 detected in the cerebrospinal fluid, reported to the US National Enterovirus Surveillance System in 2005 [78]. The majority of cases with acute flaccid myelitis due to enterovirus D68 are paediatric [30, 48, 51, 54, 119, 121, 122, 124-126, 140-158], but there are also a few reports of adult patients [30, 124, 143, 150, 151, 155, 159, 160].

Awareness and timely sampling are critical for detecting enterovirus D68 in patients with acute flaccid myelitis. In general, the workup of a patient presenting with neurological symptoms would include an analysis of cerebrospinal fluid for neurotropic patho-

gens, including enteroviruses. However, in patients with acute flaccid myelitis, enterovirus D68 is mainly detected in respiratory samples [143, 145, 147-154, 156]. Moreover, the detection of enterovirus D68 in patients with acute flaccid myelitis is challenging due to the transient presence of the virus in the respiratory tract and as the neurological symptoms may occur after the respiratory illness. Accordingly, enterovirus D68 is more likely to be detected in patients with acute flaccid myelitis if the respiratory specimen is collected close to the onset of respiratory [143, 147, 153] or neurological symptoms [150]. Detections of enterovirus D68 in cerebrospinal fluid are sporadic [78, 100, 142, 147, 150-152, 155-157, 159-161]. Also, analysis of a stool sample, which is often done as part of polio surveillance, can sometimes detect enterovirus D68 in patients with acute flaccid myelitis [140, 150, 151, 154-156, 161-164].

2.1.3 Genotyping of enterovirus D68

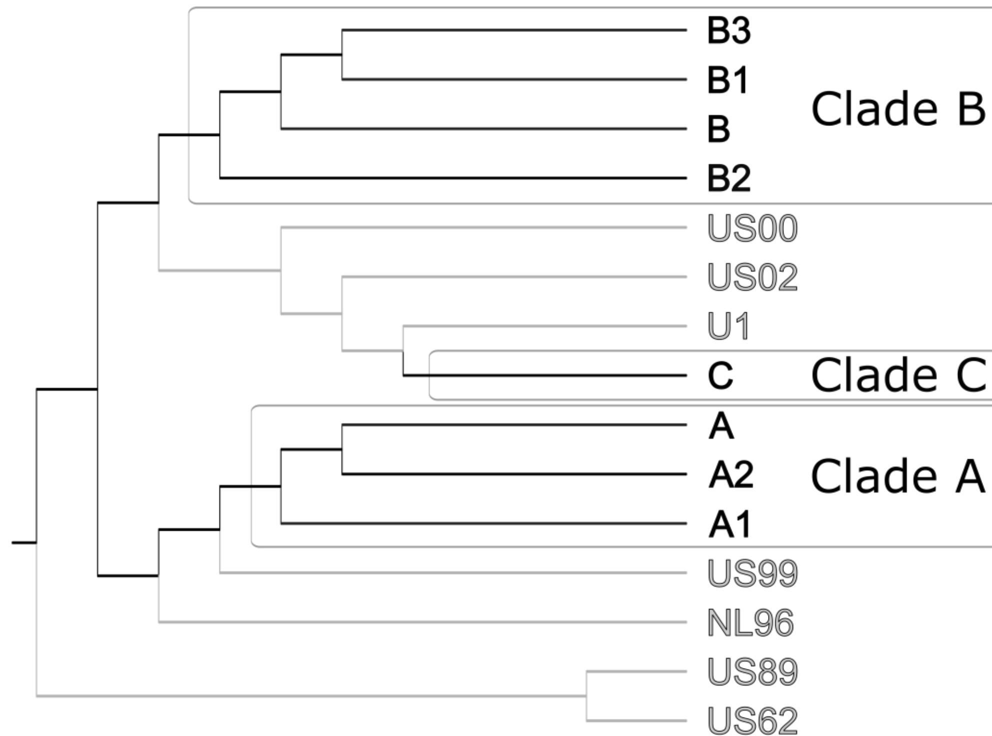
There are more than a hundred recognised genotypes of enteroviruses. The classification is based on the nucleotide sequence that encodes the capsid protein VP1. This part of the genome correlates with the cross-neutralisation of antibodies, i.e., the serotyping that was previously used for characterisation [7]. There is consensus that the threshold for identifying enterovirus is that the VP1 nucleotide or amino acid sequence differs by at least 88 or 75% from other genotypes, respectively. A genotype can be further subdivided. In contrast to the consensus on the cut-off for distinguishing genotypes, the further subdivision of genotypes is more arbitrary.

For enterovirus D68, the widely accepted subdivision is the classification by Tokarz *et al.* [28]. This classification is based on a phylogenetic tree of VP1 gene sequences. The classification identifies three clades: A, B, and C (Figure 2), and the estimated times of the most common recent ancestors of the clades are 1997, 2007, and 1999, respectively [28]. The three clades are further subdivided into subclades. The online enterovirus typing tool provided by the Dutch National Institute for Public Health and Environment [165] is a convenient and accepted resource. Currently (as of April 2023), it distinguishes the following subclades: A1, A2, B1, B2, and B3. It also annotates early clusters not included in the classification by Tokarz *et al.* [28].

2.1.4 Circulating clades of enterovirus D68 over time

Infections with enterovirus D68 are reported worldwide, but the patterns of circulation and molecular epidemiology change over time and differ between continents (Figure 3). In the reported cases from the 2000s, mainly variants outside clades A to C are found [83, 88, 99, 119, 166-170]. Subclade B2 is detected in Europe in 2009 [56, 104, 114, 170] but the circulation in the early 2010s is dominated by clades A and C [27, 28, 56, 58, 59, 104, 138, 170-173].

Figure 2. Clades and subclades of enterovirus D68

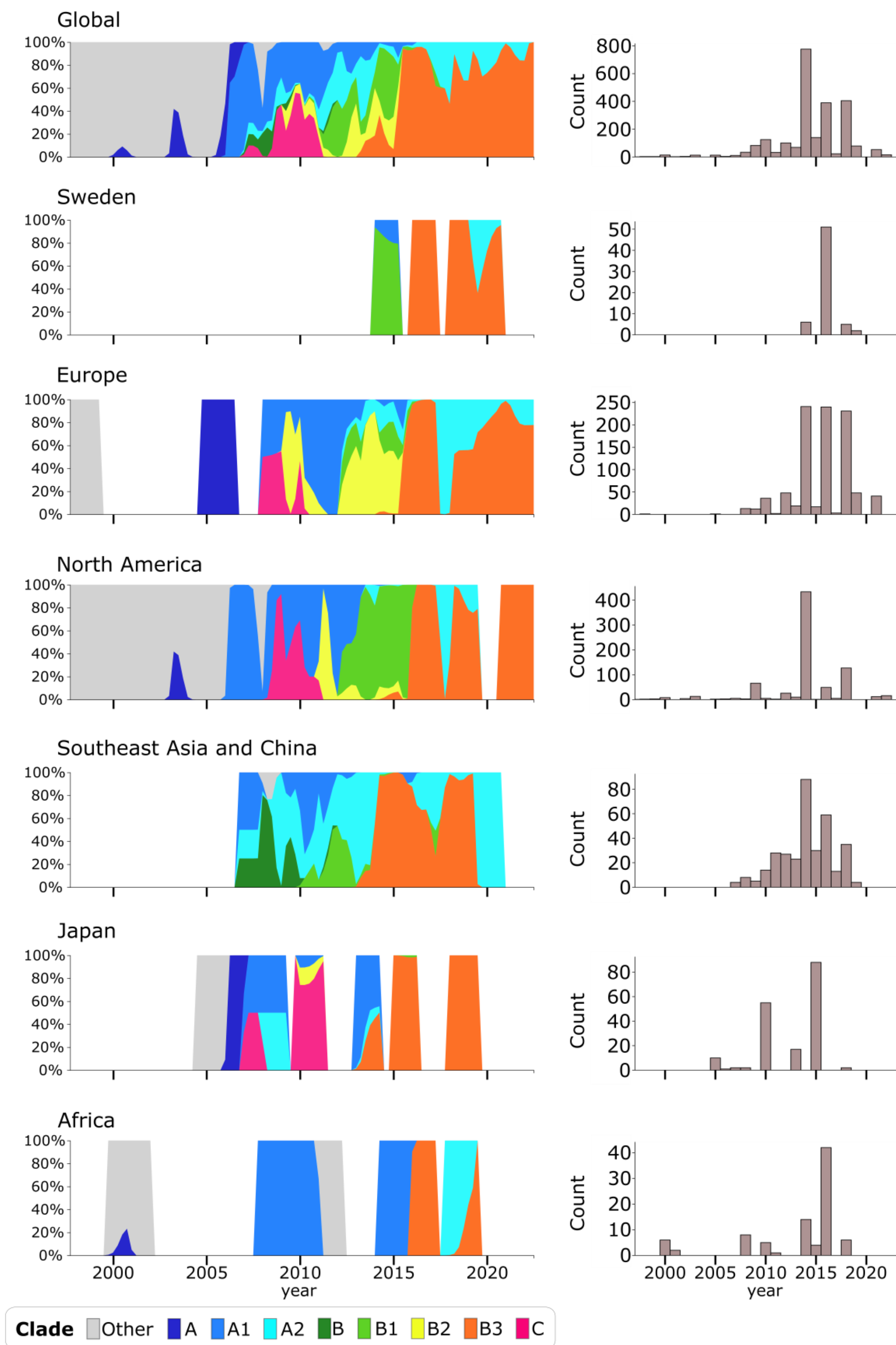


Schematic representation of the relationships of enterovirus D68 clades A, B, and C, defined by Tokarz *et al.* [28] as well as the prototypic Fermon strain, and subclades as defined by the Enterovirus typing tool provided online by the Dutch National Institute for Public Health and the Environment [165]. Early clusters not included in the classification by Tokarz *et al.* [28] are shown in grey. The Fermon strain is in US62.
The figure was generated by querying the Enterovirus typing tool for the prototype Fermon strain (accession number AY426531). Terminal nodes in the resulting Newick file were collapsed to subclade level using FigTree v1.4.4 [174], and the figure was finalised using Inkscape (Free Software Foundation).

In 2014, subclade B1 dominate during the outbreak in North America [124, 143, 145] and also in Sweden [175], whereas subclade B2 dominates in most European countries [48, 92, 176], and subclade B3 in Southeast Asia and China, [55, 167, 171, 177]. Notably, in Japan, there is no outbreak in 2014, but in 2015, there is an outbreak caused by subclade B3 [119, 169, 178].

The enterovirus D68 sequences from 2016 contain almost exclusively sequences in sub-clade B3 [48, 54, 116, 121, 138, 179, 180], and since 2016, subclade B3 predominates in a

Figure 3. Circulating clades of enterovirus D68



Circulating subclades of enterovirus D68 (*left*) and the number of enterovirus D68 sequences (*right*) over time per geographic region. Clades according to classification system by Tokarz *et al.* [28]. Data from Nextstrain enterovirus D68 VP1 build [181].

co-circulation with subclade A2 (Figure 3). Whereas subclade B3 rapidly became dominant after its first documentation in 2013, subclade A2 is detected since the late 2000s. Clade C, present in the early outbreaks, has not been found since 2010, and the last detections of subclades A1 and B2 are from 2015. Notably, there is no significant circulation reported from mainland China, despite large retrospective surveillance studies [57, 63, 66, 128, 182-184].

2.1.5 Seasonality and circulation patterns of enterovirus D68

The circulation of enteroviruses is known to have a seasonal pattern. The timing of the peak varies between the genotypes, with the species *Enterovirus D* peaking late, i.e., in September [185]. In Sweden, the incidence of enteroviruses is correlated with relative humidity but not temperature [186]. Dew point temperature, which depends on temperature and humidity, is the climate factor that shows the strongest correlation with enterovirus incidence, peaks appearing earlier as the humidity increases [185]. Furthermore, higher birth rates are associated with earlier but also lower peaks [185].

Moreover, the genotypes usually have more long-term circulation patterns, e.g., two, three, or even more years between peaks [78-80]. Enterovirus D68 has a biennial pattern in Europe and North America, with peaks in even years (Figure 3). Modelling of enterovirus circulation in Japan shows that herd immunity fits well with data as a possible explanation of the long-term patterns [47].

2.1.6 Immunity to enterovirus D68

Several studies show that antibodies confer protection against enterovirus D68 infection. In humans, individuals with lower antibody titres to enterovirus D68 have a higher risk of a symptomatic acute respiratory tract infection than those with higher titres [72]. In animal studies, mice are protected from paralytic disease and death upon live virus challenge by passive immunisation, such as sera from virus-inoculated mice [187], human intravenous immunoglobulin [188], and maternal antibodies from virus-like particle immunisation [189].

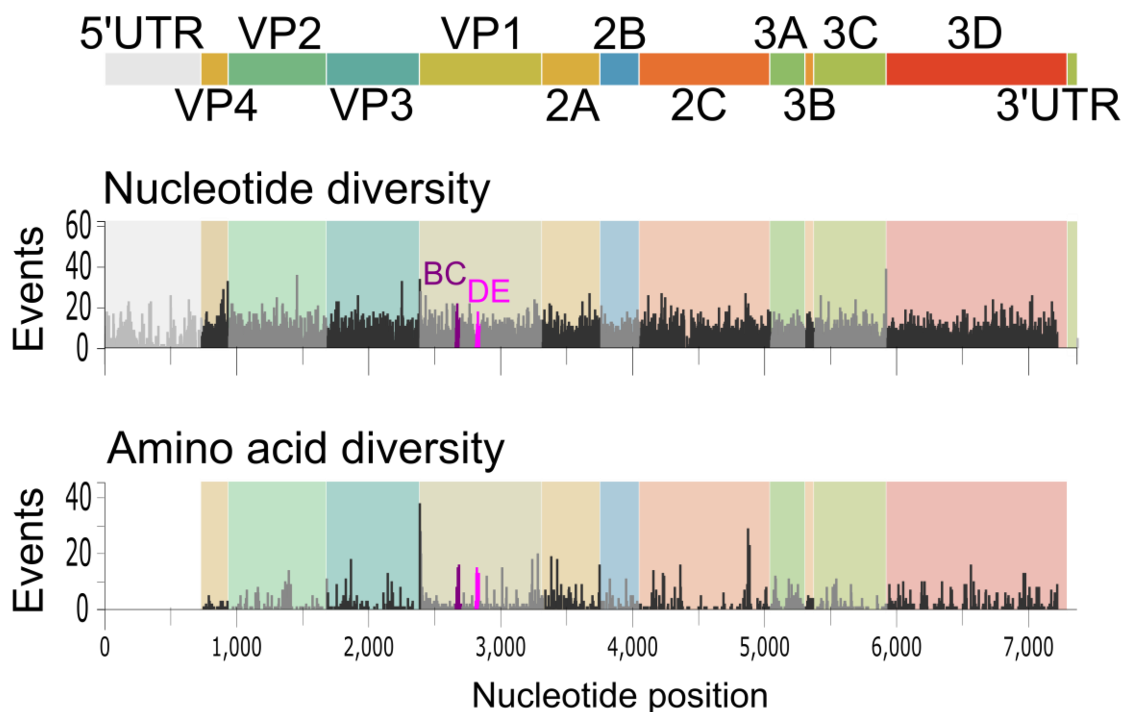
As indicated by immune escape mutations in rhinovirus 14, amino acid residues contributing to neutralising epitopes on enteroviruses are present in all the three proteins exposed on the surface of the virion, i.e., in VP1, VP2, and VP3 [6]. Nevertheless, the sensitivity of enteroviruses to neutralising antibodies correlates best with the sequence of the VP1 gene [7], indicating this protein harbours the most important neutralising epitopes. Indeed, for coxsackievirus B4, a single mutation in the VP1 gene significantly reduces the sensitivity to neutralising antibodies [190].

The putative neutralising epitopes of enterovirus D68 are inferred by structural homology with rhinovirus 14 [191]. On rhinovirus 14, four major neutralising regions are identified [6]. In enterovirus D68, two of the corresponding regions in VP1, namely the BC

and DE loops, are disordered. The lack of a fixed structure at these sites might be a mechanism for evasion of antibodies [191]. The other two immunogenic regions in rhinovirus 14 involve amino acid residues in the VP2 and VP3. Accordingly, epitopes of monoclonal antibodies are also found in the VP1 GH loop, VP2 EF loop, and VP3 C-terminus [192]. In line with this, characterisation of human neutralising antibodies showed that several of these to bind to linear epitopes in VP1 or conformational epitopes spanning all three proteins [193].

Whole-genome sequencing of enterovirus D68 shows that VP1 is the most variable gene [194], and that mutations in this gene mainly occur in the BC and DE loops [55, 170, 172, 176, 180]. However, the diversity of the genome is not limited to the BC and DE loops (Figure 4). Whereas several codons in the VP1 genes are under purifying selection, there is no significant positive selection [27, 55, 129, 172, 180].

Figure 4. Mutation events across the genome of enterovirus D68



Gene map on top. Diversity of nucleotides and amino acids as events per codon position (y-axis), based on 976 genomes sampled between September 1997 and September 2022. The codons of the BC and DE loops in VP1 are highlighted in purple and fuchsia, respectively.
Figure downloaded from Nextstrain (www.nextstrain.com/enterovirus/d68 [21]) and modified using Inkscape (Free Software Foundation).

2.1.7 Neurovirulence of enterovirus D68

The evidence from animal and *in vitro* studies on the pathophysiology of neurological disease is inconclusive. Likewise, whether any of the subclades have specific neuro-virulent factors is not entirely clear.

2.1.7.1 Pathophysiology

Passaged strains, such as the prototypic Fermon strain, do not necessarily reflect *in vivo* properties of the virus. For instance, there is a strain that is shown to have acquired a culture-adaptive mutation that affects receptor use [195]. Furthermore, in an animal study, three mutations completely (VP3:I88V) or partially (3A:H47R, VP1:L1P) attenuate the ability to cause death in mice [196]. However, these mutations are also among the acquired mutations that arise during a sequentially passage in mice [197]. Thus, it must be considered that the attenuation caused by these mutations can be due to an adaptation to mice as a host species and may not necessarily reflect their importance for the virulence of enterovirus D68 in humans.

In two-day-old Swiss Webster mice, four of five contemporary strains (one A2, two B1, one B2) cause paralysis upon intracerebral injection, whereas injection with the Fermon, Rhyne, or one of the B2 strains does not [187, 198]. For one of the B1 strains, alternative infection routes were also examined. The intramuscular injection causes paralysis in all mice, whereas intranasal or intraperitoneal infection results in paralysis in less than 5% of mice. Likewise, intramuscular injection of neonate wild-type mice (C57BL/6) with a subclade B2 strain causes paralysis [199]. Notably, also the Fermon strain caused paralysis in 4 of 5 mice upon intracranial injection in 4-week-old mice lacking the interferon α/β receptor [200]. In contrast, none of the strains tested, including a B1 strain and Fermon, causes paralysis in any of the infected 10-day-old and 4-week-old C57BL/6 mice [200].

Upon intracerebral injection of 2-day-old Swiss Webster mice, enterovirus D68 can be isolated from the spinal cord and again cause paralytic disease when injected into another mouse [187]. In a respiratory disease model using 4-week-old mice lacking the interferon αβ/γ-receptors, virus is detectable in the brain and spinal cord at 1 and 1-3 days, respectively, after intranasal inoculation [197]. Likewise, intraperitoneal injection in 10-day-old mice lacking the interferon αβ/γ-receptors causes paralysis, and the virus is detected in the spinal cord [201]. However, in 5-day-old mice lacking the interferon αβ/γ-receptors infected intranasally, the paralysis is due to myositis, and no virus is detected in the spinal cord [201]. The latter consistent with the original characterisation of the prototypic Rhyne strain [77], although in a recent study using 2-day-old Swiss Webster mice, the Rhyne strain only infrequently causes paralysis [187].

2.1.7.2 Tropism

In the only autopsy of a patient with acute flaccid myelitis, enterovirus D68 is detected in motor neurons in the spinal cord [202]. *In vitro* studies show that enterovirus D68 can infect and replicate in neuronal cells, but the details of neurotropism remain elusive.

Contemporary enterovirus D68 strains can replicate in neuroblastoma cell lines, primary human neurons [195, 198], and human brain organoids [203]. Enterovirus D68 can also replicate in a wide range of leucocytic cell lines, thus providing a means to reach secondary target tissues and a potential reservoir that may cause a prolonged viremia [90]. Moreover, in a mouse model, contemporary strains can reach the spinal cord by retrograde transport in the axons of motor neurons [199]. In an *in vitro* model with induced human pluripotent stem cells, the prototypic strains Fermon and Rhyne can also use retrograde axonal transport [199], indicating that this is not an ability acquired by contemporary strains.

In vitro, enterovirus D68 strains in clade B are independent of sialic acid as a receptor to infect motor neurons derived from human induced pluripotent stem cells, in contrast to Fermon, Rhyne, and clade A strains [199]; however, a study that uses a cell line derived from chronic myelogenous leukaemia finds sialic acid-dependent as well as sialic acid-independent strains both in subclades A1 and B2 [131]. Furthermore, contemporary strains can use ICAM-5 as a receptor [204]. However, although ICAM-5 is expressed at high levels in the brain, it is not expressed in the spinal cord (Human Protein Atlas, https://proteinatlas.org [205]) and the relevance of this finding to acute flaccid myelitis is thus unclear.

In most *in vitro* studies, the optimal growth temperature of enterovirus D68 is 33°C [87, 88, 134]. This is lower than the human core temperature and could favour replication in the upper respiratory tract. However, studies using a neuroblastoma cell line [198] and human brain organoids [203] find similar growth at 33°C and 37°C. Such ability to replicate at core temperature could allow the virus to infect tissue in the central nervous system.

2.2 Coronaviruses

Of the human coronaviruses, known as common cold coronaviruses, species 229E and NL63 belong to the genus *Alphacoronavirus*, and species OC43 and HKU1 belong to the genus *Betacoronavirus*. The genus *Betacoronavirus* also includes SARS-CoV-1, SARS-CoV-2, and MERS-CoV belong to.

2.2.1 Origins of human coronaviruses

2.2.1.1 Origins of common cold coronaviruses

Two common cold coronaviruses, 229E and OC43, were discovered in the 1960s. Two additional common cold coronaviruses, NL63 and HKU1, were found in the 2000s, following intensified research and surveillance after the outbreak in 2003 of the highly pathogenic SARS-CoV-1 [206].

It is estimated that OC43 originated in the early 20th century and 229E sometime between the 18th and the early 20th centuries [33]. It is proposed that the emergence of OC43 caused the pandemic in the 1890s called the "Russian flu" [207]. However, other studies suggest that the pandemic was due to an H3N8 influenza A virus [208].

For the origins of the common cold coronaviruses, the two *Alphacoronaviruses*, 229E and NL63, may have been transmitted to humans directly from bat reservoirs [206]. Alternatively, 229E could have entered the human population through an intermediate host, such as camels [209]. The two *Betacoronaviruses*, OC43 and HKU1, may originate from rodent coronaviruses [206], and HKU1 may have had two independent introductions into the human population [209]. For OC43, the animal coronavirus that is most closely related depends on the examined gene, with plausible ancestors including ungulate, canine, murine, and rabbit coronaviruses [209]. This pattern suggests that the evolutionary history of OC43 might include recombination between different animal coronaviruses.

2.2.1.2 Origins of SARS-CoV-2

SARS-CoV-2 emerged in late 2019 in Wuhan, China. Bats are the natural hosts for coronaviruses closely related to SARS-CoV-2. When and how the spillover from an animal to a human occurred is subject to intense investigations and discussion. The main hypotheses posit that it happened at the live-animal Huanan Market or the Wuhan Institute of Virology. Several studies favour an origin from wildlife [210], with the live-animal Huanan Market being the likely starting epicentre of the pandemic [211-213]. There is less scientific evidence for the lab leak hypothesis, but historically in 2003 and 2004, there were laboratory accidents with SARS-CoV-1, causing infections of laboratory staff [214] and minor outbreaks [215], respectively.

2.2.2 Clinical manifestations of coronaviruses

Testifying to their collective alias, the most common manifestation of the common cold coronaviruses is the common cold. Nevertheless, there are cases of severe disease [206], and NL63 is also a cause of croup [216, 217]. In surveillance studies of 229E, more than a third [218], and even a majority [219], of infections in children are not accompanied by reported illness. Similarly, a case-control study finds common cold

coronaviruses to be equally common among the cases of acute respiratory disease and in the controls [220], indicating relatively mild infections.

However, a systematic review suggests that infections with the common cold coronaviruses can also cause neurologic manifestations, such as fatigue, headache, febrile seizures, and, in rare cases, central nervous system infections [221]. Additionally, for OC43, in one report more than half of patients have symptoms from the gastrointestinal tract [222]. In contrast to the common cold coronaviruses, SARS-CoV-1 causes a severe respiratory illness, with a mortality rate of around 10% [223].

SARS-CoV-2 mainly causes respiratory symptoms with a flu-like or cold-like presentation, but fatigue, gastrointestinal symptoms, and changes in smell or taste are also common symptoms [224]. The infection fatality rate of SARS-CoV-2 increases with age, before vaccines ranging from 0.1% in the forty-year-old age group to more than 8% in the oldest age group of 80-year-olds and older [225].

2.2.3 Seasonality and circulation pattern of coronaviruses

As mentioned, the human coronaviruses 229E, HKU1, NL63, and OC43 and are sometimes called "seasonal coronaviruses" and they certainly live up to this designation. Already early studies note the common cold coronaviruses occur in a seasonal and cyclical pattern [219, 226-228]. The winter climate promotes coronaviruses' survival and facilitates transmission due to low temperature, low humidity, and low solar radiation [229]. Indeed, infections in the temperate zone are mainly detected in the winter [230].

To briefly describe the initial spread of SARS-CoV-2 in Europe during early 2020, the first cases detected were in Bavaria, Germany, but this introduction is successfully contained [231]. Subsequently, there were independent introductions of the virus from China to northern Italy [231], where the first major outbreak in Europe became apparent in late February. From northern Italy, the virus is quickly spread across Europe [232]. Further European dissemination is likely to have occurred in late February or early March due to superspreading in the Austrian Alps [233].

2.2.4 Immunity to coronaviruses

Infections with the common cold coronaviruses are common. In adults, the seroprevalence rate exceeds 90% for 229E, NL63, and OC43, and is almost 60% for HKU1 [234]. The primary infections often occur within the first years of life [235, 236], and antibodies are present in more than half of children by the age of two years [236-238],

Immunity to common cold coronaviruses is not long-lasting, and the early studies note reinfections are common [218, 219, 226, 228, 239]. After a year, reinfections with common cold coronaviruses are common [240]. Based on the half-life of antibodies, the median time to reinfection by the common cold coronaviruses is predicted to be more than

three years, and for SARS-CoV-2 to around 16 months [241]. Even so, in a community surveillance study, common cold coronavirus reinfections with the same species and genotype occur during a single season [242]. Similarly, in a challenge study of 229E, re-challenge with the same strain after one year results in virus shedding and antibody boosting, but no symptoms [243]. Furthermore, despite that the antibodies to common cold coronaviruses are persistent and usually present at high levels, the risk of infection does not correlate with antibodies levels, and there is little increase of antibody levels after reinfection [244].

Neutralising antibodies to coronaviruses target the spike glycoprotein [8-12]. Phylogenetically, the trees of OC43 and 229E have a ladder-like structure, and most sites under positive selective pressure are in the receptor-binding domain of the spike glycoprotein [34, 245-248]. In addition to the topology of the phylogenetic trees, an *in vitro* study also indicates that immune escape drives the evolution. In the study, the neutralisation capacity of stored serum samples was tested against pseudoviruses carrying different variants of the 229E spike protein [249]. The serum samples more potently neutralise those spike variants that precede the collection date of the sample than spike variants that only emerged after the collection date. This indicates that novel spike variants emerge due to their ability to escape from existing antibodies.

2.3 Motivation for the studies

Enterovirus D68 caused a global outbreak in 2014. Many children were admitted due to respiratory illness, and the possible connection to acute flaccid paralysis attracted much attention. In Stockholm, we detected subclade B1 strains closely related to strains in North America [175]. In 2015, we screened respiratory samples positive for enterovirus using a laboratory-developed real-time PCR. However, we did not find enterovirus D68 in any enterovirus-positive sample during 2015; likewise, there were no reports of circulation in Europe or North America in 2015. Thus, it was still unknown whether the outbreak in 2014 would be a singular event. However, in late August 2016, we detected an upsurge of respiratory samples positive for enterovirus in the routine clinical diagnostics at Karolinska University Hospital in Stockholm. At the time, there were few reports about enterovirus D68, and therefore, in **Paper I,** we investigated the epidemiology of the outbreak in Stockholm.

At the time of **Paper I**, the genotyping of enterovirus-positive samples and phylogenetic analysis of enterovirus D68 was mainly done by Sanger sequencing of the partial or complete VP1 region or, alternatively, by partial sequencing of the VP4/VP2 region (as done in **Paper I**). Sequencing of the whole genome using next generation sequencing would add resolution to the phylogenetic analysis, allowing to detect recombination and examination of all genes. Deep sequencing would also enable the detection of minority variants of the virus and the study of virus evolution within an infected individual.

Accordingly, in **Paper II,** we developed an amplicon-based method on a next-generation sequencing platform.

The successful development of an assay for whole genome sequencing of enterovirus D68 in **Paper II** allowed a more detailed phylogenetic analysis of the outbreak in 2016 and the subsequent circulation in 2018. In **Papers I** and **II**, the generated sequences originated from patients in Sweden. However, enterovirus D68 circulates globally, and (as shown in **Paper II**) the sequences of the viruses detected in Sweden were closely related to international sequences. As enterovirus D68 outbreaks occurred in several in 2014 and 2016 seasons, for **Paper III,** we invited colleagues from the recently formed European Network on Non-Polio Enterovirus (ENPEN) to collaborate on the study of the circulation in 2018. We also used sequences available in public repositories for the analysis to be as comprehensive as possible.

In early 2020, the world was hit by the SARS-CoV-2 pandemic. A few months into the pandemic, it was clear that the virus had established itself as a significant pathogen that would not be possible to contain, and that the pandemic would sooner or later transit into an endemic state. In an endemic state, knowledge about the epidemiology and seasonality of the four common cold coronaviruses might be valuable. In **Paper IV**, we analysed the available data at Karolinska University Hospital from clinical diagnostics for the common cold coronaviruses.

To mitigate the spread of SARS-CoV-2, governments implemented unprecedented measures, such as strict lockdowns and travel bans. Despite such efforts, the virus spread globally, and Sweden was hit hard by the pandemic during the first half of 2020. In **Paper V**, we investigated the introduction and early spread of the virus in Sweden during the first pandemic wave. We used real-time PCR to retrospectively test stored respiratory samples for SARS-CoV-2 and did whole-genome sequencing of SARS-CoV-2-positive samples to generate sequences for phylogenetic analyses.

3 RESEARCH AIMS

For this doctoral project, I set out to study the epidemiology of an emerging acute respiratory infection virus, enterovirus D68. By studying the phylodynamics of the virus my aim was to advance the knowledge about the following questions: Why did this previously endemic and, seemingly, rather harmless virus emerge as a significant pathogen? Had the virus become more virulent, or had it escaped immunity? My aims for the project were thus to investigate:

• The epidemiology of enterovirus D68 infections

• The phylodynamics of enterovirus D68, i.e., the pattern of evolution and geographic spreading

• The role of cellular immunity against enterovirus D68

However, before I had time to address all of these research aims, a new virus causing acute respiratory infections emerged. The pandemic with the novel coronavirus SARS-CoV-2 brought about unprecedented consequences, not only in a medical context but even more so in everyday life in societies all over the globe. As cases and fatalities rapidly increased, it was crucial that the attention and resources of virologists urgently shifted to this new pathogen to begin to build knowledge about it and provide, at least provisional, estimates on how the pandemic could unfold. Therefore, the aims of the doctoral project on the epidemiology of emerging respiratory virus infections were extended to include:

• The epidemiology of common cold coronaviruses

• The spread of SARS-CoV-2 into and within Sweden during the initial phase of the pandemic

When I started my doctoral project on the epidemiology and evolution of enterovirus D68, I did not imagine that I and everyone else would soon have our lives overturned by a pandemic caused by an emerging respiratory virus.

4 METHODOLOGICAL CONSIDERATIONS

4.1 Data sources

4.1.1 Laboratory-based sampling

In **Papers I, II, III,** and **IV**, we used data from routine clinical diagnostics for molecular diagnostics of respiratory virus infections. The studied population was the catchment area of the Department of Clinical Microbiology at Karolinska University Hospital, which covers six of the seven emergency hospitals in the Stockholm Region and approximately half of the outpatient care in the region.

The current laboratory information system Department of Clinical Microbiology covers the results of analyses since September 14, 2009. The study period for investigating the epidemiology of the common cold coronaviruses in **Paper IV** was thus a matter of convenience but still provided a sufficient timeframe to evaluate the long-term patterns of common cold coronavirus circulation.

In **Paper V**, we performed a diagnostic SARS-CoV-2 PCR on respiratory samples. The samples had originally been submitted for molecular diagnostics by healthcare providers in Stockholm Region. These samples had not previously been tested for SARS-CoV-2. In this paper we also sequenced SARS-CoV-2-positive samples from patients diagnosed at the Departments of Clinical Microbiology at Karolinska University Hospital (Stockholm), Örebro University Hospital (Örebro), and Sahlgrenska University Hospital (Gothenburg).

4.1.2 Retrieval of sequences from repositories

In **Paper I**, we retrieved a reference dataset of enterovirus sequences with the highest nucleotide similarity of the VP4/VP2 region in GenBank (www.ncbi.nlm.nih.gov/genbank/) using the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In **Papers II and III**, we retrieved sequences and metadata of records labelled as enterovirus D68 in GenBank using the interface NIAID Virus Pathogen Database and Analysis Resource [250] (http://www.viprbrc.org/). As GenBank is not a curated database, and we would fail to obtain sequences not labelled as enterovirus D68.

In **Paper V,** we retrieved SARS-CoV-2 sequences and metadata from GISAID (https://gisaid.org/). GISAID is a semi-open database for which one needs to register a personal account to upload or access sequences. Sequences are curated on upload, but the details of this curation are not described.

4.1.3 Contact tracing information for SARS-CoV-2

In **Paper V**, we obtained data about the presumed location for contracting SARS-CoV-2 from the registry SmiNet at the Public Health Agency of Sweden (Folkhälsomyndigheten) as registered by the treating physician or local infection control units, and from contact-tracing records at the Infection Control Units (Smittskydd) in Stockholm, Västra Göta-land, and Örebro. Even though the information is likely to be correct for most cases, it is difficult to exclude that some infections have incorrectly been attributed as contracted abroad, due to recent travel history. Likewise, some infections may incorrectly have been recorded as contracted in Sweden if information about recent travel was missing.

To estimate the onward transmission of SARS-CoV-2 from introductions, we inspected the Swedish sequences in the phylogenetic tree. We considered a case to have resulted in onward transmission if there was any Swedish case with an identical or descendent sequence for which the exposure was or missing exposure data. However, note that not all domestically contracted infections would represent community transmission, as some were household members or other direct contacts to cases infected abroad. Thus, our approach should yield a conservative estimate of onward transmission.

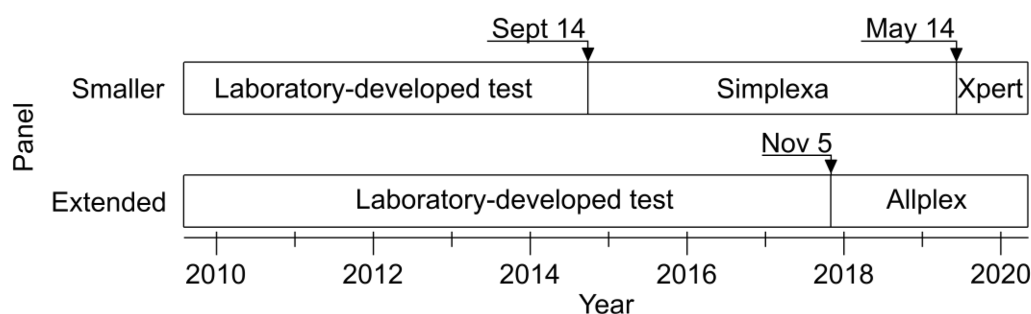
4.2 Laboratory methods

4.2.1 Clinical routine diagnostics of respiratory viruses

All data from routine clinical diagnostics were from accredited and validated assays. In **Papers I** and **II**, we used a laboratory-developed assay to detect enterovirus and rhino-virus [251], and in **Paper III,** the commercial Allplex assay (Seegene Inc.). For the detection of enterovirus D68, in **Papers I, II,** and **III**, we used a laboratory developed enterovirus D68-specific real-time PCR. This assay was based on the primers and probe by Piralla *et al.* [252]. It had been validated at the Karolinska University Hospital (unpublished data) and was used for routine clinical diagnostics.

In **Paper IV** on the epidemiology of common cold coronaviruses in Stockholm during September 2009 to April 2020, different diagnostic assays for the detection of respira-tory viruses were used during the study period (Figure 5). The assays in the routine clini-cal diagnostics at our laboratory changed over time in response to requirements to fac-tors such as capacity, cost effectiveness, and turn-around time. Two parallel multiplex panels were used in the routine clinical diagnostics: one smaller, detecting influenza A and B viruses and respiratory syncytial virus, and an extended panel that detected an additional 12-13 viruses (Figure 5), including assays for detecting the common cold coronaviruses. The laboratory-developed test by Tiveljung-Lindell *et al.* [251], which was used until November 2017, contained specific assays for each of the common cold coronaviruses, whereas the Allplex assay, which was used after November 2017, did not differentiate of the *Betacoronaviruses* OC43 and HKU1.

Figure 5. Assays for molecular diagnostics of respiratory virus infections



Diagnostic assays used in **Papers I, II, III, and IV**. The smaller panel included influenza A and B viruses and respiratory syncytial virus. The extended panel included: common cold coronaviruses (229E, NL63, OC43, and HKU1), adenovirus, enterovirus, bocavirus, metapneumovirus, parainfluenza viruses 1, 2, and 3, and rhinovirus. The Allplex assay also included parainfluenza virus 4 but did not differentiate the *Betacoronaviruses* OC43 and HKU1.
Laboratory-developed test by Tiveljung-Lindell *et al.* [251];
Allplex = Allplex Respiratory Panels 2 and 3 (Seegene Inc., Seoul (South Korea));
Simplexa = Simplexa Flu A/B & RSV Kit (Focus Diagnostics Inc., Cypress (CA, USA));
Xpert = Xpert Flu/RSV (Cepheid, (Solna, Sweden)).

4.2.2 Considerations on the molecular methods

4.2.2.1 Choice of assay for enterovirus D68-specific real-time PCR

In addition to sequencing, genotype-specific real-time PCR can be a cost-efficient and rapid alternative during outbreaks [253]. We used a laboratory-developed enterovirus D68 specific real-time PCR for this purpose in two of our studies. Thus, in **Papers I** and **III** we used the real-time enterovirus D68-specific PCR to select samples for sequencing. For the enterovirus D68-specific real-time PCR, we used the primers and probe by Piralla *et al.* [252]. We chose this assay in 2015 after an *in-silico* evaluation of published assays, using alignments of sequences from enterovirus D68 and other enteroviruses. We then chose assays with a short amplicon and an apparent good specificity for further wet-lab evaluation. This showed that the primers and probe by Piralla *et al.* [252] required the fewest cycles for target detection, which indicated that this assay had the highest sensitivity (unpublished data).

4.2.2.2 Considerations on enterovirus genotyping by Sanger sequencing

The recommended region for genotyping of enteroviruses is the VP1 region, and alternatively, sequencing of VP4/VP2 can be used [253]. In **Paper I**, we genotyped enterovirus-positive samples by Sanger sequencing of the VP2/VP4 region [254]. This protocol was validated and used at the Department of Clinical Microbiology at the time of the outbreak in 2016, which allowed for a rapid genotyping of enterovirus-positive samples.

However, most international enterovirus D68 sequences in GenBank covered the VP1 region. Since sequences can only be compared within the same region, our choice to use sequencing of the VP4/VP2 region in **Paper I** was thus a trade-off between the number of international sequences we could compare our sequences to and the timeliness of the results.

4.2.2.3 Choice of platform for whole-genome sequencing of enterovirus D68

For the whole-genome sequencing of enterovirus D68 developed in **Paper II**, we used the Illumina platform. The Nanopore and PacBio platforms were possible alternatives. Sequencing on Illumina generates much shorter reads than on Nanopore but has higher fidelity [255]. Although a consensus sequence obtained from Nanopore platform would have sufficient accuracy, intrasample single nucleotide polymorphisms would be more difficult to interpret and trust. The PacBio was an alternative that offered long high-fidelity reads. As our aims was to analyse intrasample variability, the Illumina or the Pac-Bio platform were our preferred options. Illumina was chosen because the access to PacBio was very limited, whereas we had access to a well-established workflow for Illumina sequencing in collaboration with Clinical Genomics at SciLifeLab.

4.2.2.4 Considerations for classification of enterovirus D68

The nomenclature by Tokarz *et al*. [28] is the most used classification of enterovirus D68 sequences. It is adopted by the Dutch National Institute of Public Health and the Environment, which provides the online Enterovirus typing tool [165]. It is proposed to designate subclade A2 as clade D as the genetic variation in VP1 between A1 and A2 is like that between the A1, B, and C clades [256]. Further subdivisions into subclades D1 and D2 [257] and even D3 [63] are also proposed. Nevertheless, in **Papers II** and **III,** we used the designation A2. The reason was to align with the nomenclature of the Entero-virus typing tool to facilitate communication and reproducibility.

4.2.2.5 Considerations on the classification of SARS-CoV-2

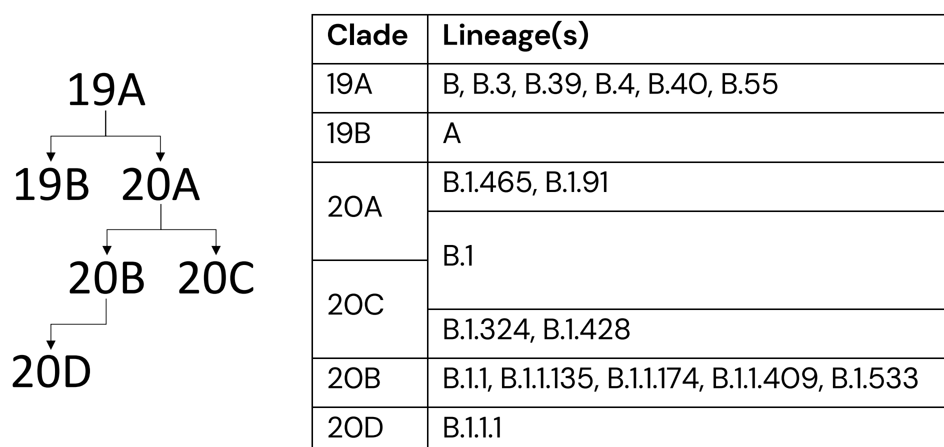
SARS-CoV-2 is categorised into variants based on whole-genome sequencing. The three major naming systems for this categorisation are Nextstrain, Pangolin, and WHO (Table 4).

Table 4. Naming systems of SARS-CoV-2

System	Naming rule	Format	Example
Nextstrain	Frequency threshold	Year + letter	19A, 20A, 20C
Pangolin	Phylogenetic criteria discerning lineages	Letter + numbers	A.1, B.1.177, B.1.1.529
WHO	Naming of variants of concern	Greek alphabet	Alpha, Delta, Omicron

In general, the Nextstrain clades and the Pangolin lineage are compliant. The Pangolin system is more fine-grained and contains more lineages than Nextstrain. Thus, in general, several Pangolin lineages would correspond to one Nextstrain clade. However, there was a crucial exception for the variants present during the first pandemic wave: sequences in Pangolin lineage B.1 were classified in Nextstrain clades 20A or 20C (Figure 6). Thus, the Nextstrain classification provided a better resolution for these early sequences. Since more than two-thirds of the Swedish sequences in the study for **Paper V** were classified as B.1, we chose the Nextstrain classification to resolve these better.

Figure 6. Classification of SARS-CoV-2 sequences



Clade	Lineage(s)
19A	B, B.3, B.39, B.4, B.40, B.55
19B	A
20A	B.1.465, B.1.91
20A / 20C	B.1
20C	B.1.324, B.1.428
20B	B.1.1, B.1.1.135, B.1.1.174, B.1.1.409, B.1.533
20D	B.1.1.1

Left: Schematic overview of the Nextstrain clades relationships. 19A is the root clade containing the reference strain Wuhan-Hu-1.
Right: Nextstrain clades and corresponding Pangolin lineages. The clades and lineages are limited to those detected in Sweden until June 2020.

4.3 Construction of phylogenetic trees

The evolutionary relationships between species or organisms can be visualised by phylogenetic trees. Table 5 lists the main approaches for constructing a phylogenetic tree [20]. Clustering methods use a distance matrix, whereas tree-searching methods evaluate the nucleotide distribution per site in an alignment of sequences. The number

of possible trees increases super-exponentially with the number of sequences. Thus, it is usually computationally impossible to perform an exhaustive search and evaluation of all possible trees with tree-searching methods; instead, heuristic search methods are used to find the “best” tree.

Table 5. Methods for phylogenetic inference

Approach	Features
Clustering method	
Neighbor joining	Computationally effective. Produces a correct tree topology if the distance matrix is sufficiently correct [258].
Tree search methods	
Maximum parsimony	Searches for the tree with maximal parsimony score, i.e., the smallest number of evolutionary events. Does not account for non-observed substitutions and unequal substitution rates between nucleotides and sites. May suffer from “long branch attraction”.
Maximum likelihood	Searches for a tree that maximises the probability of the alignment given the substitution model. Requires extensive computation.
Bayesian inference	Assumes a prior distribution of possible trees and generates posterior probabilities for the trees. Requires very extensive computation.

4.3.1 Considerations on the methods for constructing phylogenetic trees

In **Paper I**, we used MEGA for alignment and maximum-likelihood tree construction of sequences from the 2016 enterovirus D68 outbreak. In the **Papers II, III,** and **V**, we instead aligned sequences and constructed phylogenetic trees using Nextstrain’s pipeline Augur [21] in the workflow engine Snakemake [259]. In the default version of the pipeline, sequences are aligned by MAFFT [260], a maximum-likelihood tree is constructed by IQ-TREE [261], followed by the generation of a time-scaled phylogeny by TreeTime [262]. For SARS-CoV-2, there is an adaptation in the default settings such that sequences are aligned by Nextalign. As SARS-CoV-2 sequences are highly similar, Nextalign enables a quicker processing by using a codon-aware pairwise alignment to a reference sequence [263].

In **Paper V**, I modified the Augur pipeline by using MAPLE [264] for the tree construction. This allowed to construct a phylogenetic tree of all SARS-CoV-2 sequences in the dataset. However, I also constructed a time-scale phylogenetic tree that included a

small subset of the international sequences, as the size of the tree that contained all sequences only allowed generation of a divergence tree from the MAPLE output. MAPLE uses a more concise genome data representation that only annotates differences to a reference genome and is hence more condensed than the fasta and vce formats. This is computationally more efficient and allows for processing of huge datasets. Furthermore, MAPLE uses a maximum parsimonious likelihood that results in higher accuracy than IQ-TREE in large datasets of SARS-CoV-2 [264]. This is possible due to the very dense sampling of SARS-CoV-2 genomes: branches in a phylogenetic tree of SARS-CoV-2 are usually defined by a single mutation, and long branches are rare. Under such conditions, optimisations of the phylogenetic tree show a linear correlation between the parsimony score and the likelihood, i.e., a maximising of the parsimony score will also optimise the likelihood [265]. Thus, for SARS-CoV-2, a more accurate tree can be obtained using maximum parsimony since the method allows for a more extensive exploration of the tree space.

4.4 Statistical analyses

I used ordinary statistical methods in my studies, i.e., typical tests for evaluating null hypotheses (e.g., Wilcoxon rank-sum test) and regression analyses to estimate relationships between variables.

4.5 Ethical considerations

The Regional Ethical Review Board or the succeeding Swedish Ethical Review Authority approved all studies in the thesis.

4.5.1 Research performed without informed consent

We performed in-depth analyses of results from routine clinical diagnostics with informed consent waived by the ethical review authorities. An ethical issue to consider was that the samples had been submitted for a diagnostic purpose, and the patients were unaware that the samples were also being used for research purposes. Hence, the issue concerned the patients' autonomy.

If enteroviruses are detected in the stool or cerebrospinal fluid, the specimen is routinely genotyped as part of the national poliovirus surveillance program. One could argue that a similar genotyping of enterovirus-positive samples was performed on another type of specimen, namely respiratory specimen. Also, if enterovirus D68 was detected, this was reported to the patient's medical record and might thus provide additional information for the treating clinician about the cause of symptoms. Furthermore, for enterovirus D68, the additional analyses performed were directly related to the original clinical request for enterovirus diagnostics.

For the retrospective tests for SARS-CoV-2 on stored respiratory samples, testing for this pathogen had not been requested by the submitting physician. In addition, at the time of analysis, SARS-CoV-2 was a disease of public danger ("allmänfarlig"). Anonymising the samples before analysis was not feasible as the collection date and information on country of exposure were of critical interest for the subsequent analysis. We deemed that we were not in the position to decide whether revealing a positive test result would benefit or harm the patient. In accordance with the ethical approval, we reported a positive SARS-CoV-2 test result to the treating physician who decided whether to inform the patient or not. As the analyses were performed more than a year after sample collection, the results were not expected to influence patient care.

4.5.2 Protection of personal integrity

In all medical research it is crucial to protect the personal integrity of the study subjects. Therefore, it should not be possible to identify any single individual in the publications or other scientific reports. Hence, specific data or details may need to be limited to preserve personal integrity. Even so, it is challenging to ensure anonymity if a manifestation is rare, such as acute flaccid myelitis. Accordingly, in **Paper I**, the ages of the patients with severe manifestations of enterovirus D68 were provided only in age ranges, and likewise, the details regarding underlying diseases were not disclosed. Similarly, in the study of SARS-CoV-2 in **Paper V,** we needed to consider at what level of detail the data on exposure abroad, sampling date, and geographic location should be presented to preserve anonymity.

5 Results and Discussion

In my doctoral project, I have applied molecular epidemiology to study acute respiratory virus infections caused by enterovirus D68, common cold coronaviruses, and SARS-CoV-2. The studies were performed to increase our understanding of two emerging virus infections. The characteristics of enterovirus D68 and coronaviruses and their infections have both parallels and differences. Similar to the increased attention to enterovirus D68 after the 2014 outbreak, the SARS-CoV-2 pandemic increased attention to the common cold coronaviruses. In contrast to enterovirus D68, which has been circulating in humans for a long time, SARS-CoV-2 originated from a very recent introduction into humans. Furthermore, there was a huge difference in the number of available sequences in data repositories: the total number of whole-genome sequences of enterovirus D68 was surpassed by the number of Swedish SARS-CoV-2 sequences from the first pandemic wave.

5.1 Epidemiology of enterovirus D68

For enterovirus D68, in **Paper I,** we timely detected an outbreak in Stockholm in 2016 by using a real-time enterovirus D68-specific PCR, and in **Paper II,** we developed an assay for deep whole-genome sequencing. Through phylodynamic analyses in **Paper III**, we found a continuous antigenic evolution and a rapid geographic mixing. Together, these findings suggested undetected reinfections of adults (Figure 7).

In general, the severity of an infection depends on both host factors, such as immunity, and virulence factors of the pathogen, such as tropism. In the case of enterovirus D68, it is discussed whether the recent increase in severe respiratory and neurologic disease cases is due to an overall increase of enterovirus D68 infections or if the virus has come to cause more severe infections.

5.1.1 Seasonality of enterovirus D68

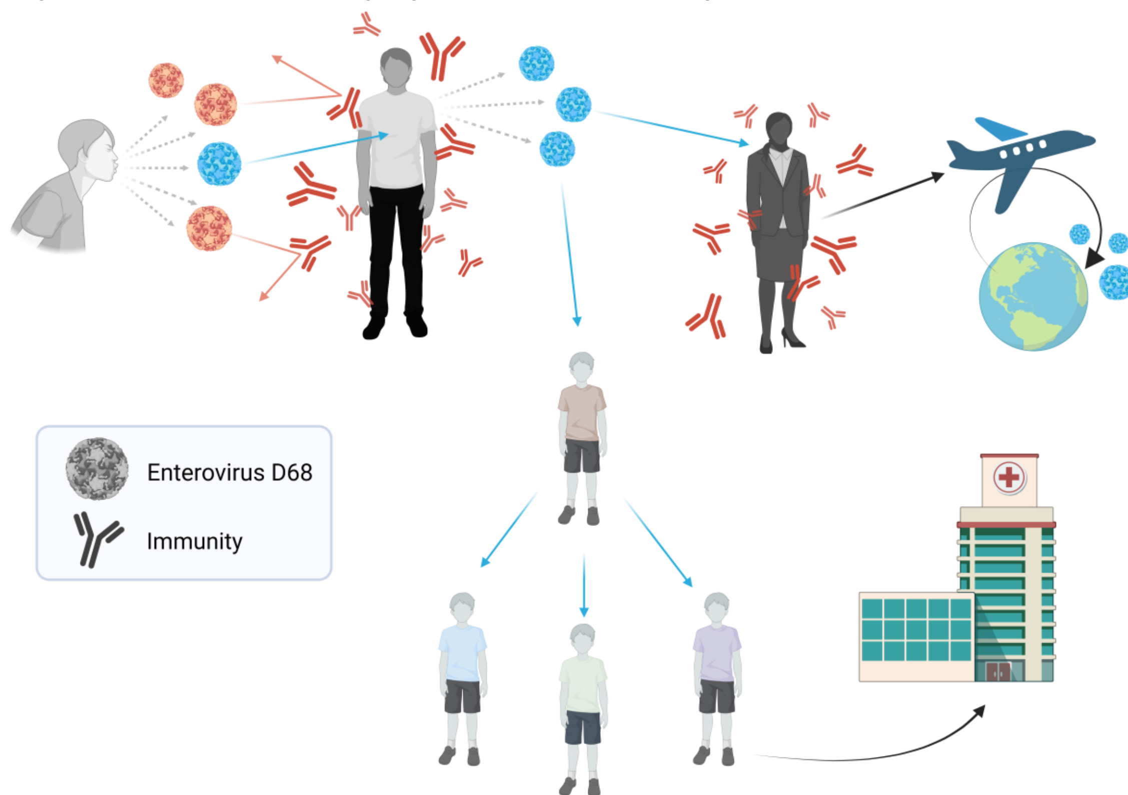
By analysing samples submitted for clinical diagnosis, we documented an outbreak in Stockholm in the early fall of 2016. The outbreak lasted four weeks and peaked in the first week of September (Paper I: Figure 1A). This peak time is consistent with several other studies that show enterovirus D68 to mainly be detected in the summer and autumn [29, 30, 48, 49, 85, 107, 116-118, 120, 123, 125, 126, 155, 168, 170, 176, 266-270]. The time of the peak may be due to a combination of favourable climatic factors and the end of the summer holidays.

5.1.2 Pattern of circulation of enterovirus D68

During my doctoral project, the circulation of enterovirus D68 occurred in even numbered years. In **Paper I** we showed that the 2016 outbreak was preceded by a high prevalence of enterovirus-positive respiratory samples in 2014, followed by few

enterovirus-positive samples in 2015, none of which were enterovirus D68 (Paper I: Figure 1B). The subsequent detections of enterovirus D68 in Stockholm were in 2018, which we characterised in our European multicentre study in **Paper III**.

Figure 7. Hypothesis for the geographic spread and antigen evolution of enterovirus D68



Immunity in adults exerts selective pressure on the evolution of enterovirus D68 by promoting the onward transmission of variants that escape existing antibodies acquired in previous infections. Travelling infectious adults disseminate enterovirus D68 geographically. Children are more prone than adults to symptomatic diseases due to a lack of previous immunity and differences in the anatomy and physiology of the respiratory tract. Thus, children are more likely to attend healthcare and be sampled, and enterovirus D68 is more likely to be detected in children than adults. Illustration created using Biorender.

The biennial circulation pattern in Stockholm was consistent with the patterns in Europe and North America (Figure 3). Accordingly, a systematic screening of clinical samples in France also shows a biennial pattern of enterovirus D68 circulation, with more positive samples in 2012 than in 2014 and oscillations of the size of the effective global population [29]. The oscillations of the effective global population could indicate repeated cycles of bottleneck events followed by rapid expansion. Such cycles could be due to the depletion of susceptible individuals followed by outbreaks due to immune escape mutations or sufficient replenishment of the pool of susceptible individuals. The prevalent circulation in 2012 is interesting because it counters the notion that there were more infections during the 2014 outbreak than in previous years.

As in Europe, in North America since 2014, the circulation has a biennial pattern, with few or no clinical cases in odd years, albeit with fewer cases than expected in 2020, presumably due to infection control measures during the SARS-CoV-2 pandemic [118, 120, 123-125, 138, 267, 268, 271]. However, despite the dominant circulation in even numbered years in Europe, the proportion of samples positive in Germany is similar [49] and even higher [48] in 2013 than in 2014, there is widespread circulation in Wales in 2015 [51], and there is circulation in Sweden and several other European countries in late 2019 [30]. Indeed, modelling of US data suggests that the biennial pattern may be a transient feature that is sensitive to stochastic demographic effects that affect population size, i.e., number of births and deaths [270]. This is in line with the modelling of enterovirus incidence that shows the birth rate to be a determinate of when the susceptible population is large enough for an outbreak of a particular serotype to occur [47].

5.1.3 Age distribution of patients with enterovirus D68

Among patients diagnosed with enterovirus D68 infection during in the 2016 outbreak described in **Paper I**, 60 of 74 patients were children, of whom 32 were in the age group 1-5 years. However, regarding the proportion of positive samples per age group, this was the highest in the age group 6-18 years (16 of 20 tested patients) and was also high in the adult age group (14 of 29 tested patients).

In **Paper III**, we found that while all subclades of enterovirus D68 were found in children, subclade A2 was overrepresented in adults, particularly in the elderly (Paper III: Figure 3A). Furthermore, subclade A1 was detected in older children than the B subclades, and it was also detected in adults. **Paper III** included all available sequences and metadata from GenBank from other researchers who had also found this predominance of subclade A2 among adults and the elderly [49, 54, 55, 63, 116, 122, 155, 272].

There may be a sampling bias that favours the detection of enterovirus D68 in children. Although enterovirus D68 was common among the sampled adults during the outbreak described in **Paper I**, children may be more likely than adults to have symptomatic enterovirus D68 disease. This could be due to differences anatomy and physiology of the respiratory tract, and a difference in pre-existing immunity. Additionally, sample practices for paediatric patients and adult patients may differ. Thus, in **Paper IV,** in which we analysed data from more than ten year of clinical diagnostics, requests for analysis of respiratory viruses with the extended respiratory virus panel was much more common in preschool children than in adults (Paper IV: Figure 4A). Therefore, there may have been relatively more undetected infections in adults. In line with this, in active community-based surveillance in Canada in 2014, all age groups have an increased and similar proportion of enterovirus D68-positive specimens, but paediatric patients under five years of age have a hospitalisation incidence due to enterovirus D68 infections that is approximately 20 times higher than that of adult patients [124].

As the subclade is unknown at the time of sampling, the overrepresentation of subclade A2 in adults and the elderly cannot be explained by a sampling bias. The finding suggests that subclade A2 infections in the elderly were either more common than infections with other subclades or caused more severe symptoms, which prompted the sampling and detection. The overrepresentation of subclade A2 disease in adults and elderly could be due to either immune escape or the phenomenon known as "original antigenic sin". The concept of "original antigenic sin" was first introduced to explain different responses to influenza vaccination [273]. In short, it refers to when an initial exposure to an antigen creates an immunological imprint, thereby influencing the subsequent immunological responses to similar antigens [274, 275]. Hence, if older people have previously been exposed to viruses that are antigenically more similar to the B clade than the A clade, they may have better protection if later infected with clade B viruses.

The emergence of strains with different antigenic properties is suggested as a possible cause of the increase in enterovirus D68 diagnoses during the 2000s [129]. Although enterovirus D68 is classified as a single genotype based on nucleotide and amino acid similarity, there are differences in the cross-neutralisation between clades and sub-clades. Thus, antibodies to clades A and B, respectively, have reduced neutralising capacity to the opposite clade, and the cross-neutralising effect against the Fermon strain is even lower [129, 192, 193, 276].

Antigenic drift of enterovirus D68 is indicated by the decline in neutralising titres to the Fermon strain in pregnant women sampled in Finland over three decades, despite sero-prevalence remaining at 100% [90]. In line with this, sampling in the Netherlands in 2006-2007 and 2015-2016 shows higher titres in children to a B3 strain than the Fermon strain, whereas adults have higher titres to Fermon than to B3 [69]. In contrast, adults in the UK sampled in 2006 have higher antibody titres to strain B3 than strains A2 and Fermon [68], whereas in children under five the titres to subclades A2 and B3 are similar. For these studies, it should be noted that the sampling in 2006 is around the estimated time of emergence of the B clade but before the B3 subclade, estimated to be around 2006 and 2009, respectively (Paper III: Figure 1A). Similarly, in Missouri, USA, seroprevalence in samples collected in 2012-2013 is 100% for Fermon and B1 strains but 91% for a subclade A2 strain [73].

Thus, the reason for more detections of subclade A2 in adults and elderly may be due to prior exposures to clade B-like viruses, which would provide a better protection against clade B viruses but worse against clade A viruses. Also, the limited cross-neutralising capacity between clades A and B may explain their co-circulation since the 2010s.

5.1.4 Acute flaccid myelitis

In **Paper I,** we describe three patients with acute flaccid myelitis in the 2016 outbreak, of which all were infected with subclade B3, and one was an adult.

It appears that there has been a recent increase in patients with acute flaccid myelitis due to enterovirus D68. In evaluating the reason for an increase, it should be considered whether there has been an actual increase of cases, or if more cases are detected due to an increased awareness. Patients with acute flaccid paralysis were also identified in the US prior to 2014, but in 2014, the cases occurred in a temporal cluster rather than irregularly as in previous years [277]. Similarly, in Sweden, already the temporal clustering of a few such cases during the autumn in 2014 caught the attention of the paediatric neurologists in Stockholm [278]. Thus, it seems more likely that the cases of acute flaccid myelitis due to enterovirus D68 have actually increased, rather than having gone unnoticed previously. Such increase could be due to either an increase in the number of enterovirus D68 infections, resulting in a parallel increase of the number of cases with rare manifestations, or due to the infecting viruses having acquired novel properties. Also, the age of primary infection should be considered, as for polioviruses the main hypothesis posits the increase of cases with paralytic diseases in the 20th century is due to an increased age of infection [279].

Whether there has been an increase in the numbers of enterovirus D68 infections is difficult to assess due to the challenges in identification before molecular diagnostics. Nevertheless, in Pennsylvania, USA, in 2009-2018, the number of cases with acute flaccid myelitis is correlated with the proportion of respiratory samples positive for enterovirus or rhinovirus that are genotyped as enterovirus D68 [138]. In retrospective testing of samples in Ohio, USA, there are more detections in 2014 than in any of the previous three years [113]. In addition, based on differences in seroprevalence, it is estimated that in the UK, infections in the age group 0.5-1 years increased by around 50% from 2001-2005 to 2012–2016 [68]. As for the age of primary infections, Taiwanese children younger than 6 years are more likely to be seropositive if they have siblings or attend day-care [70]. Thus, changes in demographics and social patterns could have impacted the number of infections and age when primary infections occur. However, due to the scarce data before molecular diagnostics it is difficult to further evaluate whether there have been any substantial changes in the epidemiology.

Mainly viruses in enterovirus D68 clade B are detected in patients with acute flaccid myelitis. Most of these are in the subclades B1 [124, 143, 145] and B3 [30, 54, 121, 125, 140, 141, 149, 154, 155, 159, 163, 164], but a few cases infected with subclades B2 [140, 146, 280] are also reported. Notably, subclade B2, which is reported in the European cases of acute flaccid myelitis in 2014 and 2015 [140, 146, 280], was not a new subclade in the region but had been present since at least 2009 (Figure 3). Nevertheless, neurovirulence

cannot be an exclusive property of clade B as there are also patients with acute flaccid myelitis in whom subclade A1 [162, 169] has been detected.

It is suggested that enterovirus D68 subclade B1 has acquired neurovirulent properties by mutations that are homoplastic, i.e., corresponding, with residues in other neuro-virulent enteroviruses [143, 281]. As also subclades A1 and B2 have been detected in patients with acute flaccid myelitis, this hypothesis would be strengthened if these mutations would be present also in the strains from these patients. However, all but one of the sequences from the patients infected with subclades A1 and B2 are short partial VP1 sequences.

In conclusion, it is difficult to separate the effects of possible epidemiologic changes that may have caused an increase in cases of acute flaccid myelitis. Thus, the increase may be due an overall increase of infections, an altered age of primary infection, and a possible acquisition of neurovirulent properties by contemporary strains. The dominance of enterovirus D68 clade B infection among patients with acute flaccid myelitis may be because acute flaccid myelitis occurs mainly in children, in whom clade B dominates.

5.2 Phylodynamics of enterovirus D68

For the phylogenetic analyses, we generated sequences from enterovirus D68-positive samples detected in clinical diagnostics and retrieved sequences available in the public sequence repository GenBank. We found that enterovirus D68 had a rapid geographic mixing and a positive selection in neutralising epitopes. These findings suggested that although enterovirus D68 was detected mainly in the preschool population, it may be asymptomatic infections in adults that explain the evolution and geographic spread.

5.2.1 Geographic dispersal of enterovirus D68

In **Paper I**, in which we investigated the 2016 outbreak in Stockholm, we did Sanger sequencing of VP4/VP2 region and found that the Swedish sequences in subclade B3 clustered with sequences from North America (Paper I: Figure 2). The whole-genome sequencing assay developed in **Paper II** allowed analyses with higher resolution and showed that the outbreak was due to multiple virus introductions with further local transmission (Paper II: Figure 6B).

In **Paper II**, we estimated the time of the last common ancestor of the subclades B1 and B3 to the first half of 2009. This estimate suggests that subclade B3, which caused the European 2016 outbreak, had been circulating for several years before it was detected in 2014 in Southeast Asia and China (Figure 3). In **Paper III**, we found that the subclade B3 strains detected in 2018 were mainly derived from the preceding 2016 circulation, in contrast to the relatively deep roots of the B subclades that dominated the 2014 and 2016 seasons (Paper III: Figure 1). One of the subgroups within subclade A2 from 2018

also had deep roots with the time of the most recent common ancestor being estimated to 2014.

Our phylogenetic analysis suggested that a significant undetected circulation maintained diversity between outbreak seasons (Paper III: Figure 2). The diversification of the European and American 2018 subgroups in the B3 subclade began approximately one year before the outbreak. Furthermore, despite the presence of hundreds of lineages during each seasonal outbreak (i.e., 2014, 2016, and 2018), the lineages derived from fewer than ten ancestral lineages at four years before each of these outbreaks. In 2018, the B3 sequences detected in Europe and North America had their closest ancestors in Asia, and the A2 sequences had their ancestors in China. Thus, the strains did not share the geographical location with their ancestors, suggesting rapid geographical mixing.

5.2.2 Antigenic evolution of enterovirus D68

In **Paper III**, we analysed the antigenic evolution of enterovirus D68. We found an increased rate of amino acid substitutions that was not limited to the putative neutralising epitopes in the BC and DE loops of the VP1.

In 2018, the North American sequences clustered into an almost exclusively North American subgroup within subclade B3. This subgroup was found to have a novel amino acid pattern in the BC loop, and the pattern in the DE loop in North American sequences had not previously been detected in North America (Paper III: Figure 4 D, E). In addition, all A2 sequences in 2018 had a novel pattern in the BC loop. In the VP1 gene, there was a relatively uniform rate of synonymous mutations across the gene; in contrast, there were spots of non-synonymous mutations, the rate being the highest in the BC and DE loops (Paper III: Figure 4C). Many substitutions also occurred in the C-terminus of VP1 and the central region of VP2. Overall, the substitution rate of surface residues was six times higher than that of buried residues or residues facing the interior of the capsid.

5.2.3 Hypothesis of reinfections with enterovirus D68 in adults

The increased substitution rate in neutralising epitopes and other surface residues was interesting in relation to the fact that most infections are detected in preschool children. Most of these preschool children are likely to have a primary infection and therefore would not have a pre-existing immunity that could drive immune escape. In addition, enterovirus D68 showed a rapid geographical dispersal. This suggests a significant impact from air travel, which is interesting as adults are more frequent flyers than preschool children. Taken together, these results suggest that undetected re-infection in adults is common, causing geographic dispersal and driving evolution by promoting immune escape.

5.3 Epidemiology of the common cold coronaviruses

We analysed the epidemiology of the common cold coronaviruses using data from routine molecular diagnostics for respiratory viruses in Karolinska University Hospital, Stockholm, Sweden, spanning from September 2009 to April 2020. We found a pattern of biennial alternation in samples positive for *Alphacoronaviruses* and *Betacoronaviruses*, respectively. Our results also indicated that infections with these viruses had likely been underdiagnosed among adults and the elderly.

5.3.1 Characteristics of patients with common cold coronavirus infections

In **Paper IV**, we found the highest proportion of samples positive for any common cold coronavirus among children up to five years of age; nevertheless, the viruses were found in patients of all ages, and there was an additional detection peak in the thirty-year-old age group (Paper IV: Figure 4). There was some variation in the proportion of positive samples between age groups, with NL63 and HKU1 decreasing with age, whereas 229E and OC43 were more similar across age groups. A household transmission study found that children were more likely to develop a symptomatic infection than adults [282]. However, infections are likely more common in children than adults, as another study using sequential serum sampling showed that transient increases in antibody titres increase were more frequent in children than in adults [218].

In **Paper IV**, we found that coronavirus infections may have been underdiagnosed among the elderly in Stockholm. This was indicated by the proportion of respiratory samples for molecular virus analysis that were analysed extended respiratory panel. This proportion was the lowest in patients aged over 80; however, the proportion of samples positive for 229E and OC43 were similar across age groups. Similarly, an early study of surveillance of respiratory infections show that 229E infections occur in all age groups [283]. Another surveillance study of respiratory infections in adults finds an average incidence of almost 7% of 229E or OC43 in hospitalised patients and that OC43 infections is more likely to be symptomatic than 229E [284]. In our study, OC43 was the most detected species, which is consistent with findings in other reports [285-291].

5.3.2 Seasonality and pattern of circulation of common cold coronaviruses

We found that the incidence of *Alphacoronaviruses* and *Betacoronaviruses* peaked in alternating biennial winter seasons (Paper IV: Figure 2). Furthermore, NL63 peaks coincided with OC43 peaks and 229E with HKU1 peaks. These coincident pairs had winter peak incidences starting in even years (e.g., 2010/2011) and odd years (e.g., 2011/2012), respectively. In addition, *Betacoronaviruses* peaked earlier in the winter than *Alphacoronaviruses* (Paper IV: Figure 3). While the pattern of co-circulating pairs is not fixed (Monto 2020), an early study also notes that 229E and OC43 circulate in alternating

years [219]. Furthermore, co-circulation of 229E with HKU1 and OC43 with NL63, or alternating circulation of OC43 and HKU1, has been reported previously [29, 288, 292].

The circulation pattern may be due to an interaction of the viruses, immunology, or both. Common cold coronavirus infections in young children are more frequent at the beginning of the epidemic season, suggesting their role in the transmission dynamics as a replenished pool of susceptible individual that can initiate the circulation of a season [229]. Primary infections with OC43 and NL63 provide cross-protection against subsequent infections with HKU1 and 229E, respectively [236]. In the context of SARS-CoV-2, much research has been done on possible cross-protection by pre-existing immunity to common cold coronaviruses. Thus, a SARS-CoV-2 infection increase antibody levels against OC43, but these antibodies do not confer cross-protective immunity against SARS-CoV-2 [293]. An indication of immunological imprinting is that pre-existing antibodies against the *Betacoronaviruses* OC43 and HKU1 impair the induction of SARS-CoV-2-specific antibodies [294]. Another indication of immunological interaction is the finding that OC43 infection in early childhood induces SARS-CoV-2-reactive CD4+ T cells [295].

In **Paper IV**, we used diagnostic data on common cold coronaviruses collected over 12 years to provide insight into how the SARS-CoV-2 pandemic might evolve. Therefore, the data from this study were also used in a separate study, which estimated the seasonal forcing in simulated SARS-CoV-2 pandemic scenarios [296]. In that study, we found that is likely that SARS-CoV-2, like the other coronaviruses, would spread more easily in the winter season, although the simulation results varied depending on the assumptions about the strength of the seasonal forcing and the basic reproduction number. Based on these results, we stated that a future decline in SARS-CoV-2 case numbers in the summer of 2020 may be due to seasonal forcing rather than successful infection control, and that SARS-CoV-2 was likely to become a winter virus in a post-pandemic era. Similar attempts to model the evolution of the pandemic based on the epidemiology of the common cold coronavirus also predict recurrent wintertime outbreaks [297] and that a possible cross-protection with common cold coronaviruses would impact the interval between outbreaks [298].

During the pandemic, new variants of SARS-CoV-2 have replaced each other [299, 300], and two critical characteristics of new variants are the transmissibility and evasion of immunity [301]. Thus, projections based on common cold coronaviruses should be made cautiously, as SARS-CoV-2 was recently introduced into an immunologically naive population and likely still is adapting to the human host. Still, there may be similarities between SARS-CoV-2 and common cold coronaviruses. Thus, OC43 circulation displays a pattern of alternating genotypes [302] and an evolution of novel genotypes over time [303]. Furthermore, several studies report an increase in OC43 infections in 2007 [286, 288, 303, 304]. Interestingly, temporally this increase coincides with the emergence of a

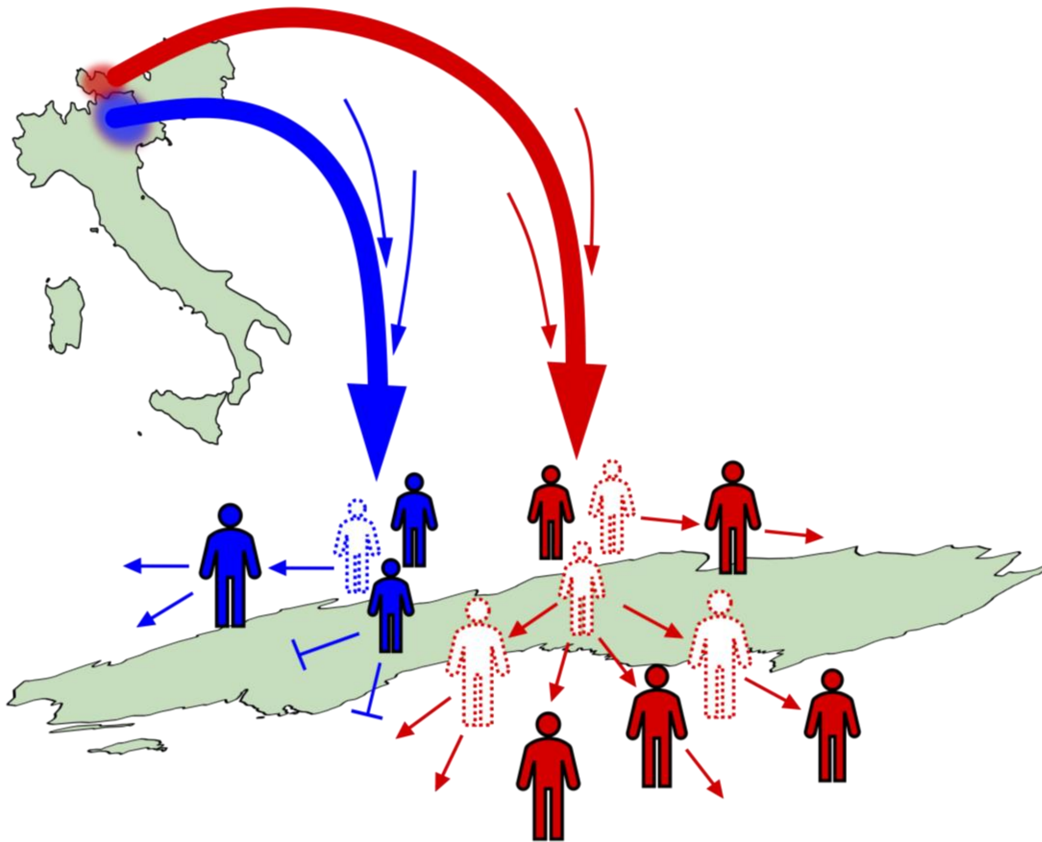
novel OC43 genotype [35, 303]. This bears similarity to novel SARS-CoV-2 variants causing new waves of infections during the pandemic. In addition, the rate of synonymous to non-synonymous substitutions in the OC43 spike decrease between genotypes the 1960s and the 2000s, which may indicate that more rapid evolution occurred after the spillover to humans as an adaptation to a new host species [35]. Similarly, the evolution of the receptor-binding domain in the spike glycoprotein in 229E may not only be due to selective pressure for immune evasion but also be caused by an optimisation of receptor binding [305].

It is worth considering that the four currently circulating common cold coronaviruses all appear to have been transmitted relatively recently to humans. It is unclear if this is due to a recent change in human behaviour that has increased the possibilities of spillover from animals or if other coronaviruses circulated previously and were replaced by the current four. The pandemic disrupted the circulation of several respiratory viruses [306, 307]. Also at my workplace, the Department of Clinical microbiology at Karolinska University Hospital, we noted a marked decline for most respiratory pathogens (unpublished data). It will be interesting to see whether the pre-pandemic circulation pattern of the common cold coronaviruses will be restored, or if SARS-CoV-2 will replace one or several of the current common cold coronaviruses.

5.4 The spread of SARS-CoV-2 into and within Sweden during the first pandemic wave

In **Paper V**, we investigated the introduction and early spread of SARS-CoV-2 in Sweden by retrospective PCR testing and phylogenetic analyses of whole genome sequences. By integrating the phylogenetic analyses with metadata and epidemiological data, we found that community transmission was established earlier than appreciated and that most sequences in Sweden belonged to a clade likely introduced from Austria. As there was a period of cryptic circulation of SARS-CoV-2 in the Austrian Alps, this likely caused mitigation measures targeted at travellers returning from risk regions to be insufficient in preventing onward domestic transmissions. We found that such undetected introductions significantly impacted how the first wave of the pandemic unfolded in Sweden (Figure 8).

Figure 8. Spread of SARS-CoV-2 into and within Sweden



Infection control measures targeting travellers returning from known risk areas, such as northern Italy, at least partially prevented onward domestic transmission of the SARS-CoV-2 clades 20B and 20A (blue). Such targeted measures were insufficient to detect and prevent onward transmission of SARS-CoV-2 introduced by travellers returning from areas of cryptic circulation, such as viruses in clade 20C from the Austrian Alps (red).

We approached the question how SARS-CoV-2 spread to and within Sweden during the first wave of the pandemic by retrospectively testing stored respiratory specimens for SARS-CoV-2 and by phylogenetic analysis. For the phylogenetic analysis, our team in Sweden sequenced stored samples positive for SARS-CoV-2 in Stockholm, Örebro, and Gothenburg, and retrieved all publicly available international sequences. We found that community transmission was established earlier than previously recognised. One cluster in clade 20C accounted for a large proportion of sequences viruses in the first wave in Sweden. Furthermore, early undetected introductions likely affected the course of the first pandemic wave.

5.4.1 Community transmission of SARS-CoV-2

We found that community transmission had been established in Sweden earlier than recognised (Paper V: Figure 1). This early community transmission was found by retro-spectively testing nearly 2,000 stored respiratory specimens in Stockholm that previ-ously had not been tested for SARS-CoV-2. This testing identified 12 new cases. None of

these cases preceded the already known cases; however, the earliest sample among these cases was collected on 3 March 2020, i.e., one week before community transmission was recognised. Importantly, this infection was probably community acquired as the case did not have any travel history or any identified link to a travel-related case. The underestimation of how early community transmission is established is not unique to Sweden and probably occurred in several countries [308].

5.4.2 Country of infection with SARS-CoV-2

In our study, information about the country of exposure was obtained for 83% of cases, either from the national SmiNet case registry or from local contact tracing records in Stockholm, Örebro, and Gothenburg (Paper V: Figure 3). Of these cases with information, 23% had exposure abroad, with the most common countries being Italy and Austria, accounting for 65% and 22% of cases exposed abroad, respectively. Most patients with exposure abroad occurred early in the pandemic, with a median sample date of 9 March. Among cases exposed in Austria, clade 20C was found in 55% of cases. In contrast, clades 20B and 20A were detected in 58 and 37% of cases exposed in Italy, respectively.

5.4.3 SARS-CoV-2 clade 20C in Sweden

Clade 20C was the most common Nextstrain clade in Sweden during the first pandemic wave (Paper V: Figure 4). In addition, most Swedish 20C sequences formed a cluster defined by the D936Y spike substitution conferred by the G24368T mutation. In our dataset of Swedish sequences, the root of this cluster (20C:G24368T) accounted for 15% of the sequences and was the overall most common haplotype, suggesting a superspreading event may have contributed to the initial dissemination. This hypothesis was supported by the fact that 20C:G24368T variants were rare in other countries, except Finland where it appeared much later than in Sweden, and likely due to spread from neighbouring Sweden.

The 20C:G24368T variant was first detected in Sweden in a sample collected on 8 March, in Denmark and the UK on 9 March, and within a week also in Saudi Arabia, Faroe Islands, Germany, Norway, Colombia, and the US. The almost simultaneous appearance of the variant in several countries suggests an introduction from a common source. Among cases with exposure abroad, most (74%) patients with clade 20C were exposed in Austria. Hence, we argued that Austria was also the likely source of 20C strains with the G24368T mutation. Nevertheless, it must be acknowledged that no 20C sequences with this mutation were detected in any of the cases with an exposure in Austria, nor in available sequences from Austria.

In vitro, the D936Y substitution, which is encoded by G24368T, does not increase replication capacity [309]. Although the D936Y mutation show a positive selection in

Sweden [310], this does not have to be due to the properties of the virus itself. Instead, it is possible that D936Y mutation passively hitchhiked on a variant that increased in proportion due to epidemiological factors, i.e., a founder effect. Specifically, during the initial phase of the pandemic, infection control efforts were targeted at travellers returning from designated high-risk areas. These areas included northern Italy and, later, the Austrian Alps. However, our study and those of Popa *et al.* [233] and Gudbjartsson *et al.* [311] indicate that there was a period of cryptic circulation in the Austrian Alps before the region was considered a risk area, allowing for undetected influx of infected travellers.

5.4.4 Undetected introductions of SARS-CoV-2

The molecular epidemiology allowed us to investigate the impact of control measures. Northern Italy was identified early as a high-risk area, and clades 20B and 20A were detected in cases exposed in Italy. In contrast, Austria, which was recognised as a risk area with some delay, was the main source of clade 20C. Clade 20C sequences could therefore be used as a proxy for lineages introduced from Austria, i.e., early undetected introductions, not targeted by mitigative measures.

To estimate the impact of undetected introductions, we used measurable proxies for the actual infection burden. These outcomes were the cumulative numbers of reported cases, admissions to intensive care units, and mortality due to COVID-19 up to 7 June 2020. On a regional level in Sweden, we found a positive correlation between the proportion of clade 20C sequences in a region and the per capita number of intensive care admission and deaths due to COVID-19 (Paper V: Figure 6). The correlation was weaker, and non-significant, for the cumulative number of diagnosed cases, which likely is a poorer proxy for true infection burden because testing capacity was very limited during the first pandemic wave.

These two findings, that a community transmission started earlier than recognised and a significant impact of undetected introductions, are relevant for future mitigation strategies. As a result of the late identification of geographical regions with ongoing transmission, prevention measures targeted at limiting influx from specific risk areas are likely to be implemented too late to be effective. Moreover, efforts focusing primarily on preventing influx will not be sufficient if community transmission already is established. Indeed, no travel restrictions stopped any later variants of concern, such as Alpha, Delta, or Omicron, from rapidly disseminating worldwide. Furthermore, travel bans are also criticised for causing stigmatisation and other adverse effects, which may limit open and timely sharing of data and thereby be counterproductive [312].

5.5 Considerations on limitations of the studies

The studies included in my thesis have several limitations that need to be considered. As discussed below, the most obvious limitation is the risk that the collected and analysed data are not representative, i.e., the selection bias referred to as sampling bias.

The **Papers I-V** were all based on patients sampled in routine clinical diagnostics. There is a risk that the viruses among sampled patients were not representative of the viruses that circulated in the community.

In our studies, and in general in hospital-based sampling, only patients with symptoms severe enough to present to health care are available for sampling, and not all will be sampled for testing. This can cause a sampling bias that favours detection of severe cases. For example, a population-based study of upper respiratory tract infections in Sweden shows the proportion of samples positive for picornaviruses (i.e., enteroviruses and rhinoviruses) and common cold coronaviruses to be higher in active surveillance than in hospital-based sampling [313]. This finding suggests that these viruses cause mild symptoms that do not prompt sampling in healthcare settings. In line with this, for enterovirus D68, there is a similar proportion of samples positive for enterovirus D68 in children and adults in active community-based surveillance, whereas 80% of positive samples in hospital-based sampling belong to paediatric patients [124].

It is essential to consider whether the enterovirus D68 sequences analysed in **Papers I-III** represented the strains circulating in the community. A strength of **Paper III** was the multisite sampling in several European countries and the extensive use of data in public repositories. Nevertheless, the sequences generated in **Papers I-III** and almost all the sequences in GenBank were obtained from symptomatic patients. As such, the studies suffer from the same potential sampling bias as all other studies based on routine clinical diagnostics. However, the reference dataset in **Paper III** also contained 27 wastewater samples from the UK; these sequences belonged to the A2 and B3 subclades and were well mixed in the phylogenetic tree with the clinical sequences included in the study. Since virus detection in wastewater is not biased by the symptomatic status of infected individuals, the routine clinical diagnostic samples included in this study seem to reflect community circulation reasonably well. In line with this, closely related strains of enterovirus D68 are detected in a parallel sampling of patients and wastewater [117, 314]. Similarly, in the UK, the enterovirus D68 sequences found in environmental surveillance of wastewater in 2015 and 2021 are closely related to concurrently strains from hospital-based sampling [161, 315]. Collectively, these results suggests that the results obtained in **Paper III** reflected the circulating strains of enterovirus D68.

A further a bias was introduced by sampling practices. For instance, practices to request an extended respiratory virus panel might vary between clinics. Thus, in **Paper IV**,

in which we analysed data from more than ten years of clinical diagnostics, the proportion of requests for the extended respiratory virus panel, i.e., analysis of 15-16 respiratory viruses instead of 3, was much higher for paediatric patients than for adults and the elderly.

In **Papers III** and **V**, we used genomes from sequence repositories as reference data, but these carry a similar risk of sampling bias as our own sequences and in addition are subject to additional biases due to different practices and sequencing efforts across countries. For enterovirus D68, sequences mainly originate from centres that perform research on this virus. For SARS-CoV-2, the number of sequences from the UK or the US outnumbers those from any other country. Therefore, inferences about the origin of a particular virus variant must be very cautious if based solely on the geographical location of similar sequences [231, 316].

In **Paper V**, which investigated the early SARS-CoV-2 events in Sweden, an obvious sampling bias was introduced by the initial recommendations to target PCR testing to symptomatic persons returning from travel in specific risk areas (in particular northern Italy). One way of mitigating this bias was our retrospective SARS-CoV-2 PCR testing of stored samples that had not been tested for this pathogen. This could have revealed cases with exposures in "non-risk" areas, but no such cases were identified. Moreover, it seems unlikely that a putative region with cryptic circulation would not have been identified by now. Indeed, although the Austrian Alps was initially such an area with cryptic circulation, it is quickly revealed through diagnoses among travellers returning to Iceland [233, 311].

6 CONCLUSIONS

In this doctoral project, I have used real-time PCR and next-generation sequencing to study the molecular epidemiology of acute respiratory virus infections. The Ph.D. project began with the study of enterovirus D68, a ubiquitous cause of infections, and ended with SARS-CoV-2, a novel pathogen that caused a pandemic.

By molecular diagnostics, we could timely detect the outbreak of enterovirus D68 in Stockholm in 2016. The further development of a whole genome sequencing assay for enterovirus D68 in **Paper II** enabled further international collaboration in Europe. Complementing the analysis with sequences in public repositories, we moved from the initial investigation of a specific outbreak in Stockholm in **Paper I,** to a global study of the phylodynamics of enterovirus D68 in **Paper III**. We found that although most of the sequences came from paediatric patients, the evolution of the virus could be driven by asymptomatic or mild infections in adults, which could also explain the rapid geographical dispersal of the virus.

In **Paper IV**, we retrospectively analysed data for the common cold coronaviruses from routine clinical diagnostics. At our laboratory, data about the circulation of the common cold coronaviruses at the species level had been gathered during more than a decade. In this data, we found a pattern of alternating biennial circulation of the four species of the common cold coronaviruses as well as that infections with these viruses may have been underdiagnosed in the adult and elderly.

Finally, in **Paper V**, we investigated the introduction and spread of SARS-CoV-2 in Sweden during the first pandemic wave. By retrospectively analysing stored samples using molecular diagnostics, we found that community transmission in Stockholm started earlier than previously recognised. Using phylogenetic analyses, we researched the likely origins of the viruses introduced into Sweden. Through the combined analysis of sequences and metadata together with epidemiologic data, the investigation suggested that early introduction from areas with unrecognised ongoing transmission had a critical role in introduction intro Sweden as well as the further evolution of the pandemic in Sweden.

My thesis has illustrated how two respiratory viruses, an old and a new, emerged as relevant new pathogens. Of course, these two viruses are not the last to emerge, and new threatening viruses will continue to appear. However, this project also illustrates how molecular techniques have allowed to obtain timely detections and epidemiological knowledge of such viruses.

7 FUTURE PERSPECTIVES

In the 2000s, the outbreak of SARS-CoV-1 stimulated research that led to the discovery of several respiratory viruses. In the 2010s, multiplex molecular diagnostic panels enabled rapid detection of respiratory viruses in clinical settings, and advances in sequencing technology allowed elucidation of their molecular epidemiology. Finally, the 2020s began with a pandemic that was met with unprecedented mitigation efforts and the application of molecular diagnostics and molecular epidemiology on an unparalleled scale.

The critical role of undetected infections has been a common theme in the studies of my thesis. Thus, in the case of enterovirus D68, we proposed that undetected infections among adults may drive the evolution and geographic dispersal; for the common cold coronaviruses, we suggested that infections among adults and the elderly may have been underestimated; and in the study of the first wave of SARS-CoV-2 in Sweden, we found early undetected introductions may have had a significant impact on how the pandemic unfolded in Sweden. The focus of these future perspectives will therefore be the aspect of undetected infections.

At the time of writing, the Department of Clinical Microbiology was implementing metagenomic sequencing as a diagnostic method. In short, this is sequencing of the entire nucleic acid content in a sample, and hence an unbiased approach that ideally can detect any microbe. Therefore, novel as well as known but unexpected pathogens can be detected [317]. In addition to implementing this as a diagnostic method, we will conduct a study by applying this method in the workup of patients with lower respiratory infections.

The SARS-CoV-2 pandemic has been unprecedented in many ways. One aspect is the generation of an enormous number of viral whole-genome sequences. This has been essential for detection of novel virus variants and immune escape mutations. In comparison, we have much less information about the molecular epidemiology of several other respiratory viruses [65]. For example, when writing, just the number of Swedish whole-genome sequences of SARS-CoV-2 from the first pandemic wave exceeds the total number of whole-genome sequences of enterovirus D68, metapneumovirus, or any of the common cold coronaviruses. At the time of writing, circa 250,000 Swedish SARS-CoV-2 sequences are publicly available. As the threat from SARS-CoV-2 will hopefully recede soon, it would be valuable to extend this capacity and infrastructure for molecular surveillance to other pathogens. For example, extended surveillance of enteroviruses in respiratory specimens in Denmark has led to the detection of rare genotypes [318].

Strategies such as sampling of returning travellers [319] or wastewater from aircrafts [320] are approaches to obtain early warnings about introductions of pathogens from

abroad. Nevertheless, globally available, and affordable, diagnostics and surveillance would address the underlying cause for such sampling. As effective distances worldwide have shrunk and air travel becomes increasingly important for the geographical dispersal of pathogens [321], modern surveillance must therefore be global to be useful and provide timely information. Molecular epidemiology offers powerful tools in this regard, but its true value will be obtained first when the knowledge it generates is translated into timely action.

As illustrated by my doctoral project, the epidemiology of acute respiratory viruses is a global issue. Therefore, there is added value in conducting epidemiological studies in broader international collaborations, such as European coordinated efforts on enterovirus D68. Community or environmental surveillance could complement molecular epidemiology studies to avoid the sampling bias inherent in routine clinical diagnostic samples. Indeed, during in parallel sampling of patients at hospital and of sewage, in the environmental surveillance enterovirus D68 is detected even at times with no clinical cases [314]. With next-generation sequencing, enterovirus D68 in wastewater can be assigned to the level clade or subclade [117, 161, 314, 315, 322]. In addition, wastewater surveillance is can monitor other pathogens such as adenoviruses, hepatitis A and E viruses, and noroviruses [314, 323], and is also valuable for the surveillance of SARS-CoV-2 [324, 325].

Finally, virological research has mainly focused on viruses for only a few host species other than humans [326]. However, with the expansion of human activities and ongoing climate change, the risk of novel pathogens being introduced is escalating [327-330]. Surveillance should, therefore, not be limited to the human context, but should ideally be approached from a One Health perspective, i.e., also including animal and environmental health. Indeed, I would hope that this would lead not only to a swift response to novel pathogens but also to a proactive prevention of viral emergences and emergencies.

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