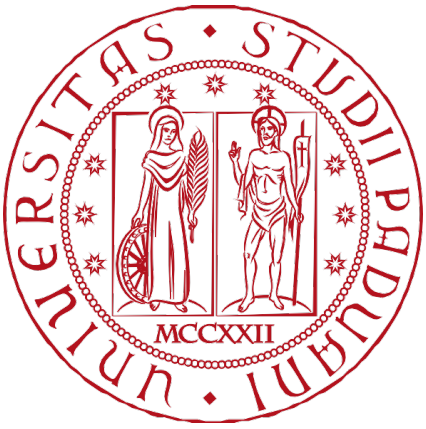
University of Padua

Master's Degree Programme in Medical Biotechnologies

Department of Molecular Medicine



Surveillance of respiratory viruses in 2024/2025 season in the province of Treviso (Italy)

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Abstract

After SARS-CoV-2 pandemics, national and regional surveillance of respiratory pathogens gained particular attention to control and prevent emerging and existing viral epidemics outbreaks. The genome of influenza viruses is subjected to seasonal antigenic drift and to antigenic shift over the years, which causes changes in its genome sequence and requires update of diagnostic methods. RespiVirNet is an important surveillance network which collects epidemiological data at regional and sub-regional level to provide seasonal national reports. This study aims at providing the number of positive cases of respiratory pathogens among Treviso province's hospitalized patients by following the RespiVirNet surveillance setting. Influenza A pdm09, A H3N2, A H1N1 virus, Respiratory syncytial virus A and B, Metapneumovirus, human Adenovirus, human Enteroviruses, human Rhinoviruses, human Coronavirus OC43 are the targeted viruses. The pathogen detection was performed in nasopharyngeal samples, bronchial aspirates and broncho-alveolar lavages through the tagging oligonucleotide cleavage extension (TOCE) and multiple detection temperatures (MudT) technologies. The innovation of this method consists of a multiplex one-step real-time nested RT-PCR to detect respiratory viruses and influenza subtypes. The thesis results report about the most prevalent viruses and the positivity percentages by demonstrating their targeted population and their seasonal circulation. Human Rhinovirus positives prevailed over the other viruses, followed by human Coronavirus OC43 and Influenza pdm09 virus circulating during winter months till April, while metapneumovirus reached its peak in spring. The data collected were compared to the ones registered by the RespiVirNet national reference to provide two different sample-populations. Data about Influenza vaccination are included in this project thanks to Treviso's Sentinel doctors' samples, collected at Treviso hospital and analyzed in Padua. Positive vaccinated patients for influenza virus represent a small percentage over all the vaccinated in the provincial sample-population. The epidemiological situation of Treviso province's hospitalized patients can be considered as a sample-population mirroring the national overview to monitor the trend of respiratory viruses. The data collected during this thesis work underline the importance of a surveillance plan to follow up the frail conditions, to control the possible outbreaks of new diseases, and to update the seasonal vaccine formulation.

Introduction

This thesis collects and analyzes the epidemiological data about the seasonal circulation of respiratory viruses subdivided into 3 panels, among hospitalized patients of Treviso province. This study is related to the RespiVirNet network, a national surveillance project about respiratory viruses and bacteria circulation during the flu period. The first panel includes Influenza A virus (Flu A), Influenza B (Flu B), Respiratory syncytial virus (RSV A), Respiratory syncytial virus (RSV B), Flu A-H1N1, Flu A H1pdm09, Flu A-H3N2. The second panel detects Adenovirus (AdV), Enterovirus (HEV), Parainfluenza virus 1 (PIV 1), 2 (PIV 2), 3 (PIV 3), 4 (PIV 4), metapneumovirus (MPV). The third panel marks Bocavirus (HBoV), Rhinovirus (HRV), Coronavirus NL63 (CoVNL63), Coronavirus OC43 (CoV OC43).

In this Introduction, the most relevant viruses in terms of positivity cases reported in this study, are described underlining their most important features.

1. From global to national surveillance

From the experience of past respiratory pathogens pandemics, such as COVID-19 and influenza A (H1N1) pdm09, World Health Organization (WHO) has highlighted the future threat represented by emerging respiratory viruses and bacteria. For this reason, WHO has pointed out the need for a surveillance plan in the document *"Preparedness and resilience for emerging threats (PRET) module 1: planning for respiratory pathogen pandemics"* (2023) [(1) (2)].

PRET module underlines the presence of serious gaps in the world's prevention and defense against respiratory pathogens pandemics by driving countries to get ready for the next possible outbreak. This document illustrates a plan that should be implemented by both regional and global stakeholders to manage emergencies. The framework of this project beside showing how to contain community transmission, it firmly sustains the importance of prevention and preparedness through surveillance at sub-national, regional, national and global level. In fact, the emergency coordination is based on four main factors: collaborative surveillance, community protection, access to countermeasures and clinical care.

Surveillance consists not only in the detection and monitoring of pathogen, but it also supports evidence-informed operational decision making and it shares information

routinely, before and during respiratory pandemics. It's a useful tool to provide key information for countermeasures to emerging respiratory viruses.

In PRET, recommended actions to strength surveillance and laboratory services according to *International health regulations (IHR, 2005*) are described. Among them, this document mentions the need to review and strengthen the existing surveillance systems for respiratory pathogens, in particular the integration of multi-pathogen laboratory testing within sentinel Influenza-like Illness/Acute Respiratory Infection and Severe Respiratory Infections case-based surveillance system. By adopting this measure, it could also provide data about targeted population groups and understand the disease spread during pandemics.

In 2005 due to Influenza pandemics outbreaks, in particular the 2003 avian H5N2 diffusion, WHO recommended the Member States to set up and constantly update a Pandemic Plan for Influenza viruses.

In Italy in 2006, the development of the *"Italian Pandemic Plan of 2006"* replaced the previously existing one *"Piano italiano multifase d'emergenza per una pandemia influenzale"* of 2002.

With the outbreak of SARS-CoV2 pandemics in 2019 the flu plan was amplified to other respiratory pathogens other than influenza virus. The *"Piano strategico-operativo nazionale di preparazione e risposta a una pandemia influenzale (PanFlu) 2021-2023"* [(49)] is committed to set a national plan to tackle influenza pandemics, with the awareness that potential threats are not just limited to influenza viruses, but they are extended to other pandemic potential respiratory pathogens, such the COVID-19 experience has taught. In this regard, in Italy the well-established Surveillance RespiVirNet system, previously called InfluNet, is the national fundamental tool to monitor respiratory pathogens circulation through diagnostic and epidemiological data. [(1)]

1.1 RespiVirNet

RespiVirNet is the Integrated Surveillance System (epidemiological and virological) of flu-like syndromes and of respiratory viruses [(3)], coordinated by Istituto Superiore di Sanità (ISS) with the support of Ministero della Salute. It engages a network of General Practitioners, primary care Pediatricians and Regional Reference Laboratories. They deal with sample collection of patients affected by flu-like symptoms, according to the clinical definition, while their analysis and reporting involve the reference laboratories. The clinical definition of "flu-like syndrome" has been modified from 2014-15 season

to adapt it to the European one by ECDC (European Commission Decision 28/IV/2008). [(1)] A subject affected by flu-like syndrome is the one who manifests the quick and sudden onset of at least one of these general symptoms: fever, fatigue, headache, myalgia, and at least one of these respiratory symptoms: cough, sore through and wheezing. [(1)] Data collection of RespiVirNet surveillance began the 42th week of 2024 (Monday 14 October 2024) and ended the 17th week of 2025 (Sunday 27 April 2025). Although, it's highly recommended to extend the surveillance up to the whole summer/fall period (up to the 41st week of 2025). The data analysis is performed by ISS and the results will be published weekly in the epidemiological RespiVirNet report of Ministero della Salute internet website.

The targets of 2024-25 season for RespiVirNet laboratories analysis are: influenza viruses with their subtypes specification, Respiratory Syncytial Virus (RSV), Rhinovirus, Parainfluenza viruses (PIV), Adenovirus, Metapneumovirus, Bocavirus and nonhuman Coronaviruses different from SARS-CoV-2. General objective of RespiVirNet surveillance is to build a data bank to evaluate the incidence of flu-like syndrome cases of the current season. The specific goals are:

1. To describe flu-like syndrome cases from a sample of "sentinel doctors" of the National Health Service.
2. To estimate the week of beginning, the duration and the intensity of the seasonal epidemy.
3. To estimate incidence rates per week during the season.
4. To estimate incidence rates according to age.
5. To use incidence data to create mathematic models for the estimation of the impact and of countermeasures adopted.
6. To share surveillance data with ECD in the European data bank TESSy (The European Surveillance System).

The requirement to guarantee the surveillance parameter is to involve as many "sentinel doctors" as possible to monitor at least the 4% of the national population. The number of pediatricians recruited must ensure the surveillance of at least 4% of syndromes in 0-4- and 5-14-years old population.

Virological surveillance system is aimed at:

1- Monitoring the circulation of A and B influenza viruses and their subtypes in the specific geographic areas and during the different periods of the epidemic season.

The surveillance is also aimed at controlling the circulation of other respiratory viruses such as SARS-CoV-2, RSV A and B and possible coinfections.

2- Evaluating the antigenic homology among epidemic and vaccinal strains through serological and molecular analysis on flu-like symptoms patients.
3- Evaluating the susceptibility of influential viruses to antivirals, in particular neuraminidase inhibitors.
4- Evaluating the number of SARS-CoV-2 infections and of other respiratory viruses in the "RespiVirNet" system.

Antigenic proteins of influenza virus are highly variable, and this causes the emergence of new variants able to overcome antibodies barriers in the population by leading to epidemic influenza viruses. The identification of these variants is possible thanks to antigens characterization and molecular analysis of viral strains. The analysis has a fundamental role mainly in the initial and final phases of the epidemic season, to evaluate the effectiveness of vaccines, and to eventually implement them. The National Influenza Centre (NIC) will receive the results of RespiVirNet selected influenza viral cases from regional laboratories, which will be sent to WHO Collaborating Centre for Reference and Research on Influenza, The Francis Crick Institute of London.

National viral results will be published, together with the epidemiological ones, on the weekly updated on the Ministero della Salute, WHO, and ECDC website. Antigenic and molecular data of viral strains national data will be discussed in Ginevra (WHO), to update the vaccinal composition for the season 2025-2026.

In this thesis project, we collected samples from hospitalized patients of Treviso Ca' Foncello Hospital, Conegliano's and Vittorio Veneto's Hospital to analyze them and to obtain epidemiological data about the incidence of each pathogen included in the respiratory panel within the flu season, starting from the Monday, 14th October 2024 to 4th May 2025. Analysis for the bacterial panel is also available for external patients such as samples sent from Treviso general practitioners. The results of Influenza and RSV cases are randomly selected and directed to the regional report of RespiVirNet surveillance.

2. Viral respiratory panel

2.1 Influenza virus

Influenza viruses A and B cause seasonal epidemics of infectious respiratory disease in humans, characterized by about 1 billion infections, 3-5 million cases of severe illness, and 300,000 – 500,000 deaths per year worldwide as reported by WHO reported. [4]

2.1.1 Classification and evolution

Influenza viruses belong to *Orthomyxoviridae* family which is subdivided into influenzavirus A, B, C and D *genera*. Influenza A virus (IAVs) infects avian and mammal species including humans, birds, ducks, chickens, turkeys, pigs, horses and dogs, while influenza B virus (IBVs) targets are humans and seals.[5] Influenza C and D viruses do not cause substantial disease in humans, however cases of influenza C have been reported in particular instances.[6] Influenza D virus instead has been isolated from pigs and cows in 2011 and infects mainly cattle, with no reported human cases. [7][8] Influenza A viruses are responsible for severe mortality, clinical, socio-economic consequences in the population. This occurs when a new influenza strain arises from an animal origin and spreads rapidly among people that are no immunized, by causing excessive mortality and morbidity such as a real pandemic. Influenza A viruses have 18 hemagglutinin- (HA-) and 11 neuraminidases (NA-) known subtypes. 16 HA and 9 NA were found in aquatic birds such as ducks, geese, and shorebirds. Avian influenza viruses infect poultry, and a few of them have established infections also in other species, such as pigs and humans (H1N1, H2N2 and H3N2). These animal reservoirs provide a source of different HA and NA genes that can be exchanged between viral strains by the antigenic shift mechanism. It consists of genome reassortment of two different viral strains after the coinfection of the same single host. This process induces cross-species transmission because the reassortment of gene segments between different influenza A virus strains forms a novel virus able to infect a new species. [8] This phenomenon is responsible for the influenza virus evolution of last decades together with another important mechanism, the **antigenic drift**. The antigenic drift is related to the accumulation of mutation in the viral genome during viral replication (around 1.5×10^{-5} per nucleotide per replication) mainly due to the lack of proofreading activity of viral RNA-dependent RNA polymerase. [9] Mutations establish within the antigenic sites of HA and NA envelope proteins, which are fundamental for the host cell invasion.

In fact, this mechanism leads to evasion of host acquired immunity to previously circulating strains. [10]

Influenza B viruses are classified according to their antigens (HA) and genome characteristics into two lineages: B/Yamagata/16/88-like lineage and B/Victoria/2/87-like lineage. First Influenzas B virus isolation was performed in 1940s by *Sharp et al.* while the antigenic divergence into the two lineages was reported later, in 1990 by Rota et al. In 2000s the replacement of B/Victoria lineage with B/Yamagata was described [11], and from that moment both lineages start co-circulating globally. [12]

Pandemic influenza occurs every 10-50 years. [8] There have been four influenza pandemics in modern times *(fig.1)*. In 1918-19 the so called "Spanish" flu represented the most severe pandemic, which killed around 50-100 millions of people worldwide. The viral sequence was characterized by Taubenberger only in 2005 and this virus originated mostly from an avian host. [13] [14] 1918's influenza A H1N1 virus circulated until 1957, when established a new lineage derived from its mutations, the 1957's "Asian" flu virus (H2N2 subtype). This virus circulated in humans for a decade. It was the result of the reassortment between HA, NA, PB1 vRNA segments of an avian influenza virus, and the remaining five vRNA deriving from the previously circulating H1N1 virus. In 1968 another genetic reassortment caused a new influenza virus H3N2, responsible for the Hong Kong influenza outbreak. Its genome was formed by six vRNAs from the previously circulating H2N2 virus and it possessed HA and PB1 vRNAs derived from avian H3 influenza virus found in pigs. In 1977 the 1950-like H1N1 virus reemerged and circulated in humans together with the H3N2 virus, until they were replaced by a new pandemic virus, the 2009 H1N1 pandemic influenza virus, called the "Swine influenza". It was created by multiple reassortments events occurred in pigs. This virus had PB2 and PA polymerase vRNA segments derived from avian influenza viruses, NP, M, and NS vRNA segments derived from previously circulating H1N1 swine influenza virus, while HA, NA, and PB1 vRNA segments from human influenza viruses. This triple reassortment caused sporadic infections at first but did not become adapted to humans. Then this triple-reassorted virus reassorted together with a Eurasian avian-like swine virus resulting in the novel 2009 pandemic H1N1 virus. Nowadays this novel virus represents a stable lineage in humans and co-circulates with H3N2 influenza viruses. [14]

Humans can also be infected sporadically by animal influenza viruses, this type of infections are called zoonotic, mostly avian and swine flu. For example, avian H5 and H7 influenza have caused hundreds to thousands of cases in humans. [10] Some subtypes such as influenza A H1N1, H3N2 and H1N1 Pdm09 are still circulating in humans causing seasonal influenza, that are targets of this surveillance project.

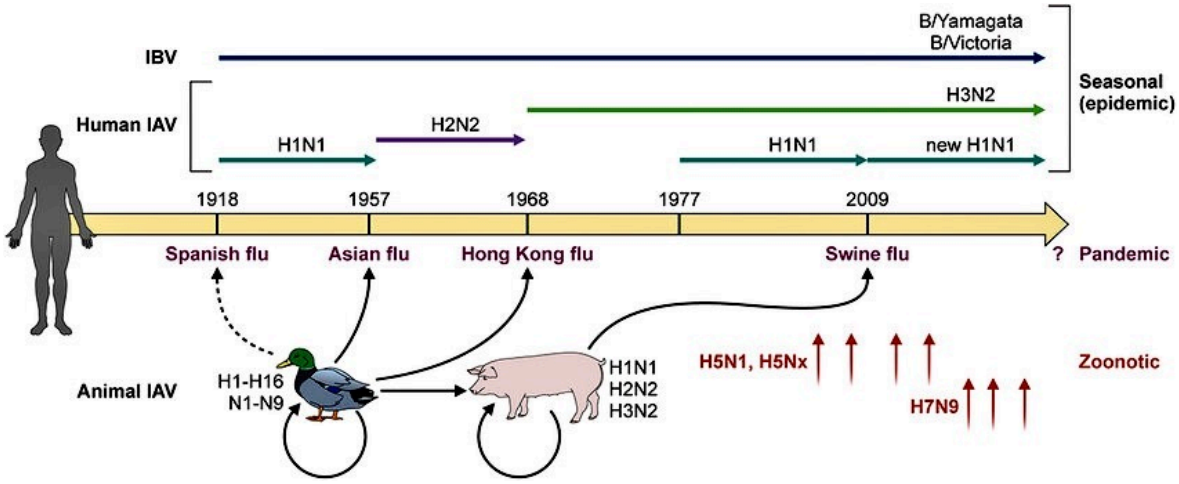

***Figure 1**. Influenza virus chronological epidemic outbreaks. Source: Liang Y. Pathogenicity and virulence of influenza. Virulence. 2023 Dec.*

2.1.2 Influenza virus genome

IAVs are enveloped RNA viruses constituted by eight single-stranded segments, with a genome of about 13.5 kb. The envelope includes two major glycoproteins, haemagglutinin (HA) and neuraminidase (NA), and the transmembrane protein M2. The matrix lattice is located just beneath the viral membrane, and it's formed by M1 proteins. Each RNA segment (vRNA) is represented by a viral ribonucleoproteins complex (vRNP) formed by the vRNA, encapsulated by nucleoproteins (NPs) and associated with PB1, PB2, and PA polymerase complex proteins. Each vRNA segment encodes for 1-3 genes (and proteins), through alternative splicing and frameshifting. [10] Ten viral proteins are essential for viral infection. They are PB1, PB2, PA, NP, HA, NA, M1, M2, NEP/NS2 *(fig.2)*. The three subunits of the viral RNA-dependent RNA polymerase (PB1, which contains an RNA-dependent domain, PB2 containing a cap-domain, and PA formed by an endonuclease domain) are encoded by the three largest RNA segments. They are responsible for RNA synthesis and replication in infected cells. Two viral segments encode for the haemagglutinin (HA), which binds the host receptor and

mediates viral entry, and for the neuraminidase (NA). NA facilitates the release and the spread of virion progeny by cleaving sialic acid on the cell surface. M1 is involved in the viral particle assembly and budding from the infected cell plasma membrane. M2 is an ion channel transmembrane protein required for viral replication and cellular homeostasis. NS1 acts against the host antiviral response, while NS2/NEP facilitates the export of vRNAs from the nucleus to the cytoplasm for virion assembly. (10)

NP is encoded by RNA segment 5, while segments 6 and 8 encode more than one protein, M1 and M2, non-structural protein N1 and the nuclear export protein (NEP). (4)

Influenza B virus, as IAV, is an enveloped virus formed by 8 negative-sense singlestranded RNA segments. They encode for at least 11 proteins: polymerase subunits (PA, PB1 and PB2), non-structural protein (NS1), nuclear export proteins (NEP), matrix proteins (BM1), ion channel (BM2), surface glycoproteins (HA, NA, NB) and the nucleoprotein (NP). The number of encoded proteins is different in influenza A, which is higher (at least 17 proteins) than in influenza B virus. Moreover, NB is the only protein unique to influenza B viruses, encoded in the same segment as NA (segment 6), however its function is not fully understood yet. (15)

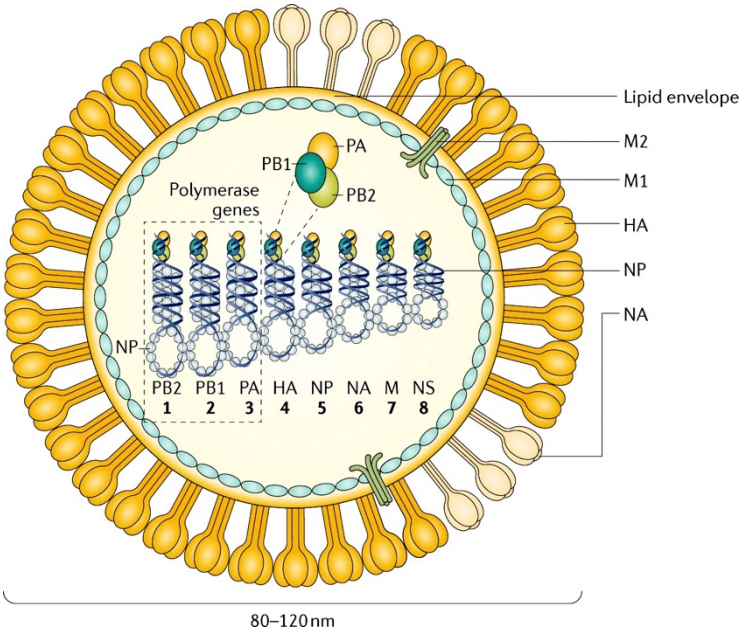

***Figure 2**. Influenza A virion structure. Source: Krammer, F., Smith, G.J.D., Fouchier, R.A.M. et al. Influenza. Nat Rev Dis Primers **4**, 3 (2018).*

2.1.3 Influenza virus life cycle.

Infection initiation is mediated by the binding of hemagglutinin HA to its cell surface receptor-binding site (RBS), such as sialic acid residues of glycoproteins or glycolipids. Viruses are then internalized within early endosomes which maturate into late ones, where the drop of pH causes conformational changes. As a consequence, the virus exposes a fusion peptide which leads to viral and endosome membranes fusion. M2 ion channel protein triggers H+ influx into the viral particle by determining the uncoating process *(fig.3)*. At this point, viral ribonucleoprotein complexes (vRNPs) are released from M1 to cell cytoplasm. The vRNPs are then imported into the nucleus through importins way, where viral RNA replication and transcription occurs. Viral RNA synthesis is mediated by the heterotrimeric polymerase complex PB1/PB2/PA. Each vRNA segment has a terminal promoter sequence recognized by the polymerase complex, which snatches the first 10-13 nucleotides and transcribes them into a complementary RNA positive-strand (cRNA). This will be used to generate more copies of vRNA. [10] [16] The mRNA is transcribed through a cap-snatching mechanism: PB2 binds to host mRNA 5' mG cap sequence cleaved by PA endonuclease and used by the virus as primer to initiate viral mRNA synthesis. [17] Furthermore, influenza mRNAs are 5'-capped and 3'-polyadenylated, these last steps of capping and polyadenylation are performed by a stuttering polymerase, which transcribes a nucleotide several times without processing further the mRNA chain. At this point, primary and processed viral transcripts are exported to the cytoplasm for translation by the host ribosomes, while cytosolic viral proteins PB1, PB2, PA, NP, M1 and NEP are imported into the nucleus, where they will increase the viral RNA synthesis and vRNP formation *(fig.3)*. Transmembrane viral proteins HA, NA, M2 are translated in the ER and trafficked to Golgi where HA is activated by proteolytic cleavage and transported to the plasma membrane where it encounters lipids rafts by forming the bud-zone. Here newly synthesized vRNPs are exported from the nucleus for the assembly of new viral particles. [18] These new viral RNPs are assembled in a 1+7 configuration, with one central vRNP surrounded by the other 7. When the virions formation is completed, they aggregate at the cellular surface level, because of their binding of HA to sialic acids. [17]

2.1.4 Influenza virus pathogenesis and clinical features.

Human influenza viruses are transmitted through respiratory route, while avian influenza viruses are spread among birds through fecal-oral, fecal-fecal and fecal-respiratory way. According to the route of transmission, influenza virus infects epithelial cells of respiratory tract or intestinal tract. Avian and human influenza viruses have preferential cell targets: the formers target alveolar type II cells and ocular epithelium, the latter infect mainly the upper respiratory tract epithelium, which increases the risk of transmission, while viruses infecting the lower respiratory tract are associated with more severe inflammation and complications. For instance, 2009 H1N1 flu was highly transmissible but with moderate virulence. As opposed, H5N1 and H7N9 were low transmissible but highly virulent. [(4)] The viral replication peaks at 48 hours, incubation period, after the inoculation, and then declines slowly. Human influenza virus sheds in respiratory secretions for 5 -10 days, while the onset of symptoms is sudden. Respiratory symptoms are cough, sore throat, and runny or stuffy nose. Systemic symptoms include fever, chills, headache, malaise, and myalgia. Vomiting and diarrhea may also occur. Recovery is rapid, fever in 3-4 days, while other symptoms within 7 days. Groups such as elderly, patients with chronic pathologies (heart disease or diabetes), pregnant women and young children are at most risk to develop influenza complications. These include secondary bacterial pneumonia (Streptococcus pneumonia, Staphylococcus aureus), exacerbations of underlying respiratory conditions, otitis media, laryngotracheobronchitis and bronchitis. Primary pneumonia, encephalitis, aseptic meningitis, transverse myelitis, myocarditis, pericarditis, Reye Syndrome can also be settled after influenza infection. Most deaths due to influenza typically affect people older than 65 years. [(4)(19)] Influenza viruses induce epithelial cells death, while the infected ones release cytokines and chemokines (IL-6, IL-1, TNF-alpha and CCL) to recruit infiltrating inflammatory cells such as neutrophils and macrophages. This explains the fact that pathogenesis of severe influenza is not only related to viral cytopathic effect but also to excessive inflammatory response. [(4)] Influenza B viruses cause indistinguishable symptoms from influenza A virus, including fever, cough, headache, although complications could be different. It has also been studied that influenza B viruses are responsible for most deaths caused by influenza in

children, which are commonly associated to bacterial pneumonia and cardiac injury. [20]

2.1.5 Treatment and prevention

Vaccines and antiviral drugs are used for prevention and treatment of influenza virus

infections. Antiviral drugs such as oseltamivir and zanamivir are neuraminidase inhibitors that prevent the spread of virions from infected cells to other host cells. Oseltamivir is considered the first-line treatment of influenza in children, while zanamivir administered by inhalation route is approved for patients > 7-year-older patients. Older antivirals, inhibitors of M2 protein such as amantadine and rimantadine have showed treatment failures caused by increased global resistance and are no more recommended since 2006. [4] Another antiviral drug approved by FDA in 2018 is baloxavir marboxil, which targets the endonuclease function encoded by PA subunit of the viral polymerase. It has demonstrated a broad antiviral activity against all known types of influenza, approved in USA and Japan. However, OSM and ECDC suggest using antivirals for influenza treatment only in specific cases due to their inconsistent clinical relevance,

related to their collateral effects and patients' resistance. [21] Vaccination is instead the primary mode for influenza prevention. It must annually revised because its effectiveness is determined only by the matching with circulating viral strains. Annual deadlines for vaccines are important also because flu season are different in diverse hemispheres: October-May in Northern Hemisphere and May-October in the Southern hemisphere. Vaccines are manufactured in embryonated eggs or other systems. Nowadays there is trivalent and quadrivalent formulations availability. Trivalent influenza vaccines include two inactivated influenza A strains (H1N1 and H3N2) and one influenza B strain. The quadrivalent vaccine targets H1N1, H3N2 and both influenza B lineages, which offers a broader coverage. Commercially available vaccines include egg or cell-based inactivated influenza vaccine (IIV), a live attenuated influenza vaccine (LAIV) and a recombinant HA vaccine produced in insect cells. [22] [23]

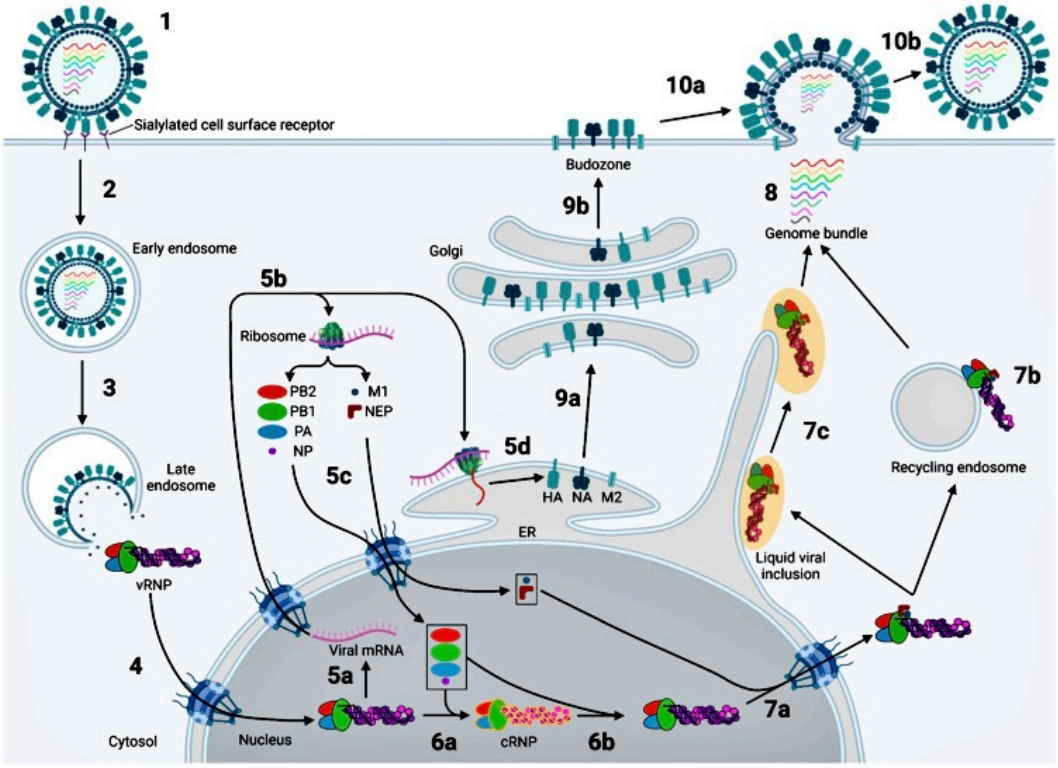

***Figure 3**. Influenza virus life cycle. Source: Carter T, Iqbal M. The Influenza A Virus Replication Cycle: A Comprehensive Review. Viruses. 2024 Feb 19;16(2):316..*

2.2 Respiratory syncytial virus (RSV)

Respiratory syncytial virus is the second leading cause of infant death worldwide and the first leading cause of acute lower respiratory tract infections.

2.2.1 Classification and genome

Respiratory syncytial virus is part of *Pneumoviridae* family*, Orthopneumovirus* genus. It's a seasonal highly contagious pathogen responsible for Lower Tract Respiratory infections (LTRI) with severe consequences in children, elderly and immunocompromised people. [(24)] RSV is an enveloped virus with a diameter ranging from 120 nm to 300 nm. Its genome is formed by a positive single-stranded non-segmented RNA molecule of about 15.2 kb, which encodes 11 proteins. They are: matrix protein (M), nucleoprotein (N), phosphoprotein (P) which interacts with the L protein during replication, RNA polymerase (L), non-structural proteins (NS1/NS2) involved in the evasion of innate immune response, regulatory proteins (M2-1/ M2-2), small hydrophobic protein (SH), glycoprotein (G) that causes the docking in the cell surface receptor, and glycoprotein F (fusion

protein) *(fig.4)*. G and F proteins are responsible for the viral pathogenesis. F glycoprotein triggers the fusion between the virion and the targeted cell by causing direct cytoplasm infection. [25] The virus has one serotype, but it's divided into two strains: RSV A and B, according to the heterogenicity of G protein. The genetic drift of G gene has been identified as the main driver for the emergence of new epidemics. [26] [25] [27] As Shi T. *et al* described, RSV has caused 33.1 million clinical cases in 5 years old younger children worldwide in 2015. [12] In the Northern hemisphere RSV peaks during winter months, January and February, while in summer there are no outbreaks normally. RSV A and B co-circulate during the same season, with different geographical and temporal prevalences. [26] The risk of hospitalization caused by RSV is higher in infants born "in season" compared to those born outside.

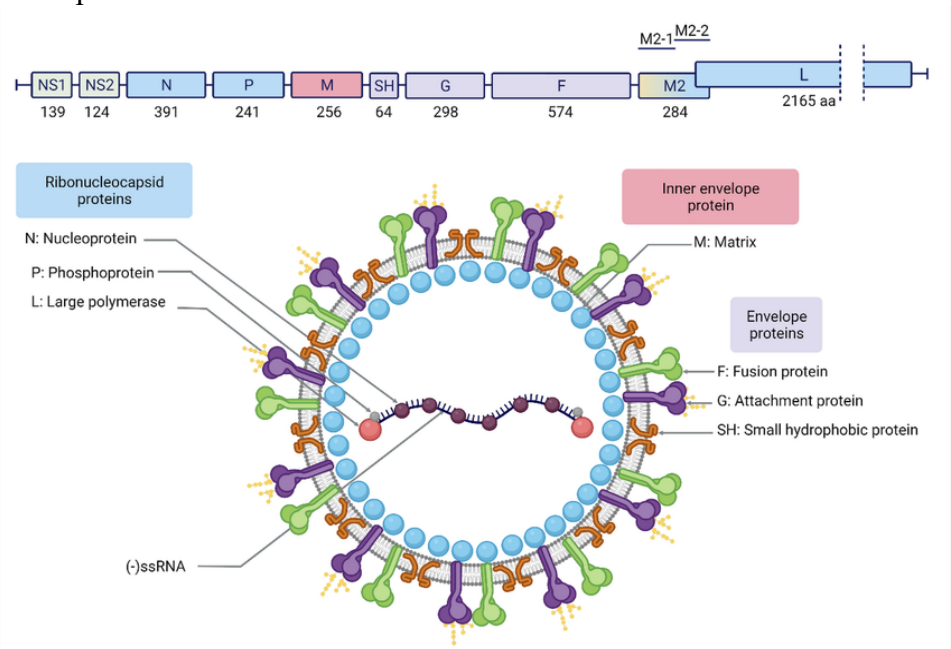

Figure 4. *Virion structure and genes of RSV. Source: L. E. Cordova-Davalos, A. Hernandez - Mercado et al; impact of genetic polymorphism related to innate immune response on respiratory syncytial virus inflection in children; Virus Gene (2022) 58:501-514.*

2.2.2 Replication cycle

Infection initiation is determined by the attachment of the virus on host cell surface mediated by G and F glycoproteins that interact with cell receptors (CX3CR1, TLR4, Heparin, ICAM-1). The virion changes into pre-harping conformation and it is endocytosed. The viral envelope fuses with host cell membrane and nucleocapsid is delivered inside a cytoplasmatic inclusion body (IB). This space contains some host proteins such as poly A binding protein

(PABP) and the eukaryotic translation initiation factor EIF4G. The viral genome, embedded within cytoplasmatic inclusion bodies, is transcribed by preformed RNA-dependent RNA polymerase (primary transcription) into a full-length positive sense antigenomes and into the 10 viral subgenomic mRNAs. Both the genomic and antigenomic RNAs are encapsulated by the nuclear protein (N). In fact, nuclear protein (N), phosphoprotein (P) and the matrix protein (M) are translated in the cytoplasm, and they wrap the newly synthetized viral genomes. Small hydrophobic protein (SH), glycoprotein (G) and fusion protein (F) mRNAs undergo a secondary transcription in cytoplasm with the addition of methylated guanosine cap in 5' and of polyA tail in 3'. Then, the RSV glycoproteins are translated into endoplasmic reticulum and successively glycosylated in the Golgi apparatus. RNP complex loaded with RSV genome is assembled within filaments and transferred to plasmamembrane to bud new viral particles *(fig.5)*. When the RSV is released, its morphology is filamentous.

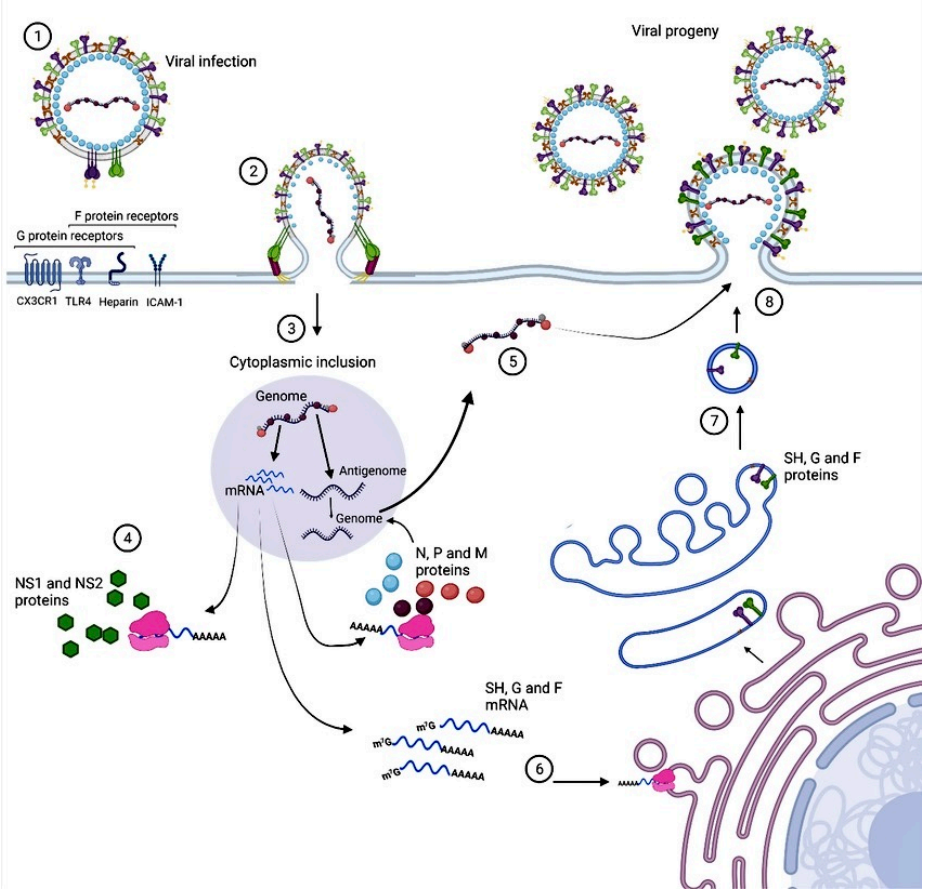

Figure 5. *Life cycle of RSV. Source: L. E. Cordova-Davalos, A. Hernandez -Mercado et al; impact of genetic polymorphism related to innate immune response on respiratory syncytial virus inflection in children; Virus Gene (2022) 58:501-514*

2.2.3 Pathogenesis and clinical manifestations

RSV is transmitted through direct contact with saliva or mucus droplets.

This virus infects mainly respiratory epithelial cells, which appear usually normal; however, they can also show cytopathological signs such as the presence of inclusion bodies. The primary infection occurs through the upper respiratory tract, and then the virus further spreads to lower respiratory epithelium, the damage caused by RSV infection is in fact located at the level of bronchi and bronchioles. Bronchiolitis is the most common LRTI caused by RSV, and it leads to necrosis and sloughing of the small airways with increased mucus secretion, which obstructs the lower airway. In 5-10% of hospitalized infants, the disease onset requires admission to the intensive care unit (ICU). Reinfection by RSV occurs in 30-70% of children 2 years-old-younger, who experienced RSV infection during their first 12 months of life. Even the secondary infection, the disease is symptomatic in young children, but it's less severe. In adult population symptoms are generally absent or confined in the upper respiratory tract. Clinical manifestations occur after 3 to 7 days from the infection, and it includes: fever, runny or stuffy nose, cough, chest tightness, wheezing and dyspnea. [25]

RSV involves lower respiratory tract infection in immunocompromised patients, in those with chronic cardiopulmonary disease, in the frail and the elderly, with significant risk of morbidity and mortality. The well-established risk-factors for hospitalization are preterm birth, chronic lung disease of prematurity, congenital heart disease, low birth weight, trisomy 21 or other chromosomal abnormalities, diabetes mellitus complications, chronic liver disease. [28] The host immune response is mediated by neutralizing antibodies and by cytotoxic T cells. The immune reaction increases the damage at the level of respiratory airways by exacerbating the clinical condition of the patient.

2.2.4 Treatment and prevention

Acute bronchiolitis management varies among different countries and centers and consists in supportive care. Ribavirin is a broad-spectrum antiviral agent approved by FDA used for children and infants with severe bronchiolitis at high risk, but not for routine treatment. [28] Vaccines for RSV are available and are considered an important tool to protect the most at-risk groups. CDC recommends a single

dose of vaccine to adults 75 age and to adults aged 60-74 years old at risk. This vaccine was approved by European Medicines Agency (EMA) in 2023. Moreover, the Centre of Disease Control suggests the maternal vaccine for pregnant woman to protect women during 32-36 months of pregnancy. This type of vaccination is usually formed by a recombinant RSV F protein antigen (both RSV A and B subtypes), stabilized in the prefusion conformation (preF). [30] For babies after birth and for young children 8-19 months-old a monoclonal antibody Nirsevimab is used for immunized. [29] Another significant monoclonal antibody approved by FDA is Palivizumab which targets the RSV F protein. It's administered to premature children and to those at high risk to develop LRTIs. [30] It doesn't completely immunize patients, but it reduces hospitalizations by preventing lower respiratory tract infections on this targeted population.

2.3 Adenovirus

Adenoviruses (AdVs) were discovered over 80 years ago through their isolation from adenoid tissue [31], as their name suggests, *adénos* means gland. Since then, these viruses demonstrated broad clinical roles and were exploited for different applications. Adenovirus infects both immunocompetent and immunocompromised patients, with high morbidity and mortality in these last ones. AdVs aren't just infectious agents, they are also related to important discoveries, such as the mRNA splicing mechanism (Nobel price, 1993) and their application to develop vectors, due to AdVs broad cell tropism. Moreover, these viruses' genome contains oncogenes, whose tumor growth potential was demonstrated in animal models [32].

2.3.1 Genome and viral structure

Human Adenovirus (AdV) has a diameter of about 70-100 nm marked by a non-enveloped icosahedral protein shell, which contains a dsDNA non segmented linear genome of about 26-48 kb [33]. The genome transcription leads to different-time products: early (E), intermediate (I), late (L) transcripts. Early genes, from E1 to E4, are involved in viral replication, E3 determines the HAdVs variability and influences the host immune response. Late genes, from L1 to L5, encode for structural and non-structural proteins which are involved in mature virions production [34]. AdVs are characterized by a complex structural organization which consists of 252 capsomeres including 240 hexons and 12 pentons. The 12

vertices are delimited by penton base proteins and each capsomere supports a trimeric fiber protein. Fibers' most apical domain interacts with the host cells receptor (CAR, DSG2, CD46). Penton base, fiber, and hexon proteins are part of major capsid proteins, while minor capsid proteins are involved in the stability of hexon shell and contribute to events of internalization. (34)

The viral **core** of icosahedral adenovirus consists of DNA associated with proteins. These core proteins are pVII, pV, Mu, that are core condensing nucleoproteins complexes, pIVa2, an intermediate protein, TP, engaged in the genome replication, AdV protease (AVP) which cleaves precursors proteins *(fig.6)*. (35)

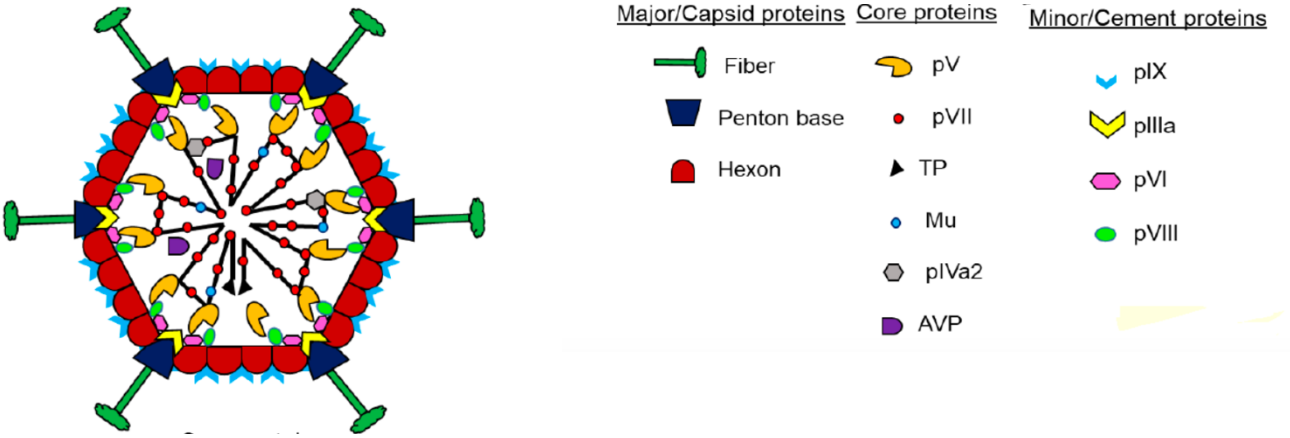

***Figure 6**. Adenovirus virion structure. Source: S. Kulanayake, Adenvirus Core Proteins: Strutcure and function, Viruses 2021, 13 (3), 388.*

2.3.2 Classification

Human AdV are part of genus *Mastadenovirus*, further classified into seven species, A to G. The species subdivision depends on different factors such as the phylogenetic distance, genome organization (particularly E3), nucleotide composition, and on G-C content. In the past, AdVs were characterized by the combination of serum neutralization (SN) and hemagglutination inhibition assay (HI) which identified 51 serotypes. This classification is based on the identification of hexon proteins loops for the SN, and of fiber proteins for the HI typing. Since 2007 HAdVs were identified by computational analyses of genomic data, which leads to 67 total HAdVs characterization. The evolutionary variation of HAdVs genomes is driven by homologous recombination (HR) and mutation. HR was already demonstrated in 1970 within tumorigenic adenoviruses of same

species and A, B, D subtypes generation involved mainly penton base, hexon and fiber proteins genes. The condition for HR requires the coinfection of same cell by at least two different adenoviruses and long-term viral persistence infection. [36] The AdV associated with human disease world-wide are part of C, B, F, E subtypes, while the AdVs affecting mainly immunocompromised patients are of C and B species majorly. [37]

2.3.3 Viral life cycle

Adenovirus infects target cell by binding its fiber proteins to the host cellular receptors CAR, desmoglein-2, or CD46. The virus is endocytosed and transported to the nucleus. During transport the virus is released from endosomes by its membrane proteins and endosome acidification. Through nuclear pore complex AdV enters the nucleus where the early phase replication begins. In this phase the virus transcribes the early genes (E1-4) encoding for the transcription factors and their expression will contribute to complete DNA replication by late genes expression. The assembly of structural and non-structural proteins occurs, and the new viral particles are released through cell lysis *(fig.7)*. Adenovirus DNA replication proceeds bidirectionally, by single strand displacement and it's catalyzed by adenovirus-encoded DNA polymerase, pTP, adenovirus E2A protein, and host proteins. [38]

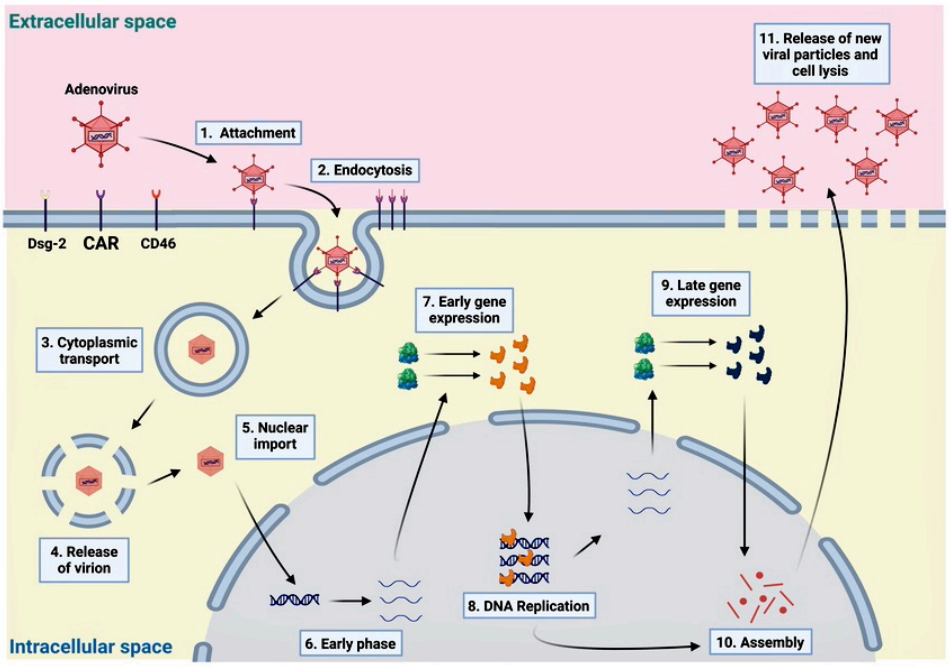

***Figure 7**. Adenovirus life cycle. Source: L. Scarsella et al. Advances of Recombinant Adenoviral vectors in preclinical and clinical applications, Viruses 2024, 16, 377.*

2.3.4 Pathogenesis and clinical manifestations

AdV transmission occurs via aerosol droplets or conjunctival infection, but also fecal-oral way through infected tap water, recreational freshwater and airflow filters.

Due to their lack of envelope they are very resistant to disinfectants and can be a threat in hospital surfaces for immunocompromised patient who will receive transplantation. In fact, adenovirus infects mainly immunocompromised population, with fatality rates of about 55% [39] and solid organs transplant recipients. Specific adenoviruses have a targeted tissue tropism, showing tissue-related symptoms in patients, even though the same clinical manifestations can be caused by other adenovirus types. For example, gastroenteritis is associated with HAdV -F and -G, pneumonia to HAdV -B, -C, -E, hepatitis HAdV -C, cystitis HAdV -B, and keratoconjunctivitis to -B and -D. [34]

In immunocompetent people HAdV infect mainly children below 5 years old. It can also set a latent state as was demonstrated by Garnett in human tonsils [40].

2.3.5 Treatment and prevention

In immunocompetent individual adenovirus infections are mild and they resolve by themselves, while immunocompromised patients, particularly allogeneic transplant recipients, showed the greatest risk of life-threating clinical course. The preemptive treatment includes reduction of immunosuppressive therapy, use of antiviral, such as cidofovir, and immunotherapy. Cidofovir is the primary antiviral agent for preemptive therapy against all AdV species and it consists in a nucleotide analog of cytosine able to inhibit DNA polymerase and viral replication. Another possible antiviral is Ribavirin, used in specific situations. [34]

2.4 Metapneumovirus

Human Metapneumovirus is a recently identified virus isolated in 2001, even though its antibodies were already circulating during 1958 as serological studies demonstrated. [41] HMPV infection is seasonal, with winter epidemics occurring from December to April in the northern hemisphere, simultaneously with RSV epidemics.

2.4.1 Classification and genome structure

HMPV belongs to *Pneumovirinae* family, previously classified into *Paramyxovirus* family. Metapneumovirus differs from *pneumovirus* by the absence of two non-structural proteins, NS1 and NS2 and in gene order. [43] From phylogenetic analysis two genotypes were identified, HMPV A and B, divided into their respective subgroups A1, A2 and B1, B2, according to the sequence variability of the G and F surface glycoproteins. [44] Human metapneumovirus is an enveloped non-segmented negative sense ssRNA virus, with a genome of approximately 13.3 kb in size, constituted by 8 genes encoding for 9 structural proteins. The MPV genes order is: 3-N-P-M-F-SH-G-L-5 with ORFs of M2 coding for proteins M2-1 and M2-2. [43] These are: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), M2/1 protein, small hydrophobic (SH), glycoprotein (G) and the large polymerase (L). N is responsible for encapsidation and genome protection, P is a co-factor of polymerase protein L, both proteins induce inclusion bodies formation. M protein is aimed at viral assembly and budding, while F protein induces host-cell binding and membrane fusion together with G glycoprotein. [45] The replicative cycle resembles the *Paramyxoviridae* family one, which consists in the transcription of the negative sense RNA viral genome into viral mRNA and the further translation into viral proteins.

2.4.2 Pathogenesis and clinical manifestation

HMPV is transmitted by contact with respiratory droplets or contaminated saliva, with an incubation time of 4 to 6 days. The virus is inoculated in the nasopharyngeal mucosa and spreads into the respiratory tract. Metapneumovirus infects mainly 5 years old children, with subsequent reinfections throughout life. Adult patients are asymptomatic or manifest mild symptoms, such as cough, nasal congestion and dyspnea, while in elderly and immunocompromised patients' complications can be more severe. Gastrointestinal symptoms can also be included, such as diarrhea, nausea, vomiting.

In young children HMPV represents the second most common cause of lower RTI after RSV, leading to pneumonia, bronchiolitis or acute asthma exacerbations. [41]

Infants less than one year old of age show the highest rate of infection, while children older than 5 years old demonstrate seroprevalence.

2.4.3 Treatment and prevention

The HMPV treatment is mainly supportive, some innovative approaches such as fusion inhibitors and drugs based on RNA interference are under evaluation in vitro e in animal studies. Ribavirin for example is a nucleoside with inhibitory activity against RNA and DNA viruses, including HMPV. Furthermore, humanized monoclonal antibodies used to treat paramyxovirus infection such as RSV, have shown a similar approach for the protection from HMPV.

Vaccination is not currently available; however, several animal studies are investigating a possible vaccine against HMPV. [46]

2.5 Enterovirus

2.5.1 Classification and genome organization

Human Enteroviruses (EVs) are part of *Picornaviridae* family belonging to *Enterovirus* genus. Enteroviruses were subdivided according to their biological properties into *Polioviruses* (PV), *Echoviruses (E)* and *Coxsackieviruses A (CVA)* and *Coxsackieviruses B (CVB)*. [42] The most recently classified types are defined by the sequence analysis of the viral protein 1 (VP1), named "Enterovirus" with their species letter and a consecutive number. [47] Enteroviruses identified in humans are included into Enterovirus A, B, C or D, while rhinoviruses infecting humans belong to the species Rhinovirus A to C. As Simmonds *et al*. described in 2020, Coxsackie A viruses are distributed across three different Enterovirus species: EV A, B and C. The poliovirus is assigned to the Enterovirus C species. The classification was discontinued in the late 1960s, and subsequently new serotypes were named by consecutive numbering. Recently the numbers were prefixed by A, B, C or D to indicate the species assignment. [47] In recent years, outbreaks of enterovirus D68 and A71 have been associated with respiratory infections and neurological complications have been reported worldwide.

Enterovirus A71 (EV-A71) is the major cause of hand-foot-and-mouth-disease, a children disease manifesting blisters on hands, feet and buttock, oral vesicles and fever. It's considered the most neurotrophic non-polio enterovirus (NPEV). The

risk of infection is registered mainly during summer and fall, but infections can occur year-round. [48]

The enterovirus D68 (EV-D68) was isolated from children with respiratory infections in United States in 1962 and during 2008 it was responsible for outbreaks of respiratory diseases. [49] These infections cause mild respiratory disease but also result in severe bronchiolitis or pneumonia, occasionally to death among children. Certain EVs, including EV-D68 and Enterovirus C viruses, share some biological properties with rhinoviruses, however they exhibit distinct respiratory tropism, the "respiratory" enteroviruses replicate in the epithelial cells of the respiratory tract. [50] EVs showed high mutation and recombination rate, by increasing the risk of emergence of new pathogenic strains. The development of new vaccines for EVs associated with sever diseases has been indicated as an important measure to contain their spreading. [51]

Enteroviruses are nonenveloped, icosahedral, with a diameter of 27-30 nm viruses. They contain a single positive-sense RNA virus of about 7.2-8.5 kb, encoding a polyprotein.

Enterovirus capsid is composed of 60 asymmetric units made of four viral polypeptides known as VP1 to VP4. VP4 is embedded inside the capsid of the viral particle. The genome of EV D-68 virus consists of the genome-linked protein VPg at 5' end, the long untranslated 5'-UTR, which contains the internal ribosome entry site (IRES), a single ORF coding for a polyprotein, a 3'-UTR, and a poly (A) tail *(fig.8)*. The polyprotein is processed by the viral proteases into three precursor proteins P1, P2, and P3. P1 region encodes for the structural polyproteins, while the P2 and P3 regions for the nonstructural proteins are associated with the replication. [49] The structure of EV-D68 virion has considerable similarities to those of rhinoviruses.

2.5.2 Replication process

EV viral particle attaches to specific host cellular receptors and co-receptors leading to their endocytosis. [52] EV-D68 binds to sialic acid (SA) found mainly in the upper respiratory tract cells. [53] The endosomes pH changing induces the virus uncoating and the release of the viral genome into the cytoplasm. Different enteroviruses species use diverse receptors to bind to the host cell, however the post-entry-steps are highly conserved among species. Following viral entry, the

viral genome is translated into a single large polyprotein. This is processed by viral proteinases 2A, 3C and 3CD into ten proteins (structural and non-structural proteins). The genome replication takes place at replication organelles (ROs). The RNA-dependent RNA polymerase 3D synthesizes a negative-strand copy of the incoming viral genome to generate a double stranded RNA. The negative strands are used as a template to create a new positive strand. The structural capsid proteins VP0, VP1 and VP3 form the enterovirus particles by assembling into protomers and pentameres. The RNA-induced processing of VP0 into VP2 and VP4 leads to mature virions. Viral proteinases are also involved in the cleavage of host proteins, to optimize the translation and virus spreading. This process results in the cytopathic effect of the virus in the host cell. The virion particles once assembled can egress either by cell lysis or by membrane-bound structures. (52) The translation of viral proteins is conserved among enteroviruses, and it's guided by an internal element called internal ribosome entry site (IRES). The replication requires a primer 3B (known as VPg) coupled to two uridines and is performed by the viral RNA-dependent RNA polymerase 3D. Besides the viral proteins several host RNA-binding proteins are involved in genome replication.

2.5.3 Transmission and clinical manifestations

Enterovirus capsids proteins grant resistance to acid environments by infecting the gastrointestinal tract, and by spreading through fecal-oral way. However, enteroviruses spread also via oral and infected respiratory droplets. (54)

Enterovirus infections occur most frequently in children under the age of 10, during summer and fall in temperate regions, while throughout the year in tropical regions.

The clinical presentation is associated with age: central nervous system (CNS) manifestations are associated with 5–15-year-olds, myocarditis in 20-40 year-old, severe infections (sepsis-like illness) in neonates and infants, and hand-foot-and-mouth disease in children aged <5 years. Enterovirus infections are related with low socioeconomic status, associated with poor sanitation and close living quarters.

Non-polio Enterovirus transmission occurs generally by fecal-oral contamination or by respiratory droplets. In most cases enteroviruses are asymptomatic however they can also be life-threating. Infants, children and teenagers are more likely than

adults to get infected and become sick because they have not yet developed the immunity from previous exposures to the virus. Infected adults have milder symptoms. Symptoms of non-polio enteroviruses include fever, runny nose, sneezing, cough, skin rash, mouth blisters, body and muscle aches, such as influenza-like symptoms. Some non-polio viruses cause viral conjunctivitis, hand, foot and mouth disease, viral meningitis, viral encephalitis, myocarditis, pericarditis, acute flaccid paralysis, inflammatory muscle disease. [55]

EV-D68 causes both upper and lower respiratory tract infections by representing a threat for children with asthma, which can be the target of more severe complications. [49]

Polioviruses are the enteroviruses of most public health concern, which we are trying to eradicate, together with EV-71 and CVA viruses can cause neurological diseases like poliomyelitis.

2.5.4 Treatment and prevention

Inactivated poliovirus vaccines (IPV) and live, attenuated oral poliovirus vaccines (OPV) have been instrumental in preventing poliomyelitis worldwide.

Each individual contracts EV infection during lifetime, which can be symptomatic or asymptomatic. If a new vaccine will be approved for clinical use, the prioritization will be to both elderly and young people because the infections have more sever clinical manifestations where the immune system is more vulnerable.

There is no specific treatment for people with EV-A71 infection, however patients with severe disease require hospitalization to receive supportive therapy.

Another recent strategy to target enteroviruses is the inhibition of viral capsid proteins using the so-called capsid binders. [54] Liu *et al.* reported Pleconaril as a capsid-binding compound able to act against rhinoviruses and some enteroviruses such as EV-D68, however the CDC do not approve their efficacy at clinically relevant concentrations. [53] Other antiviral compounds against EVs have been tested exclusively in vitro, due to the lack of animal models for this virus.

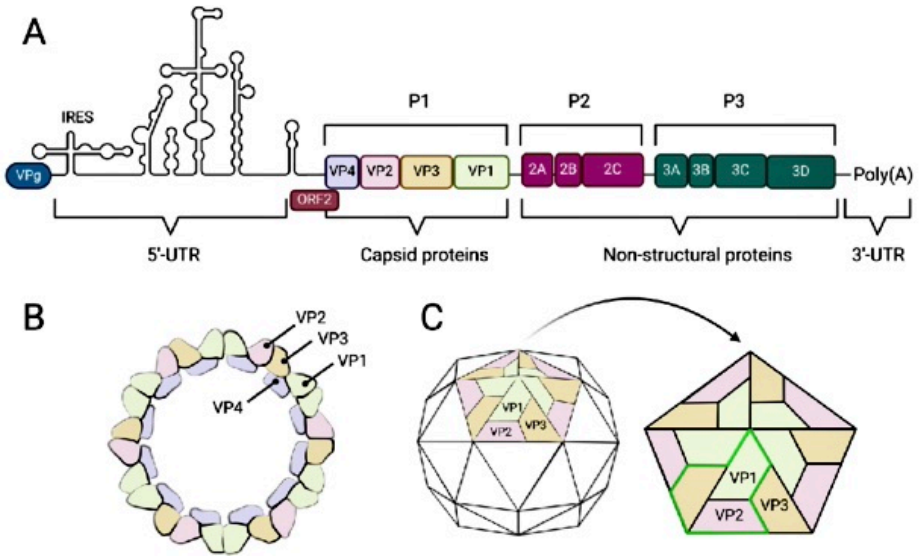

***Figure 8**. Enterovirus structure and genome. Source: M. Jartti et al. Enteroviruses: epidemic potential, challenges and opportunities with vaccines, Journal of Biomedical science (2024) 31:73.*

2.6 Rhinovirus

Human rhinoviruses were discovered in 1950s by understanding the etiology of common cold. They are distinguished into three HRV groups: A, B and C within the genus *Enterovirus* and the family *Picornaviridae.* [56]

2.6.1 Genomic organization

HRVs are positive-sense single-stranded RNA viruses of approximately 7,200 nucleotides.

The viral genome is composed of a single gene encoding for one protein which is cleaved by viral proteases into 11 proteins. HRV has a single open reading frame joined to a 5' untranslated region and a short viral priming protein (VPg), followed by a capsid -coding region (P1). The P2 and P3 regions encode for the non-structural proteins, two viral proteases and the RNA-dependent RNA polymerase. These regions are followed by a short 3'- noncoding region and poly A. The four viral proteins VP1, VP2, VP3 and VP4 form the capsid, while the remaining nonstructural proteins are involved in genome replication and assembly *(fig.9)*.

As for Enterovirus Rhinoviruses, HRV virions are made by 60 copies of each four capsid proteins, providing the virus of an icosahedral structure, with a canyon in VP1 as site of attachment to cell surface receptors. [56]

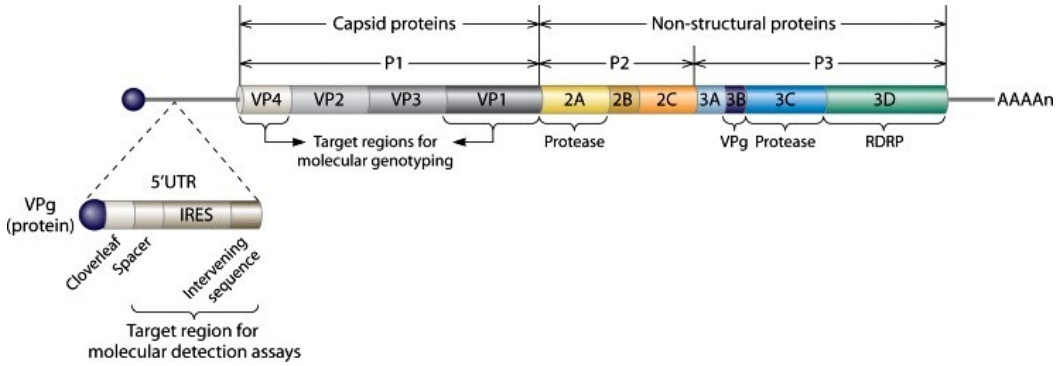

***Figure 9**. Rhinovirus genome organization. Source: Jacobs SE, Lamson DM, St George K, Walsh TJ. Human rhinoviruses. Clin Microbiol Rev. 2013 Jan;26(1):135-62.*

2.6.2 Virus life cycle and replication

Once the viral particles attach to the host cell receptor, they are internalized via different endocytosis routes, such as micropinocytosis or clathrin-dependent or independent endocytosis. It follows the change of the viral conformation within the endosome and, due to the acidic environment formation, the release of subviral particles. The replication process takes place in the cytoplasm. The genome is firstly translated by the host ribosomes into a polyprotein (P0), and then copied into the complementary "negative strand" RNA molecules. The P0 is cleaved in cis and trans by viral proteases (2A protease, 2Apro and 3C protease, 3Cpro) into 11 proteins. This is used to generate a larger number of viral genome copies. They act as messenger RNAs for viral protein synthesis. The RNA replication takes place within virus-induced membranous replication organelles and the (-) ssRNA is transcribed by the viral RNA dependent RNA polymerase into new (+) ssRNA. The final stage involves the assembly of structural proteins into capsids and the release of infectious virions via lysis or non-lytic mechanisms. [57]

2.6.3 Pathogenesis and clinical manifestations.

In respiratory tract, HRV replicates in the ciliary epithelial cells of nasal mucosa where it finds the optimal replication temperature of about 33°C, and more rarely in the oral cavity and throat. More than 90% HRV serotypes, called "major group", utilizes the cell surface receptor intercellular adhesion molecule 1 (ICAM-1) or heparan sulfate, while the "minor group" attaches the host cell via the low-density lipoprotein receptor (LDLR). [56] HRVs cause upper respiratory tract infections,

otitis media, and sinusitis, but also lower respiratory infections in patients with asthma, in infants, in elderly and in immunocompromised hosts. Symptoms of LRT infection are cough, shortness of breath, chest tightness and wheezing. In non-asthmatic individuals, symptoms of RV infection are generally limited to the upper respiratory tract. They include rhinorrhea and nasal obstruction, due to neutrophilic inflammatory response associated to vascular permeability and stimulation of mucus hypersecretion. When RVs infect cells of lower respiratory tract, they usually cause bronchial infections. The most important aspect of RV pathogenesis is its capacity to predispose to concomitant or subsequent infection with other respiratory pathogens. While influenza viruses and RSVs destroy the airway epithelial barrier, RV doesn't cause cytopathology of the cells, however it disrupts the function of the epithelial barrier, by impairing its tight junction's activity causing vascular leakage and mucus secretion. This effect facilitates the translocation of pathogens and their soluble products. [58]

2.6.4 Treatment and prevention

There are no currently approved antiviral therapies for HRVs, and treatment remains primarily supportive. There are three lines of antiviral products promising at the moment. The mechanism by which these compounds block the infection is the slipping into the lipid pocket, a hydrophobic molecular cave under the receptor canyon in the viral capsid, by stabilizing physiochemical bonds with the amino acid residues. This will prevent conformational changes of the capsid structure that would lead to the uncoating and release of the genomic RNA at the onset of infection. Pleconaril is considered an efficient agent against picornavirus infections. Another potential intervention involves the blocking of the virus-receptor interactions, and inhibitors of the viral proteases.

2.7 Human coronavirus

All coronaviruses are members of the *Coronaviridae* family belonging to the order *Nidovirales*. Based on the sequence homology they are further classified into four genera including alpha, beta, gamma and delta coronaviruses. Seven human coronaviruses 229E, OC43, SARS, NL63, HKU1, MERS, SARS-CoV-2 can cause both a mild cold and an epidemic of large-scale deaths and injury. Human coronavirus OC43 (HCoV-OC43) is one of the endemic strains of low-risk coronaviruses and it's

used as alternative research to study coronavirus in the low-risk research setting. Endemic strains of human coronavirus such as 229E and NL63 are members of alpha coronaviruses. The genus *beta* is divided into 2a (OC43 and HKU1), 2b (SARS), and 2c (MERS). HCoV-229E, HCoV-NL63, HCoV-HKU1, and HCoV-OC43 are four typical co-circulating low-risk human coronaviruses. They are associated with mild infections of the upper respiratory tract. These viruses have zoonotic origin, and they are thought to have broken the interspecies barrier, resulting in their spillover by infecting humans. [59]

2.7.1 Genome structure

Coronavirus genome consists of a single-stranded RNA genome, which encodes for two open reading frames (ORF1a and 1b), which generates 16 non-structural proteins necessary for viral replication. Viral structural proteins include spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins. The genome sequence of SARS-CoV shares about 50% homology with that of HCoV-OC43. [60] However, OC43 contains extra NS2a and hemagglutinin-esterase (HE) genes in front of the S gene. The HE is unique of group 2a coronaviruses, and it possesses specific characteristics that are absent in SARS-CoVs. It has a dual function of receptor-binding and receptor-degrading activity. Spike protein is responsible for hemagglutination by HCoV-OC43, while the envelope protein leads to the formation of ion channels. The membrane protein determines the virion morphology, and the nucleocapsid protein is responsible for the helical nucleocapsid formation. On the other hand, human coronavirus NL63 genome sequence is characterized by the presence of the ORF3, which encodes for the accessory N-glycosylated protein. The ORF3 gene has a unique nucleotide composition: U-rich and A-poor.

2.7.2 Pathogenesis and clinical manifestations

The airway epithelium of nasal, bronchial and alveolar cells represents the major target of most CoVs. Like influenza virus, OC43 utilizes sialic acid entry receptor mediated by the S protein. HCoV-OC43 employs caveolin-1-dependent endocytosis for viral entry and dynamin-dependent budding for viral exit, while SARS-CoV2 mainly engages angiotensin converting enzyme 2 (ACE2) for its infection process, as well as NL63 coronavirus. Human coronaviruses are mainly

responsible for common cold; however, some patients show symptoms of diarrhea and enterocolitis. According to surveillance data, HCoV-OC43 and HCoV-NL63 have been regarded as the most frequently found strain responsible for coronavirus-associated mild colds. These infections are self-limited, however more severe respiratory tract infections including bronchiolitis and pneumonia have been reported in high-risk groups. They include infants, elderly and immunocompromised patients.

2.7.3 Life cycle

HCoV-NL63 virion interacts with the viral receptor ACE2 on the surface of targeted cells. This attachment triggers the receptor mediated endocytosis followed by the acidification of the endosome. Spike protein priming is carried out by host cells transmembrane protease serine 2 (TMPRSS2) in the early endosome and cathepsins in the late endosome. Nucleocapsid is degraded and the viral particle releases its genome in the cytosol. This is translated to produce two viral proteins pp1a and pp1ab that are cut by proteases into non-structural proteins (Nsp). Nsp3 and nsp4 zip the RER membrane to generate double membrane structures (DMS). Within these DMS the viral genome and nsps initiate the replication and release the sgRNA out of them to translate the viral structural proteins. N protein accumulates in the inclusion bodies (IB), while other structural proteins are processed into the endoplasmatic-reticulum-Golgi intermediate compartment (ERGIC). The viral genome released from the DMS is coated by N protein and directed to ERGIC and Golgi where new virions are formed. These are released either via exocytosis or through cell lysis *(fig.10)*. [61] Since HCoV-OC43 belongs to beta coronavirus genus it shares similar replicative cycle to the one of SARS-CoV-2.

2.7.4 Treatment and prevention

There is not available vaccine to protect population against human endemic coronaviruses and there is no specific treatment for illness caused by human endemic coronaviruses.

Antivirals that have been approved by the FDA for use against COVID-19, such as Paxlovid, have demonstrated inhibitory activity against the main protease (MPro) of multiple human CoVs, however it cannot target them in vivo. New

antivirals are required to target the four common human CoVs that are currently circulating (229E, OC43, NL63, HKU1). The similarity between HCoV-NL63 and SARS-CoV allows the block of the binding between ACE2 and S protein to prevent the infection via similar ways. For example, antisense oligonucleotides, chitosan chlordie and Thymoquinone are molecules able to inhibit the entry of the virus inside the target cells. Among new targets of human coronaviruses, the heptad repeats of S protein have been identified due to their crucial role of conjunction with TMPRSS2. [61] Another molecule is the host defense peptide (HDP) which demonstrates broad-spectrum antiviral against HCoV-OC43, 229E, NL63 and SARS-CoV-2 by blocking virus attachment and early entry targeting heparan sulfate proteoglycans. [62] The viral replication is also mediated by proteases cleavage. HCoV-NL63 protease shares substrates with SARS-CoV, however some differences are reported which are crucial for the development of selective inhibitors. 37 compounds screened for SARS-CoV-2 inhibit the NL63 protease too. Furthermore, other inhibitors target the main protease nsp5 conserved among CoVs. Regarding replication blockers, inhibitors of cellular poly (ADP-ribose) polymerase (PARP) blocks SARS-CoV-2 and HVoV-NL63 replication by reducing the virus entry and completing the inhibition of plaque formation. [61] Among host factors, the interferon-inducible transmembrane (IFITM) proteins inhibit the entry of most CoVs including HCoV-NL63, HCoV229E and SARS-CoV, probably through cholesterol accumulation on late endosome. [63] Neutralizing antibodies against common human coronaviruses like OC43, 229E and NL63 showed not to be protective against SARS-CoV-2 in young children. Even though SARS-CoV-2 infection or vaccination boosted the level of NL63 neutralizing antibody, the vaccination supports protection against SARS-CoV-2 but not to HCoV-NL63. The vaccination against SARS-CoV-2 in fact showed partial protection against endemic seasonal coronavirus like OC43, NL63 and 229E. [61] [64]

Aim of the study

The global aim of this thesis is to provide a report related to the epidemiological patterns of selected respiratory pathogenic cases of Treviso province hospitalized patients with particular focus on influenza virus. The period of interest corresponds to the flu season designated by the RespiVirNet project, from the 14th of October to the 4th of May. The trends of hospitalized patients are then compared to those obtained from the national surveillance project to prove the validity of these data and to underline similarities and differences. Each pathogen circulation is associated with its specific seasonality during the year, which is illustrated for the selected viruses of this project, by underling the importance to trace it year after year.
Two sample groups of individuals are involved in this thesis project results.

- The first group concerns the incidence of each respiratory pathogens positive cases of Treviso province hospitalized patients processed at Ca' Foncello Hospital's Microbiology laboratory.
- The second group includes the results of positive respiratory viral cases among the samples sent from Treviso Sentinel doctors analyzed in Padua University Hospital's Microbiology laboratory. In this case, Treviso Hospital was commissioned to collect the samples and to send them to Padua's Hospital. For this sample group this thesis provides useful information about Influenza A and B trends.

To sum up, this thesis project's aim is to provide the epidemiological trend of respiratory pathogens to contribute to the national surveillance of respiratory diseases that may represent a possible future threat.

Materials and methods

1. Sample collection

The type of samples adopted for this epidemiological study are mainly naso-pharyngeal swabs, suitable for the molecular analysis of viral nucleic acids, and in a smaller number also bronchial aspirates and broncho alveolar lavage (BAL).

According to RespiVirNet guidelines, the samples collection must be performed by doctors during the acute phase of the disease (fever symptoms), while it must be avoided after 7 days from the beginning of symptoms. For the hospitalized patients of Treviso province two types of swabs were used: the eNat and the eSwab by Copan, able to collect, transport, and preserve clinical specimens to be analyzed by nucleic acids amplification techniques. The eNat medium [65] stabilizes and preserves RNA/DNA for prolonged periods and it is compatible with commercial nucleic acid extraction and amplification platforms. This medium consists of guanidine thiocyanate, Tris-EDTA, HEPES, and a detergent, which inactivates nucleases and stabilizes RNA and DNA of viruses and bacteria. Moreover, eNat completely inactivates microbial viability within 30 minutes to ensure safe specimen handling, processing and transport. These media preserve nucleic acids up to 4 weeks at room temperature and 4°C and up to 6 months at -20°C to -80°C. eNat can be applied for different analysis other than for respiratory pathogens, such as gastrointestinal and HPV. The downstream processes suitable for these swabs are molecular-based assays and next generation sequencing. eNat system consists of 1 ml transport and preservation medium tube and a mini-tip applicator swab with red mark printed break point and flocked nylon fiber tip. *(fig. 10).* After the insertion of the swab in the nasal-pharyngeal site of collection, the doctor inserts this swab inside the tube until the breaking point reaches the opening level where it will be broken and then closed. The eSwabs by Copan [66] are very similar to the eNat samples. Their medium is intended for the collection and transport of clinical samples containing aerobes, anaerobes, bacteria, viruses and *Chlamydia*. eSwab preserves the viability of all the organisms tested for 48 hours at both controlled room and refrigerated temperature, except for *Neisseria gonorrhoeae*, which must be processed within 24 hours. eSwab is also able to preserve bacterial, viral or chlamydial antigens and nucleic acids from swab specimens for five days when stored at room temperature (20-25°C), 7 days if stored and 4°C and up to 6 months when stored at -20°C. They are constituted by the same components of the eNat

and the specimen sampling is similar. Each tube is labeled with the personal information of the patient and its identification barcode.

Bronchial aspirates are collected by direct aspiration of material from the large airways of the respiratory tract by means of flexible bronchoscope, while BAL is a safe and minimally invasive bronchoscope sampling method recommended for patients with severe lung medical conditions. The procedure to collect it consists in the administration of a saline solution in small airway, then the bronchoscope aspirates the fluid with cells and bacteria.

Figure 10. *eNat sample tube. Source: eNAT Nucleic acid collection and preservation medium,Copan, https://mediadelivery.copangroup.com/wp-content/uploads/2023/09/eswab_JMKPF001R02.EN_DIGITAL.pdf*

2. Commercial kits

The STARMag 96 X 4 Universal Cartridge Kit has been used for the extraction of nucleic acids, while for the amplification 3 different kits have been employed: the Allplex Respiratory Panel 1, 2, 3. The first panel includes Influenza A virus (Flu A), Influenza B (Flu B), Respiratory syncytial virus (RSV A), Respiratory syncytial virus (RSV B), Flu A-H1N1, Flu A H1pdm09, Flu A-H3N2. The second panel detects Adenovirus (AdV), Enterovirus (HEV), Parainfluenza virus 1 (PIV 1), 2 (PIV 2), 3 (PIV 3), 4 (PIV 4), metapneumovirus (MPV). The third panel marks Bocavirus 1/2/3/4 (HBoV), Rhinovirus A/B/C (HRV), Coronavirus NL63 (CoVNL63), Coronavirus OC43 (CoV OC43).

2.1 STARMag 96 X 4 Universal Cartridge Kit - Seegene

STARMag 96 X 4 Universal Cartridge Kit (384 tests) is intended to be used for isolation of nucleic acid from tissue, cells, bacteria, serum, plasma, nasopharyngeal

swab, nasopharyngeal aspirates, bronchoalveolar lavage (BAL), urine, stool, sputum, whole blood, genital swabs etc. by using automated liquid handling instruments, such as Seegene STARlet which was adopted in this study.
This cartridge is applied to nucleic acids automatic extraction and purification system with the handling of magnetic beads. The purification procedure comprises four steps: sample lysis, nucleic acid binding to magnetic beads, washing away debris and purified nucleic acid elution.

The kit reagents are:

- Lysis Buffer Universal (LB)
- Binding Buffer Universal (BB)
- Wash Buffer Universal 1, 2, 3 (WB 1, 2, 3)
- Elution Buffer Universal (EB)
- Universal Magnetic Beads
- Lysis Buffer Universal LB (200 ml)
- Universal Proteinase K (lyophilized)
- Proteinase Buffer Universal (PB)

This kit permits the reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions. Cells are lysed with SDS/proteinase K solution (Lysis Buffer LB). The binding buffer BB is added to the lysate to adjust the binding conditions under which the nucleic acids bind to the magnetic beads. After magnetic separation the beads are washed two times to remove contaminants and salts using Wash Buffers WB1, WB2 and WB3. Finally, highly purified nucleic acid is eluted with low-salt Elution Buffer EB and can directly be used for downstream applications.
The kit provides reagents for the purification of up to 20 µg of pure nucleic acid from suitable samples (up to 20 mg tissue or up to 1 x 10^7 cells) with an $A_{260/280}$ ratio ≥1.6 – 1.9 and typical concentration of 20/50 ng/µL. Depending on the elution volume used, concentrations of 10 -150 ng/µL can be obtained. Yields and concentration of nucleic acids depend on sample type.

2.2 Allplex Repiratory Panel 1, 2, 3, 4 – Seegene

Allplex Respiratory Panel 1, 2, 3, 4 - Seegene are used for multiplex one-step real-time RT-PCR assay for essential screening of the respiratory pathogens illustrated above.

Each kit includes:

- The 5X MOM. It's the oligonucleotides MuDT mix which contains the primer sequences, the RNA oligonucleotides complementary to the RNA sequence to be detected, and the probes.
- RNAse free water. Used as negative control.
- Buffer. It contains the dNTPs.
- Enzyme. The RTase transcribes the RNA into cDNA and the DNA polymerase add the dNTPs during amplification.
- Positive controls. Mix of pathogenic clones and the IC.
- Internal controls. It's an exogenous control constituted by the MS2 gene phage assembly protein.

In the respiratory panel 4 the enzyme and the buffer are contained in one unique vial. Before submitting the report, it's necessary to check the positive and negative controls for each sample, provided by the All plex Respiratory Panel kit and dispensed in the PCR plate by the StarLET machine. The quality reports are fundamental parameters to assess the validity of each run. Negative control (NC) must not emit any signal, and it's distributed in the last well of each panel. It consists of RNAase free water. Positive control (PC) is constituted by the targeted sequences contained in plasmid. Its signal must be detected to validate each run. Each panel must contain the positive control in the last well of the PCR plate. The internal control (IC) validates all the process phases, and it must be present and positive in each well of the PCR plate filled with the patient specimen.

3. Workflow

Sample processing consists in the extraction of the nucleic acid and in their amplification to detect the presence of the specific viruses included in the selected panels.

The technician begins the workflow by opening all the sample tubes under the fume hood and displays them on the 32-sample racks where they will be processed by the

StarLET - Seegene machinery. The instrument must be prepared and set with all the reagents of the STARMag 96 X 4 Universal Cartridge Kit (the buffer cartridge, manually filled by 48 microliters of ethanol, the magnetic beads vial and the proteinase K) and the Allplex Repiratory Panel 1, 2, 3, 4 (MOM, buffer, water, enzyme, viral and bacterial internal control). Moreover, the 96-deep well plate must be placed within the instrument to allow the magnetic separation of nucleic acids from the paramagnetic beads, together with the PCR plate and the empty vials where the mix will be formed. The StarLET uses 1000 µl and 200 µl tips for all its operations.

3.1 Nucleic acid extraction and PCR plate preparation

The nucleic acids extraction protocol is the following.

- Lysis of the samples. The instrument adds the Proteinase K and the internal control, and then the Lysis Buffer (LB) to 96 deep well plate. The samples are then transferred into the 96 deep well plate and mixed by pipetting up and down.
- Bind nucleic acid to magnetic beads. The machinery dispenses the resuspended beads to 96 deep well plate and the binding buffer BB to lysed sample. It separates the magnetic beads against the side of the well by placing the 96 well plate on the magnetic separator. It waits until all the beads have been attracted to the magnets and removes the supernatant by pipetting.
- WB1 wash. The instrument removes the 96 deep well plate from the magnetic separator and adds the Washing Buffer 1 and resuspends the beads by shaking. It separates the beads by placing the magnetic 96 deep well on the magnetic separator again. And repeats the pipetting and the discarding of the supernatant.
- WB2 and WB3 wash. The operations of the washing buffer 1 are repeated for the WB2 and 3.
- Elution. Add Elution Buffer EB to each well of the 96 deep well plate and resuspend the beads by shaking. Incubate the suspension at 56°C and separate the magnetic beads by placing the 96 deep well plate on the magnetic separator. Wait until the beads have been attracted to the magnets. Transfer the supernatant containing the purified nucleic acids to the PCR plate.

3.2 Nucleic acids amplification

The PCR plates are closed with a film by the technician to avoid evaporation or contaminations, spinned and manually transferred to the C1000 Thermal Cycler CFX96 real-time system - BIO-RAD to perform nucleic acids amplification and analysis. This instrument performs PCR and the detection of the targeted viral nucleic acids.

The purified nucleic acid is reverse transcribed by using the MOM, the buffer and enzyme reagents (RTase, DNA polymerase, UDG and buffer containing dNTPs) into cDNA which is subsequently amplified by CFX96 ™ IVD, CFX96™ Dx or CF96 Touch ™ real-time PCR system. To perform the multiple target amplification and detection with superior accuracy in a single reaction well, this assay kit employs Seegene's innovative proprietary DPO ™, TOCE ™ (Tagging Oligonucleotide Cleavage and Extension), MuDT™ technologies.

Multiple Detection Temperature (MuDT) technology allows the detection of multiple targets in a single fluorescent channel by providing individual Ct values for multiple targets *(fig. 11)*.

For example, if we need to detect the presence of Flu A and Flu B, we know that the first emits a robust signal at less than the relative temperature of 65°C, while Flu B target at less than 75°C. We assume to select the detection temperature at 60°C and 72°C, that are the temperatures at which the fluorescent signals are collected. At 60°C signals for both Flu A and B are overlapping whereas only the signal of Flu B can be read at 72°C. MudT works on this step, it subtracts the fluorescence signal for Flu A from the overlapping signal collection at 60°C and it then converts the subtracted fluorescence signal for Flu A into a Ct.

During the process, the TOCE ™-Pitcher probe anneals to a specific target sequence located between the DPO ™ – forward and DPO ™ – reverse primers. During the extension of the PCR cycle, the 5' nuclease activity of Taq polymerase cleaves target-bound Pitcher tagging probe and releases the unlabeled extender in the Pitcher probe which specially serves as a primer for an artificial template, a quenched-fluorescent molecule, the TOCE ™ – Catcher. Annealing and extension of the extender on the catcher generates Duplex Catcher by DNA polymerase, and it separates the reporter molecule from the quencher, by allowing the signal generation.

These signals are analyzed by real-time amplification curve or by Melting Temperature curve.

The first challenge is high multiplex target detection by the real-time PCR. Since each Catcher has a designated Melting Temperature, if the target is present in the sample, it will be amplified, and its expected unique Tm value is obtained. The Tm value can be controlled by adjusting the length and the sequence of the Catcher. In this way the Tm vale is constant, and it's not affected by the sequence variations of target sequence. Since the Tm value of the Catcher is controlled by the design of the assay, multiple targets can be detected very easily in a single channel. TOCE technology allows the detection of five targets per channel, which results in the detection of up to 20 targets on an existing 4 channel real-time instrument.

The second challenge is the high multiplex quantitation in the real-time PCR. Melting temperature can be measured repeatedly at specific cycles during the PCR process, the specific cycles are called cyclic-CMTA points. Quantitation of the sample can be determined at cyclic-CMTA points based on known concentrations of standards.

One of the key features of TOCE technology is the wide range of tolerance in annealing temperature, which allows, for example, the high multiple detection of single point mutation related to multi-drug resistance.

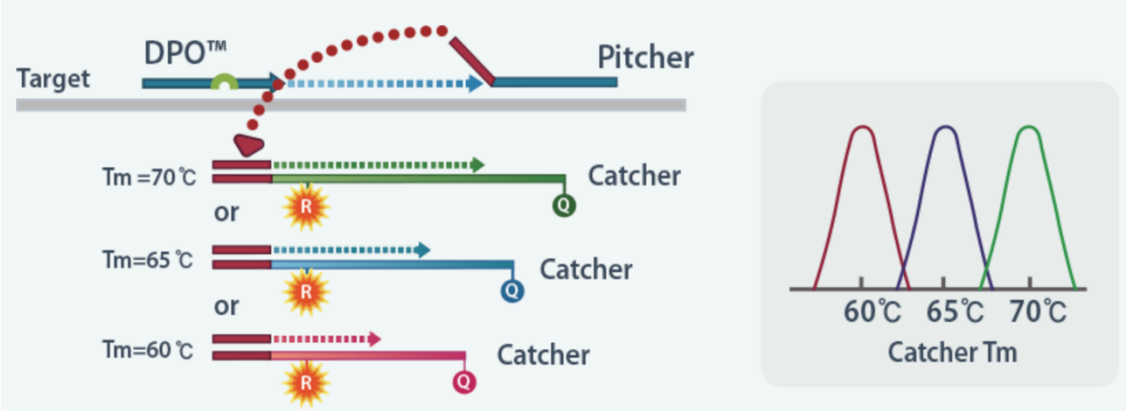

***Figure 11**. TOCE technology. Source: https://www.seegene.com/technologies/toce*

Furthermore, this PCR is a nested PCR. It involves two sets of DPO primers used in two successive run of polymerase chain reaction, the second set intended to amplify a second target within the first run product, in this case the subtype sequences of influenza.

The steps of the nucleic acid amplification reaction are the following:

- Retro-transcription of the viral RNA into cDNA at 50°C for 20 minutes.

- Denaturation of the RNA/cDNA hybrid at 95°C for 15 minutes. The reverse transcriptase thanks to its ribonuclease H activity degrades the RNA filament.
- Annealing of the templates with the primers at 60°C for 1 minute. Forward and reverse primers, bound to their specific probes, hybridize to the target sequences.
- Extension of the target sequence at 72 °C for 10 minutes. The DNA polymerase extend the target sequence by adding the dNTPs, provided by the mix.
- Denaturation, annealing and extension are repeated for 45 cycles.

Once the amplification is completed, results need to be analyzed. To correctly evaluate them it's necessary to know the parameters according to which the positivity, negativity and invalidity is defined.

C100 Thermal Cycler CFX96 real-time system, thanks to its specific software Seegene Viewer, monitors the thermal cycles and their real-time amplification curve. Furthermore, at the end of the run, the software provides the visualization of each plate well with the respective results (positive, negative, invalid) that are subsequently checked by the personnel. Seegene Viewer software allows identification and differentiation for Ct value of multiple targets in a single channel as well as melting curve analysis. Seegene Viewer displays a convenient readout of multiple sample results by a color-coded interpretation of each sample reported in a 96-well plate type template. This software is interlocked with the hospital's LIS to easily transfer the results.

3.3 Nucleic acids detection results and interpretation

The fluorescent reporter molecules associated with the Catcher probes are:

- FAM for RSVA, Flu A, PIV4, MPV, OC43 and HBoV of viral panels. SP and LP of bacterial panel.
- HEX for RSVB, Flu B, PIV2, PIV1, 229E and NL63 of viral panels. HI and BPP of bacterial panel.
- Cal Red 610 for Flu A pdm09, Flu A H1N1, AdV, HEV and HRV of viral panels. MP and BP of bacterial panel.
- Quasar 670 for Flu A H3N2, PIV3 and internal control of viral panels. CP and BP of bacterial panel.

The positivity of the results is confirmed by interpretation of the amplification curves of the samples. When the PCR run ends, we validated the results by checking

the curves. The validation consists in the observation of Ct value at which the curves peak.

Results

From the 14th of October 2024 to the 4th of May 2025 a total of 7.353 samples coming from Treviso province hospitalized patients were analyzed, among them 82 are broncho lavage samples (BAL) and 45 broncho aspirates. The percentage of positivity is 40.1% over the total amount of samples. During this period the highest number of positive cases was registered for the human rhinovirus with a total of 1005 cases by representing the 34.03% of all positives, which peaks in February. This virus is followed by the human coronavirus OC43 (275 cases) and by influenza pdm09 virus (267 cases) *(fig. 12)*. The infection of metapneumovirus was significant too with 251 positives.

	RSV A	RSV B	Flu A H1N1	Flu A H3N2	Flu Pdm09	Flu B	MPV	PIV-1	PIV-2	PIV-3	PIV-4	AdV	HBoV	OC43	229E	HRV	NL63	HEV
14/10/2024 a 20/10/2024								2		1		4	1	3		44		39
21/10/2024 a 27/10/2024									1	1		4	1	2		29		29
28/10/2024 a 03/11/2024					1			2		1		1		4		30	2	29
04/11/2024 a 10/11/2024	2						1	1	1	1		4		1		65	3	18
11/11/2024 a 17/11/2024		1					1	3	1			3	2	4		28	2	5
18/11/2024 a 24/11/2024					4	1	1	3	1	1		5	3	9		47	5	10
25/11/2024 a 01/12/2024	1	1			2		1	1	4		1	7	5	12		41	7	4
02/12/2024 a 08/12/2024					4		1	2		2	3	5	4	11		36	4	4
09/12/2024 a 15/12/2024	3				4			2	3	1		5	2	10		32	1	3
16/12/2024 a 22/12/2024	8	4		1	9		2	5		2		10	5	18		36	9	1
23/12/2024 a 29/12/2024	6	7		2	10	4	6	1				5	4	22		26	6	1
30/12/2024 a 05/01/2025	7	10		3	18	6	7	1	1			4	4	25	1	40	7	1
06/01/2025 a 12/01/2025	8	8		7	34	3	7	1		1		3	1	20	1	25	6	
13/01/2025 a 19/01/2025	6	5		16	21	5	6	2	1			2	4	14		27	10	
20/01/2025 a 26/01/2025	7	4		24	28	10	5			1		3	8	23		29	2	3
27/01/2025 a 02/02/2025	10	11		40	25	8	4	3			1	4	2	18		34	1	
03/02/2025 a 09/02/2025	16	12		24	26	10	13	1				1	6	15	1	26	7	
10/02/2025 a 16/02/2025	11	16	1	20	25	5	7			1	1	1	3	7		24	3	
17/02/2025 a 23/02/2025	10	18		11	15	8	11	1		1		5	4	6		30	4	
24/02/2025 a 02/03/2025	11	9		18	10	12	22	1		1	2		5	3	1	36	2	
03/03/2025 a 09/03/2025	10	13		19	6	8	19			3	1	2	4	8		20	5	1
10/03/2025 a 16/03/2025	9	17		18	4	6	11				1	2	4	10	2	23	1	
17/03/2025 a 23/03/2025	4	8		12	3	2	18			1		1	1	4		24	3	
24/03/2025 a 30/03/2025	4	8		2	2	1	18					6	5	5	2	26	2	
31/03/2025 a 06/04/2025	3	8		4		3	18	1		1	1	4	1	2	2	34	2	
07/04/2025 a 13/04/2025	5		1	3	3		15	1		2	1	3		4		36	1	
14/04/2025 a 20/04/2025	1	1			3	2	10			3	2			2	2	24		
21/04/2025 a 27/04/2025	3	3		1	1		23			6		2	2	4	2	34		1
28/04/2025 a 04/05/2025		3		2	1		22	1	1	11	4	6	3	3		34		1
	145	167	2	227	259	94	249	35	14	42	18	102	84	269	14	940	95	150

***Figure 12**. Nasopharyngeal positive samples per each week. Source: author's elaboration (Excel) from Treviso Hospital BASE software data.*

The most prevalent subtype of influenza A virus is the pdm09 influenza A virus which represents 53 % of all influenza A subtypes. Its cases rise around December, and they peak in the second week of the year *(fig.13),* with a significant drop between February and March. Influenza virus A H3N2 has a similar pattern compared to the pandemic one, but in the hospitalized population it's steady till March and it drops in April. RSV A and B show similar trends, however after their peak in February, RSV A cases tend to decrease, while RSV B maintains a constant rate until March. The metapneumovirus is characterized by a distinct pattern compared to the previously described viruses, it reaches its peak in March with a constant increasing of cases and it circulates mainly during spring months.

Among the total number of nasopharyngeal samples processed for viral detection 140 samples belong to the reanimation care units of Treviso province. 47 of these were positive for at least one virus, for the majority for influenza A virus, while 23 % of all positives belonging to this care unit were affected by human rhinovirus. On the total amount of the nasopharyngeal swabs 554 samples were pediatric patients, among them 364 were positive. The more frequent infections detected in pediatric samples are human rhinovirus (53 %), adenovirus (12%), RSV A (11%) and the human coronavirus OC43 (11%).

The second group of patients analyzed in this project provides samples collected by Sentinel doctors of Treviso Province and analyzed at Padua Hospital.

The analysis of samples in Padua adopts Panther Hologic machine, a fully automated system which performs nucleic acids' extraction and amplification by saving time and costs.

170 samples were analyzed and investigated about the presence of influenza virus and its subtypes. The percentage of positivity is 38%, influenza A H1N1 cases represents the 34% of all positive cases, while the subtype A H3N2 are the 30%, the influenza B Victoria lineage is the 31% of all positives.

Among these patients, about 50% are < 10 years old and 37 are positive for at least one influenza A and B virus subtype, while the elderly represent only the 16.4% of the entire group of patients. The highest rate of positivity among children is detected for IBV: 43.2% of their total number of positive cases. The 59% of all the patients were vaccinated for influenza virus. Only 16% get infected despite they received the vaccination. Patients > 60 years old with chronic diseases represent 25% of this percentage, while the 14% of the positive vaccinated is made of children under 10 years old. We compared these data with the ones performed by The Health company ULSS2 Marca Trevigiana. 10.3% on the total amount of the patients of ULSS2 has been vaccinated. We can provide an overview of the age groups. Among the total of patients between 0 and 6 years old, the 7.3% has been vaccinated, while the age group between 7 and 59 represents a low percentage of about 2.7%. The highest rate of vaccination was registered for the elderly, 11.7% detected in 60-64 years old people and the 22.5% in > 65 years patients.

To contextualize the data collected from this project, they were compared with the ones processed by the national surveillance RespiVirNet project. Two different sample populations are considered, the first made up of hospitalized patients at provincial level, the second of Sentinel doctors' patients at national level. This comparison allows us to get a national overview of the 2023-24 and 2024-25 season. As the 17th weekly RespiVirNet report mentions, in 2024-25 season estimated cases of flu-like syndrome, related to the entire Italian

population, were about 16,129,000, a number never reached in the previous flu seasons [67]. The population of surveillance patients constitutes a mean of 2.278.077 per week, the 4.0% of the regional population under surveillance.

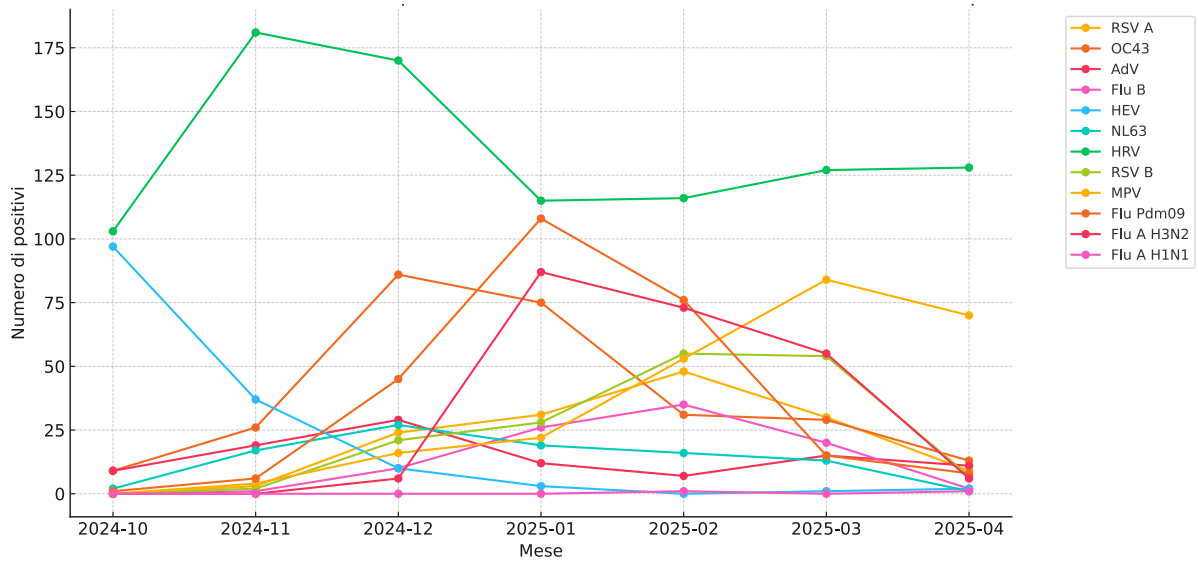

***Figure 13**. Line plot multiseries of RSV A, OC43, AdV, Flu B, HEV, NL63, HRV, RSV B, MPV, Flu Pdm09, Flu A H3N2, Flu A H1N1. Positivities of nasopharyngeal samples. Source: author's elaboration (Excel).*

Discussion and conclusions

When discussing about the percentages of positive cases between the two kinds of population analyzed, the first made of Treviso province hospitalized patients and the second constituted by the Sentinel doctors' patients, it is fundamental to consider the difference between the two types of population. Treviso patients are established sick subjects, while RespiVirNet population is randomly screened subjects showing flu-like symptoms. The positivity rate is around 40% for both groups. This demonstrates how a sample population such as the hospitalized group mirrors the situation of the province distribution by giving a reliable overview. If we consider the national population instead, cases of flu-like symptoms constitute around 26% of the national population during the surveillance season, as the 17th weekly RespiVirNet report mentions [67].

The number of positive cases of respiratory pathogens during the 2024-25 season reached higher levels than in the 2023-24 season (*fig. 14,15*). However, the incidence of the 2023-24 season was higher than the one of the following years. By observing the incidence curves, it seems that season 2024-25 lasted longer than the previous one. The peak of positive sample is also different. In 2023-24 it was reached earlier, around the 52nd - 1st week, while in the 2024-25 later, around the 3rd - 4th week. If we compare these trends with our sample population of Treviso hospitalized patients, we obtain similar statistics. Even if these graphs are based on a different number of samples processed, one on the national base and the other on the provincial range they provide two distinct groups of population by showing similar trends about influenzas viruses. In both 2024-25 graphs, national and provincial, the peak of positivity is detected around the 4th- 5th week. Both curves have long tails and early positive trends. This confirms our data validity.

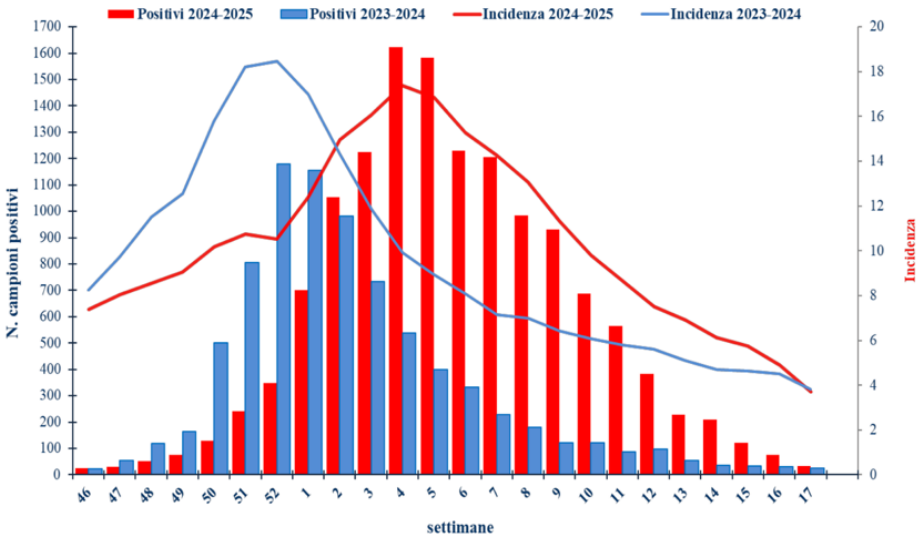

***Figure 14**. Positive cases and incidence of RespiVirNet cases reported related to 2023-24 and 2024-25 season. Source: report N 24 of 5th May 2025 RespiVirNet.*

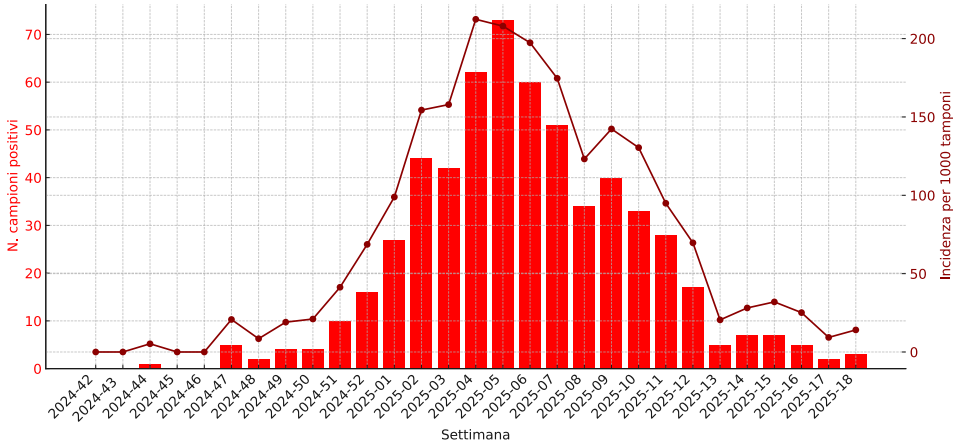

***Figure 15**. Positive cases and incidence rate registered in Treviso Hospital related to Treviso's province hospitalized patients. Source: author's elaboration (Excel).*

The seasonality of respiratory viruses changes every year. This is due to viral genome mutations, but also to environmental factors such as population immunization and lifestyle. For instance, restrictions during SARS-CoV-2 pandemic induced a drop of respiratory viruses' cases because of the use of face covering and physical distancing. [68] The human rhinovirus showed the highest percentage of positive cases with its peak reached during December and January. It is in fact a year-around virus, prevalent during colder months. Human coronavirus OC43 represents another endemic relevant pathogen with its highest infection rate registered in January, followed by a significant drop in spring.

The third most prevalent virus is the influenza A virus H1N1 pdm09, which circulated mainly in January and February months. Human metapneumovirus showed a different pattern, it tends to increase during spring months by reaching its peak in March.

We have also compared the percentage of positivity of influenzas subtypes provided by the RespiVirNet national surveillance with the ones described in our hospitalized patients group. The RespiVirNet one reported the prevalence of influenzas A (66.7%) over the influenzas B (33.3%), with the 54% belonging to Pdm09 and the 46% to the H3N2 subtype. The situation is similar among Treviso's province hospitalized patients, with the 83.8% positive for influenza A and the 16.2% positive for influenza B. In this case the 46.5% belong to flu A H3N2, while the 53.1 to flu A Pdm09 by mirroring the national data situation.

If we consider the positive viral infection cases belonging to care units patients, we can observe that 33% of reanimation care unit patients are positive for influenza virus, a remarkable percentage by demonstrating the clinical complications that this kind of virus may induce in the frail. 5% on the total amount of nasopharyngeal samples processed constitutes the percentage of positive cases belonging to pediatric care unit. Among them the most prevalent virus is human rhinovirus, it infects children because of their undeveloped immune system, since they have close contacts in nurseries and poor hygiene routine, which facilitates the spreading. The second most prevalent virus circulating in pediatric care unit is the human adenovirus which infects mainly immunocompromised people and children under 5 years old. 11 % is represented by RSV A which infects mainly infants and children under 2- years-old, with worst consequence compared to the RSV B. Human coronavirus OC43 is another relevant pathogen spread among 5-years younger children, by causing mild symptomatology.

From the data collected by the Sentinel doctors' patients we observe that the percentage of people vaccinated for influenza virus who get sick during influenza season and go to their general practitioner or pediatrician is more than the half. While the percentage of the positives despite the vaccination in this group of people is a low percentage, less than 20 %, demonstrating the effectiveness of immunization. Among these subjects, the majority is represented by patients >60 years old with chronic diseases. This confirms the recommendation of vaccination for 65-older patients and who is at risk. Another consideration is related to <10-years younger children with a chronic disease that are considered at risk and for which the vaccination is highly recommended.

To conclude, this thesis project demonstrates the importance of subregional respiratory pathogens surveillance. It's a fundamental tool to prevent possible outbreaks of new viral

diseases, to update the seasonal vaccine formulation, and to follow up frail's clinical conditions. In particular, the data collected constitutes important information to keep under control the seasonality of pathogens, which is influenced by the evolutionary and social conditions of our community.

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