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Study and development of a microneutralization assay to evaluate the immunological efficacy of vaccines against recent pandemic and epidemic viruses.

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1. Abbreviations

2019-nCoV	2019 novel Coronavirus
+ssRNA	Positive single-strand RNA
3CLpro	3C-like protease
BRC	Baby Rabbit Complement
COVID-19	Coronavirus Disease 2019
CoVs	CoronaViruses
DMVs	Double Membrane Vesicles
E	Envelope
ER	Endoplasmic Reticulum
GPC	Guinea Pig Complement
gRNA	Genomic RNA
HWs	Health Workers
ICTV	International committee on Taxonomy of Viruses
M	Membrane
MERS-CoV	Middle East Respiratory Syndrome CoronaVirus
MN-CPE	Microneutralization CPE
MPVX	Monkeypox Virus
N	Nucleocapsid
Nsps	Non-structural proteins
nsps	non-structural proteins
ORF	Open Reading Frames
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
RBD	Receptor Binding Domain
RdRp	RNA-dependent-RNA-polymerase
S	Spike
SARS-CoV	Severe Acute Respiratory Syndrome CoronaVirus
SARS-CoV-2	Severe Acute Respiratory Syndrome CoronaVirus 2
sgRNA	Sub-genomic RNA
TMPRSS2	Transmembrane Protease Serine 2

VACV	Vaccinia Virus
VoC	Variants of Concern
VoI	Variants of Interest
VuM	Variants under Monitoring
WHO	World Health Organisation

2. Abstract

Epidemics and pandemics caused by the emergence of new viruses have not been so rare in human history. The latest in timeline are the COVID-19 pandemic caused by the Sars-CoV-2 virus and the outbreak in Europe and America of monkeypox (MPXV). These new health emergencies have highlighted the crucial role vaccines play in preventing viral-borne diseases, especially for individuals considered immunologically weak.

In order for new vaccines against emerging viruses to be approved and marketed, they have to undergo several phases of clinical trials to evaluate the vaccine-induced immune response and, consequently, efficacy. To do this, clinical trials are organised in which participants undergo vaccination and subsequent biological sampling. The use of serological tests to analyse these samples makes it possible to assess antibody components in immunised and non-immunised subjects, to highlight differences in immunological responses.
In antibody quantification and evaluation, the differentiation between neutralising and non-neutralising antibodies is very useful. The Microneutralisation (MN) test makes it possible to derive neutralising antibody titres in human serum samples by observing the suppression of the cytopathic effect (CPE) in a cell substrate incubated with a standardised dose of live virus and serial dilutions of the serum sample.

This thesis work is divided into two tasks. In the first study, the MN-CPE test is used to analyse, in a population of healthcare workers (HWs) vaccinated with a double dose of BNT162b2 mRNA vaccine, the decline of the immune response 180 days after the second administration. In the second task, the MN-CPE test protocol was developed to be able to effectively assess the presence of neutralising anti-MPXV antibodies and examine possible cross-reactions in subjects previously vaccinated with the smallpox vaccine. To do this, a panel of human sera containing MPXV convalescent subjects and subjects vaccinated against smallpox virus was tested.

The CPE-based microneutralisation test proved reliable and effective in both studies to examine the presence of neutralising antibodies in serum samples from convalescent or vaccinated subjects.

3. Introduction

3.1 Sars-CoV-2

Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) is a relatively new virus detected for the first time at the end of 2019, which belongs to the highly pathogenic and transmissible Coronaviridae family,[1]. SARS-CoV-2 was directly responsible for the COVID-19 (Coronavirus Disease 2019) pandemic, an acute respiratory syndrome which led to significant health, social and economic consequences worldwide[2]. Following its appearance, this new virus was isolated, identified and sequenced at the beginning of January 2020. Phylogenetic analyses suggested that SARS-CoV-2 can be classified as a Betacoronavirus, like the (Severe Acute Respiratory Syndrome CoronaVirus SARS-CoV) and the Middle East Respiratory Syndrome CoronaVirus (MERS-CoV), viruses responsible for two of the most recent pandemics.

The origin of the latest coronavirus still remains unknown, although one of the most accredited hypotheses is that zoonotically transmission occurred, as happened with SARS and MERS[3].

COVID-19 syndrome typically manifests itself with fever, cough, asthenia and breathing difficulties, very common characteristics in many other transmissible respiratory diseases. COVID-19 shows a benign course in the majority of cases, especially in young age subjects. Symptoms begin to occur more frequently in adults over the age of 50 years old, and it considerably increases its rate of worsening when it affects patients with an already complicated clinical situation (i.e. immunocompromised patients) or in elderly subjects[4].

At the beginning of the pandemic, the containment was mainly based on an early diagnosis, with subsequent isolation of the positive patient, coupled with hygiene and health strategies[5]. The main goal was to make the environments as safe as possible from the spread of the virus between people.

Only with the rapid development of specific vaccines against SARS-CoV-2 it was possible to implement the “containment strategy” against the spread of the virus. After three years since the beginning, we can affirm that the intense vaccination campaign led to achieve a very high level of immunization of the population globally[6].

Recently, effective therapies for COVID-19 are being applied. The current clinical strategy involves treating patients with non-specific antiviral drugs and anti-inflammatory treatments that can reduce the immune response[7].

All these efforts and hygiene-health strategies contributed to the end of the emergency, which officially arrived in May 2023 when the World Health Organisation (WHO) downgraded the virus and ascertained the end of the pandemic (Statement on the fifteenth meeting of the IHR (2005) Emergency Committee on the COVID-19 pandemic, WHO, 5th May 2023)[8].

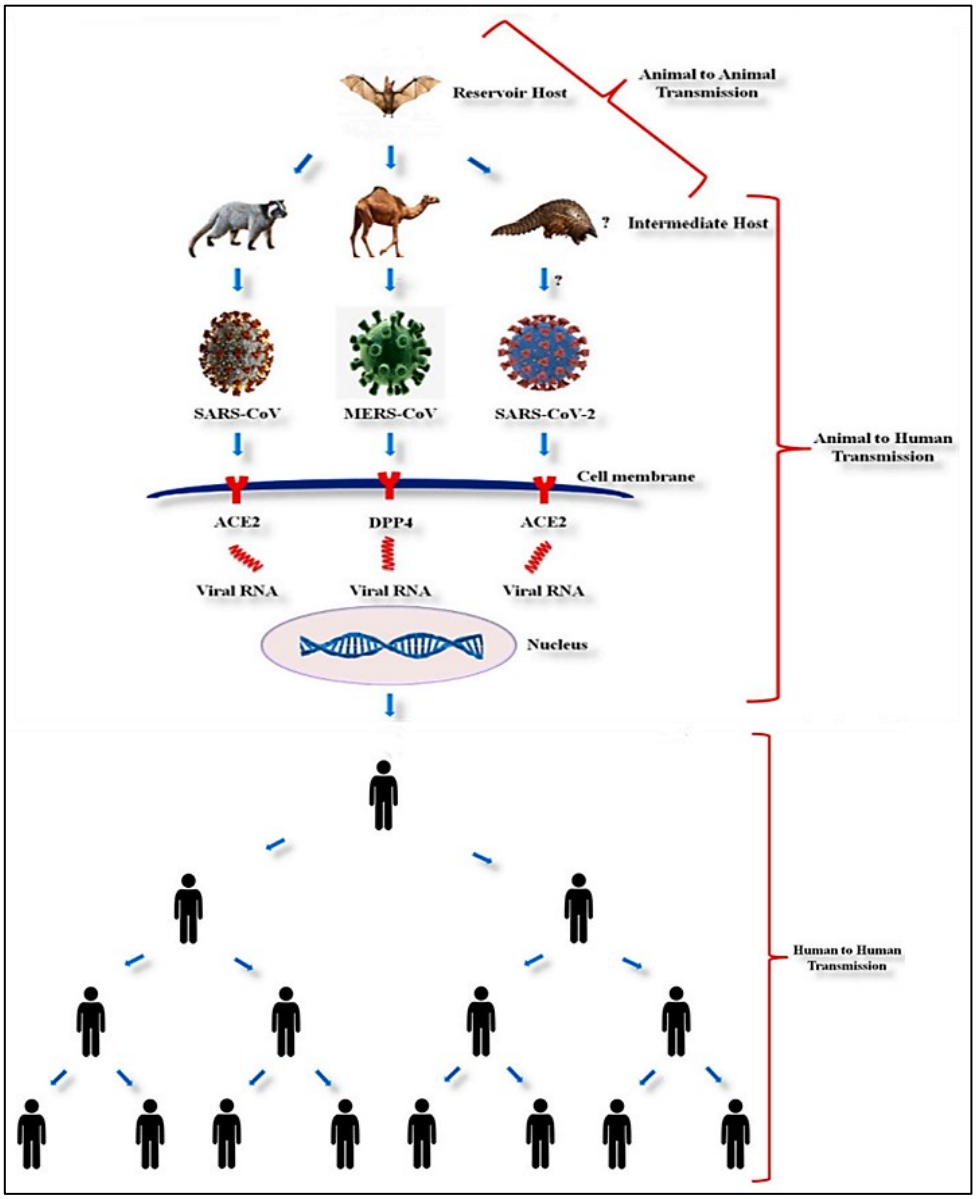
3.1.1 Epidemiology

Coronaviruses have long been known to present a high pandemic risk. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the ninth documented coronavirus that infects humans and the seventh identified in the last 20 years (Lednicky J.A., Tagliamonte M.S., White S.K., Elbadry M.A., Alam M.M., Stephenson C.J., Bonny T.S., Loeb J.C., Telisma T., Chavannes S., et al. Emergence of porcine delta-coronavirus pathogenic infections among children in Haiti through independent zoonoses and convergent evolution. medRxiv. 2021 doi: 10.1101/2021.03.19.21253391.; Vlasova A.N., Diaz A., Damtie D., Xiu L., Toh T.-H., Lee J.S.-Y., Saif L.J., Gray G.C. Novel canine coronavirus Isolated from a hospitalized pneumonia patient, east Malaysia. Clin. Infect. Dis. 2021 doi: 10.1093/cid/ciab456. Published online May 20, 2021). SARS-CoV, MERS-CoV and SARS-CoV-2 are considered as the three most dangerous viruses for public health in their family; in fact, they are directly responsible for three pandemics in the last twenty years. Common aspects regarding these viruses are a significant nosocomial transmission and a very aggressive pathogenesis, due to the replication of the virus in the lower respiratory tract and the hyperresponsiveness of the immune system of the infected host[4].

3.1.2 Origin

CoronaViruses (CoVs) are predicted to circulate for centuries in the world, but their origin remains unclear[1]. At the beginning of SARS-CoV and MERS-CoV outbreaks, civet cats and dromedary camels,respectively, were considered the natural source of these viruses[4]. Nevertheless, recent molecular genetic research studies and comprehensive phylogenetic analyses identified bats as the reservoir hosts of both SARS-CoV and MERS-CoV; instead civet cats and dromedary can be seen as intermediate in the zoonotic transmission[9].

We know that there are several theories on the origin of SARS-CoV-2. Initially, it was believed to be originated from bats, seafood and snakes, while recently it was suggested the transmission from pangolins to humans[10].



4. FIGURE 1: SCHEMATIC REPRESENTATION OF TRANSMISSION OF SEVERE ACUTE RESPIRATORY [10]

It isn't only the place of origin that is in doubt, but also when the first transmission occurred. Between all the studies conducted during the last few years, it is to highlight the research of Deslandes et al. This French group used Polymerase Chain Reaction (PCR) method to assess the presence of SARS-CoV-2 genetic material in respiratory samples. It was observed a SARS-CoV-2 infected patient one month before the first reported cases in France, suggesting that the virus may have spread to France and other European countries earlier than imagined[11,12].

For all these reasons, it is easy to understand how many doubts still remain regarding the origin of SARS-CoV-2 virus.

4.1.1 Diffusion

The first cases of COVID-19 were recorded the 31st of December 2019 in Hubei Province (China). At that time, 27 patients were admitted with a severe form of viral pneumonia associated with fever, cough, chest pain, and in the most severe cases, dyspnea and lymphocytic interstitial pneumonia[13] . Based on epidemiological data, the Huanan market in Wuhan was an early and major epicentre of SARS-CoV-2 infection. Two of the three earliest documented coronavirus disease 2019 (COVID-19) cases were directly linked to this market selling wild animals, as were 28% of all cases reported in December 2019. Overall, 55% of cases during December 2019 had an exposure to either the Huanan or other markets in Wuhan, with these cases more prevalent in the first half of that month (World Health Organization. World Health Organization; 2021. WHO-Convened Global Study of Origins of SARS-CoV-2: China Part.).

Pathogenesis and symptomatology initially suggested that these cases where the result of the infection by the two pandemic coronaviruses SARS-CoV and MERS-CoV. However, it was possible to isolate a new strain of coronavirus by metagenomic RNA sequencing on a bronchoalveolar lavage sample derived from a patient with acute pneumonia. The new strain was identified as a Betacoronavirus, belonging to the subgenus Sarbecovirus, such as SARS-CoV and MERS-CoV, justifying its high genomic homology (respectively 79.5% and50%)[13].

The new virus was temporarily named 2019 novel Coronavirus (2019-nCoV) by the WHO, only later it was identified as SARS-CoV-2 by the International committee on Taxonomy of Viruses (ICTV) (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses 2020).

The rapid increase in the number of virus-positive cases was facilitated by the coincidence with the Lunar New Year celebration, where multiple trips between cities allowed the virus to spread in more than 34 Chinese provinces in a single month.
The first death in China from SARS-CoV-2 was documented on January 11th, 2020; followed by the observation of the first cases of infection in Thailand. The ease of virus spread, and the abundance of international flights led to a sharp and sudden increase in cases worldwide, with a higher rate than SARS-CoV pandemic[10].

This generated serious concerns about global health, and with the hope of limiting the spread to other countries, the first measures to restrict airline flights to and from China were implemented. However, the intervention was not enough: after less than a month, clusters of infected patients emerged in more than 29 countries, comprehending Europe.

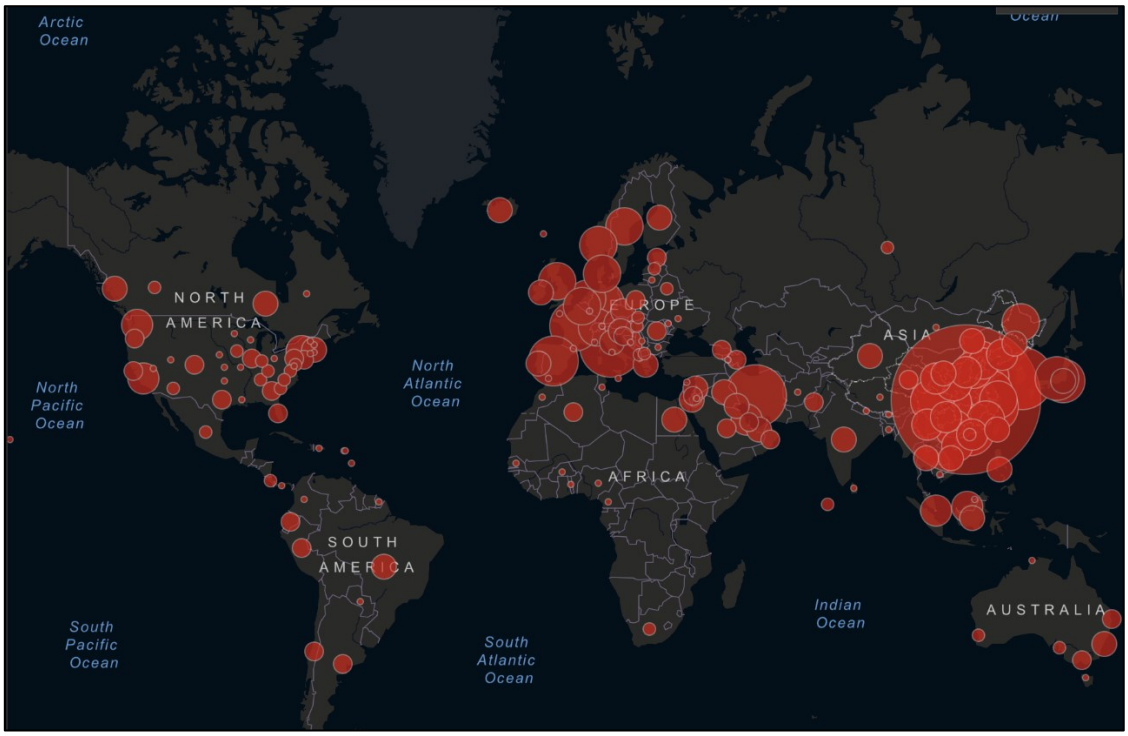


FIGURE 2: EARLY DIFFUSION OF SARS-COV-2 HTTPS://COVID19.WHO.INT

On March 11th, 2020, the WHO proclaimed the beginning of the COVID-19 pandemic.

The global impact as of December 8th, 2023 is quantifiable in 772,138,818 confirmed cases with 6,985,964 deaths, the numbers are constantly increasing despite the end of the pandemic. (WHO Coronavirus Dashboard, https://covid19.who.int).

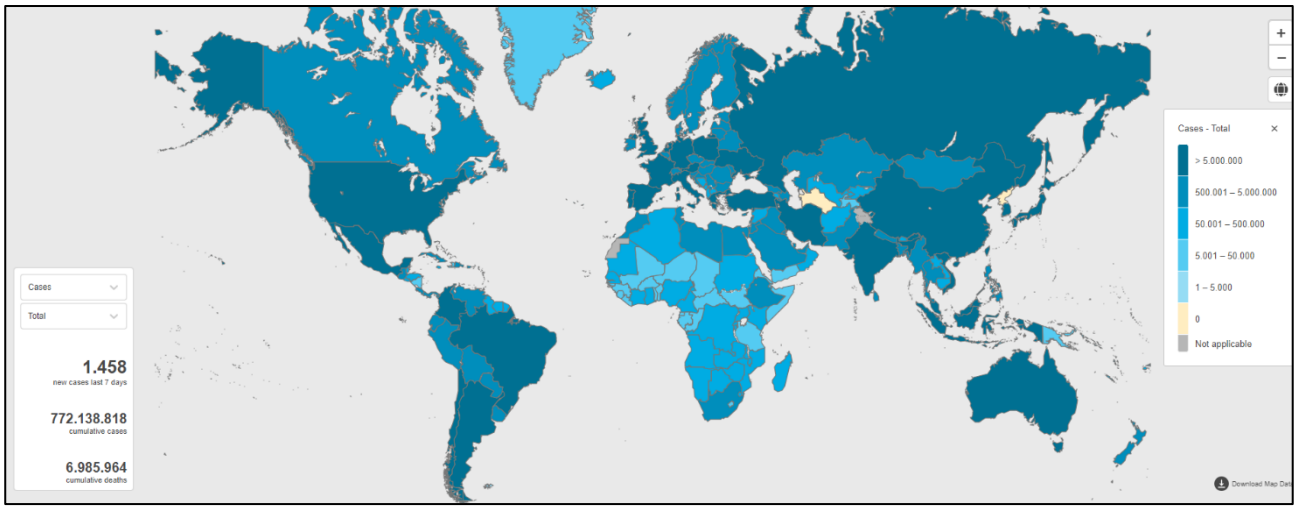


FIGURE 3: GLOBAL IMPACT FROM COVID-19,08 DECEMBER, 2023 HTTPS://COVID19.WHO.INT

4.1.2 Virology

CoVs are capsulated positive.e-sense RNA viruses, characterized by a round morphology of 100-150 nm.

These viruses belong to the Coronaviridae family (order: Nidovirales), a distinctive name that derives from the morphological arrangement of surface glycoproteins that resembles a crown. Two subfamilies reside in the Coronaviridae one, the Orthocoronavirinae and the Torovirinae. The former includes 4 genera: alpha, beta, gamma and delta[9], that differ from each other in the type of animal they infect. More in detail, alpha and beta infect mammals, gamma genus infects birds, and the delta is capable of infecting both types.

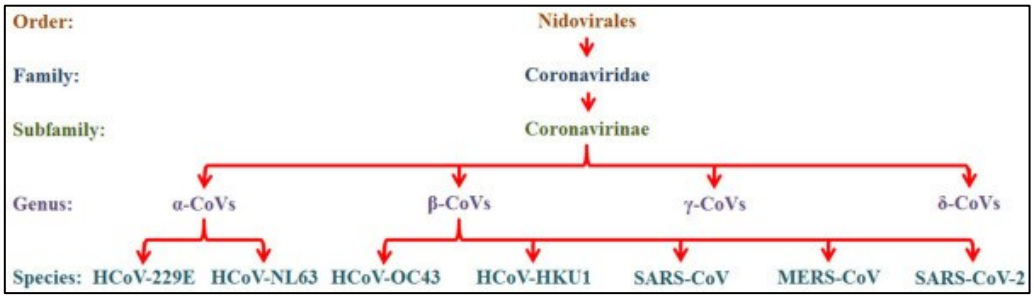


FIGURE 4: CORONAVIRUS CLASSIFICATION [10]

Many coronaviruses have a zoonotic origin and typically affect the respiratory and/or digestive tracts of mammals, including humans[14].

The first coronavirus was discovered in 1960, followed by the identification of 7 more species: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV

and SARS-CoV-2. All coronaviruses cause mild or lethal respiratory illness, depending on the strain and host condition[15].

4.1.3 Structure

Coronaviruses are spherical particles whose RNA consists of a positively coiled monofilament with a helical symmetry nucleocapsid. Their RNA is one of the largest known viral genomes, with a length between 27 and 32 kb[16].

The viruses are coated with a pericapsid, a lipid bilayer derived from the host cell membrane, in which S protein trimers are present[15].

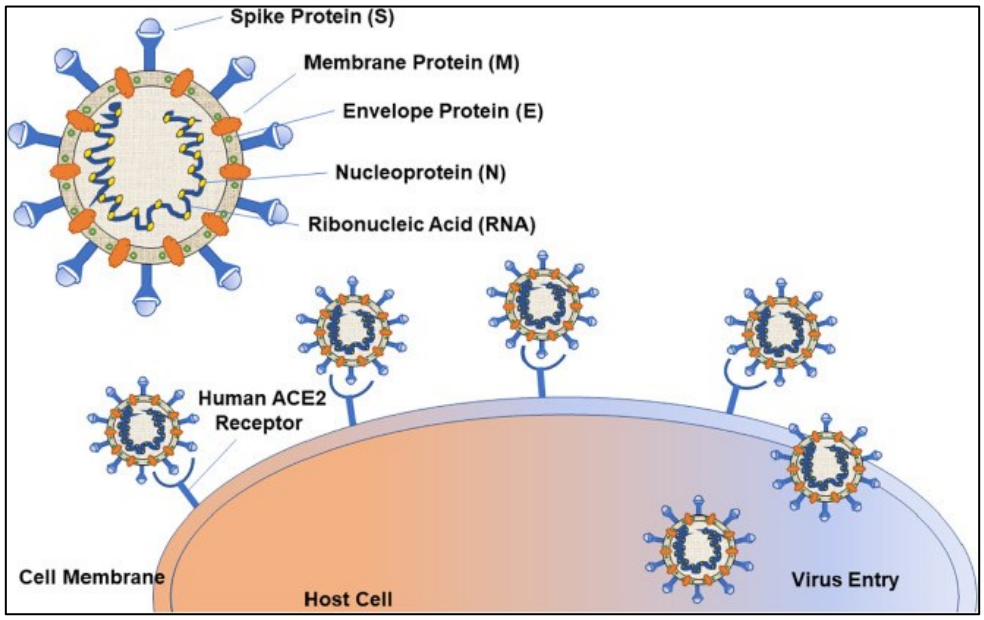


FIGURE 5: SARS-COV-2 STRUCTURE[15]

The main structure of the virus is composed of 4 proteins: membrane (M) protein, envelope (E) protein, Spike (S) protein and Nucleocapsid (N) protein. All of them are integrated in the viral envelope, except for protein N which interacts directly with the RNA located in the central part of the viral particle, forming the nucleocapsid[15] .

The S protein projects through the viral envelope, forming the characteristic spikes in the "crown" of the virus. This highly glycosylated class I fusion protein needs to be in the homotrimer form to be actively able to bind the surface of the target cells and induce the fusion with them. The binding is extremely specific, because it requires the identification of the Angiotensin-Converting Enzyme 2 (ACE-2) on the host/target cell[17].

For this reason, this protein represents the main antigenic determinant of SARS-CoV-2, being also the construction target for the developed vaccines[18].

This protein undergoes continuous evolution, over time mutations tend to accumulate throughout its length, causing conformational changes that can alter its antigenic properties, making it unrecognizable to the immune system[15].

In conclusion, we can consider the S protein as the object that directed the pandemic toward continuous progression[18,19].

The M protein (membrane or matrix) is the most abundant one and presents itself as a dimer. This protein exhibits its role in the viral particle assembly process and helps the virion to maintain its shape by nucleocapsid binding[20].

The N protein binds to the genomic material of the virion to form the nucleocapsid. Its role consists in regulating viral RNA synthesis and help the budding process by interacting with the M protein [21]. Being recognizable from some cytotoxic T lymphocytes, this protein might also be important for vaccine development[22].

Lastly, the E protein consists of two ectodomains that are located at the N- and C-terminus of its primary structure[14]. It has several functions, including facilitating virus assembly and release, but it also has an ion channel activity, useful for viral pathogenesis[15].

There are other proteins that can be identified as structural, they are encoded by a variable number of genes (from one to eight depending on the viral strain) and labelled as “accessory”. Each of these can encode for multiple isoforms of the same protein[23]. Accessory proteins have no homology in structure with other viral or cellular proteins and are not required for viral replication in cell cultures in vitro[24].

4.1.4 Genomic Organization

SARS-CoV-2 genome is a positive single-strand RNA (+ssRNA) with a length of ~ 30kb. Looking from 5’ to 3’, the genomic material contains ORF1a and ORF1b, which encode for proteins pp1a and pp1b respectively[15]. These proteins are in turn splitted into 16 non-structural proteins (nsps)[23]. Immediately after the two open reading frames (ORF) there is the genomic information for the previously described structural proteins (S, N, E, M), which permit the formation of new virion particles[25]. Between them there are sequences that encode for six accessory proteins (3a, 6, 7a, 7b, 8, 10).

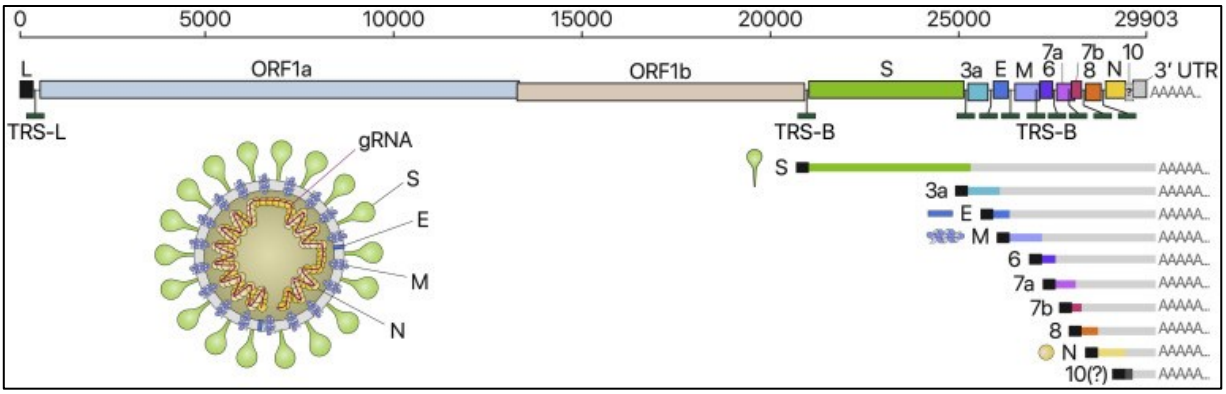


FIGURE 6: GENOMIC STRUCTURE OF SARS-COV-2 [26]

Immediately after entering in the host cell, the viral genome is translated by the host's translation machinery, in order to encode the proteins that are necessary for new genomic RNA (gRNA) synthesis[15]. The new gRNA is produced in its entirety by the synthesis of a negative intermediate strand (in the 3′→5′ direction) used as a mould to produce a new positive gRNA. Nsp12 protein is the main character in this process, due to its activity as an RNA-dependent-RNA-polymerase (RdRp)[25].

Sub-genomic RNAs (sgRNAs) that encode for N, S, M, E, 3a, 6, 7a, 7b, and 8 proteins can be obtained via a discontinuous process[27]. Each of this segment has a 5' LTR sequence of 70 nucleotides, flanked by the same upstream and downstream transcription regulatory sequence (TRS). The production of sgRNA is ensured by the pairing of the 2 TRS sequences during the synthesis of intermediate RNA[26].

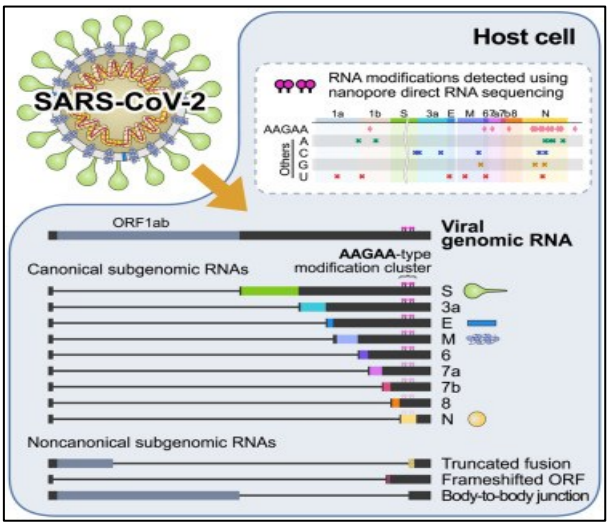


FIGURE 7: VIRAL GENOMIC RNAS[26]

The biological characteristics of CoV-2 are, to a large extent, superimposable on those of other CoVs. Nevertheless, its RNA synthesis mechanism is very complex, due to the high frequency of genes with structures that differ from the ones used for canonical transcription mechanisms.[28]. These characteristics lead to low RNA polymerase fidelity and result in a high mutation rate for SARS-CoV-2[29].

4.1.5 SARS-CoV-2 Mutants variants

The genetic evolution of SARS-CoV-2 occurred as continuous adjustment to new human hosts. As a result, mutant variants begin to occur, and many countries did undergo second or third wave of outbreaks. The main difference between new virus strains and the wild-type one is the spreading efficiency and the resistance to natural or vaccine-induced immunity in susceptible hosts[28]. All variants share the mutation D614G, here an aspartic acid is replaced by a glycine at position 614, belonging to the sequence encoding structure protein S[30]. D614G was first identified in late January 2020 but emerged only in March 2020. Main consequence of this nucleotide substitution is that prevents the hydrogen-bonding interaction with T859 of ACE-2 receptor by promoting the "up" bound conformation of the Receptor Binding Domain (RBD). This, increases virion infectivity while enhancing replication capacity in the upper respiratory tract of the host[31]. In addition, the presence of D614G in the SD2 domain, increases junction whit the target cell[32].

All variants detected worldwide are divided by WHO guideline into:

- Variants of concern (VoC)
- Variants of interest (VoI)
- Variants under monitoring (VuM)[33]

VuM are SARS-CoV-2 variants which present genetic changes that affect virus characteristics and early signals of growth advantage in respect to other circulating variants, without clear evidence of phenotypic or epidemiological impact. Consequentially, monitoring and reassessment of the evidences is required. For example, if a variant has an unusually large number of mutations in a known antigenic site, if there is evidence of community transmission in ≥ 2 countries within a 2-4 week period, but it is not possible to estimate its relative growth advantage, such a variant can be designated a VuM [34].

VoI are SARS-CoV-2 variants with genetic changes that are predicted or known to affect virus characteristics (i.e. transmissibility, virulence, antibody evasion, susceptibility to

therapeutics and detectability). To fall in this category, they must also have a growth advantage over other circulating variants in more than one WHO region, with an apparent epidemiological impact to suggest an emerging risk to global public health (i.e. increasing relative prevalence or increasing number of cases over time)[34].

VoC are VoI variants that, meet at least one of the following criteria after a risk assessment with moderate-to-high level of confidence:

- Detrimental changes in clinical disease severity;
- Changes in COVID-19 epidemiology that have a substantial impact on the healthcare field, requiring major public health interventions;
- Significant decrease in the effectiveness of available vaccines in protecting against severe disease[33]

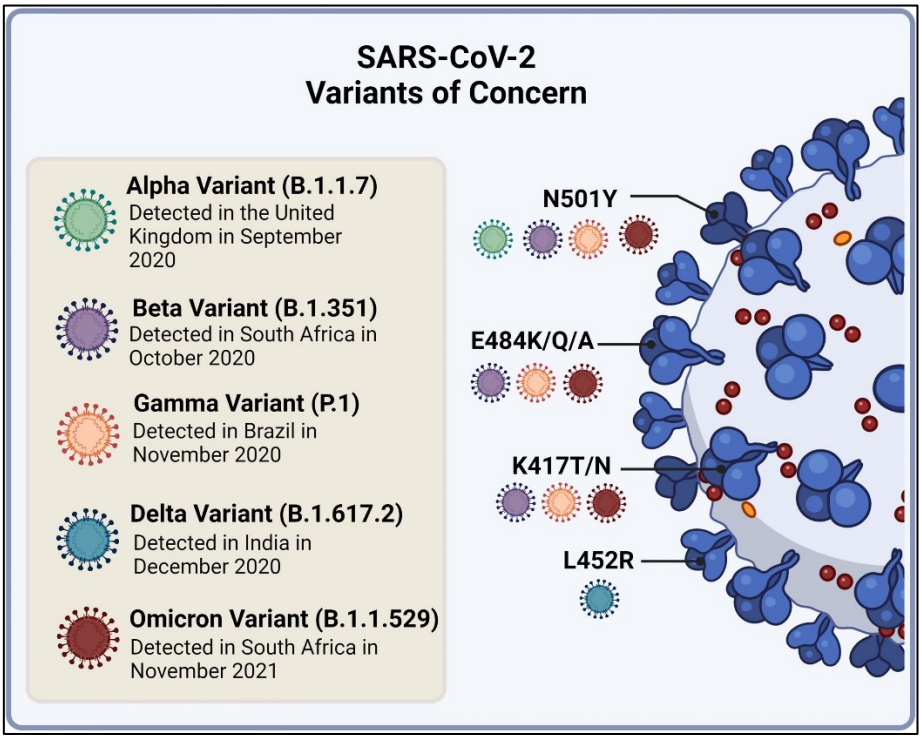


FIGURE 8: REPRESENTATION OF SARS-COV-2 VARIANT OF CONCERN[35]

The five variants of interest are Alpha, Beta, Gamma, Delta, and Omicron[36].

The Alpha variant (B.1.1.7 Lineage) started to circulate in Britain in September 2020 and was officially identified in America in late December 2020[37]. There are seventeen mutations that characterise this strain, eight of them (Δ69-70 deletion, Δ144 deletion, N501Y, A570D, P681H, T716I, S982A, D1118H) affect the spike protein. Aminoacidic changes in the B.1.1.7

S protein improve both RBD accessibility and affinity for ACE-2, which could be one of the causes of the increased transmission efficiency. The single N501Y mutation increases the affinity between RBD and ACE-2 of ~ 10-fold and alters the stability of the S protein of SARS-CoV-2, while also increasing transmission rates[32]. Studies show that a higher percentage of patients infected with the B.1.1.7 lineage variant risk to develop a severe disease and consequently death, than the ones infected with other variants[38].

The Beta variant (B1.351 Lineage) was detected in December 2020 and is responsible for the second wave of infections in South Africa throughout the following year. This strain shows 9 mutations (L18F, D80A, D215G, R246I, K417N, E484K, N501Y, D614G, A701V) in the spike protein; between them, three (K417N, E484K, and N501Y) correspond to the RBD sequence and are found to enhance the affinity for the receptors[37]. The E484K mutation improves significantly the electrostatic complementarity in the antibody binding[32]. The N501Y substitution, is the only shared mutation of the RBD detected in other three variants (i.e. alpha), proving a key role in increasing viral transmissibility. In addition, both the N501Y and E484K mutation synergically increase the affinity for human ACE-2 receptor[39].

Another peculiarity of the Beta variant is the presence of the P71L aminoacidic substitution in the viral E protein. This is the only case of mutation to this structural protein identified in the variant SARS-CoV-2[32]. The P71L mutation is known to be associated with disease severity and mortality, but its specific mechanism needs further study.

In conclusion, from genomic and epidemiological data it is possible to prove a selective advantage of this variant, as a result of increased transmissibility and/or better immune evasion[39].

The Gamma variant (P.1 lineage) was isolated for the first time in Brazil on December 2020[37]. There are thirteen mutations that characterize this strain, all of them fall in the S protein (L18F, T20N, P26S, D138Y, R190S, H655Y, T1027I V1176, K417T, E484K, and N501Y), tree in particular (L18F, K417N, E484K) are in the RBD, same as the beta variant[37].

The Delta variant (B.1.617.2 lineage), was identified in India on December 2020 and found responsible for the second wave of infection in April 2021 in the same country[37]. This strain is characterised by a high transmissibility, supplanting pre-existing variants of SARS-CoV-2 in most countries[37]. The delta variant harbors ten mutations in the spike protein (T19R, G142D*, 156del, 157del, R158G, L452R, T478K, D614G, P681R, D950N) [37].

The Omicron variant was first reported in Shout Africa on November 2021 and it spread to over 100 countries worldwide[40]. In contrast to beta and delta variants, omicron possess a lower ability in primary host-infections but expresses its high transmissibility in re-infection progressions[41].

Omicron has more than 50 mutations on the S protein. There are more than 20 new mutations in the S1 domain, 8 mutations are located in NTD and 15 mutations are located in RBD, which may directly enhance the interaction between RBD and ACE-2 and dodge binding to antibodies induced by previous infection or vaccination[42]. In addition, the insertion of the sequence ins214EPE, discovered for the first time in SARS-CoV-2, was later found to be expressed in the common cold coronavirus (HCoV-229E). This may explain the cold-like symptoms and short incubation period of around 3 days caused by omicron strain[41,43].

The Omicron viruses account for over 98% of the publicly available sequences since February 2022, and represent the genetic background from which new SARS-CoV-2 variants will likely emerge, even if the emergence of variants derived from previously circulating VoCs or of completely new variants remains possible.

The previous system classified all Omicron sub-lineages as part of the Omicron VoC; for this reason, there was no possibility to compare new descendent lineages with altered phenotypes to the Omicron parent lineages (BA.1, BA.2, BA.4/BA.5). Therefore, since 15th March 2023, the WHO variant tracking system considers each sublineage of the Omicron strain independently, and classifies each of them as VuMs, VoIs, or VoCs[36].

4.1.6 Lifecycle of SARS-CoV-2

The virus enters the human body via the upper respiratory tract, and starts its replicative cycle as soon as it finds cells that express ACE-2 receptor; usually the first ones that the virus meets are the lung cells[44].

The infectious cycle of SARS-CoV-2 begins with the binding of the RBD to the peptidase domain of the ACE-2 receptor. Upon binding, the S protein is cleaved into the S1 and S2 subunits, resulting in structural changes of the subunit S2[44] that can start the entering process. Fusion with the host cell membrane can occur in two ways:

1. transmembrane protease serine 2 (TMPRSS2) cleaves in the S2 site, leading to the exposure of the fusion peptide[44]. In this way, the heptad repeat 1 and heptad repeat 2 domains of the S2 subunit combine to produce a six-helix fusion core, which carries viral particles close to the host cell membrane[45].

2. For cells lacking TMPRSS2, at low pH the cathepsins can induce endocytoses and endosomes formation, mediating virus-cell membrane fusion at the cell surface[46].

After the fusion, the RNA genome can be released into the cytosol, translated to produce the replicase and digested by the 3C-like protease (3CLpro) and a papain-like protease to produce the 16 nsp[27]. RdRp regulates viral genome replication and sub-genomic transcription to assemble new viral particles.

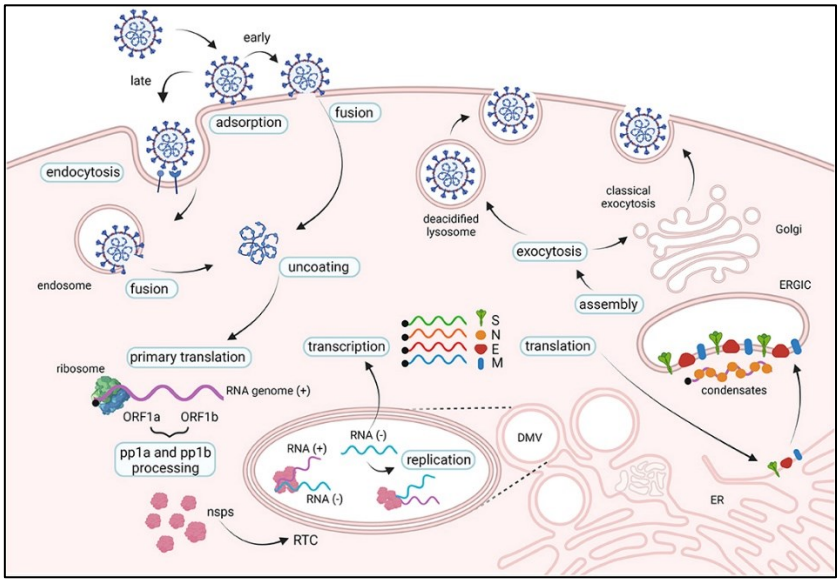


FIGURE 9: SARS-COV-2 INFECTIVE CYCLE[47]

The first step in the replication phase of SARS-CoV-2 is the formation of double membrane vesicles (DMVs) in the host cell. Nsp3 and nsp4 catalyse the rearrangement of endoplasmic reticulum (ER) into DMVs and promote the replication of gRNA and sgRNA[48]. Viral RNAs are stored into the DMVs and transported to the cytosol for viral translation and assembly[49]. Structural proteins package gRNAs to construct progenitor viral particles, while sgRNAs encode conserved structural and accessory proteins. Ribosomes in the cytoplasm translate many copies of RNA and N proteins, while S, M, and E proteins are synthesized in the endoplasmic reticulum and removed in the Golgi apparatus. The viral RNA-N complex and S, M and E proteins follow the secretory pathway to reach the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and assemble mature virions[50]. Finally, the viral particles are released through the process of budding of the Golgi apparatus and exocytosis of the cell membrane to begin a new cycle of infection[50].

4.1.7 Pathogenesis

It becomes apparent that understanding the physiological and immunological processes as well as the specific characteristics of Sars-Cov-2 infection assumes a key role in being able to develop both effective prevention strategies, such as vaccine development, and promising therapeutic interventions designed to directly target the virus or the dysfunctional immune response, for example anti-viral or monoclonal antibodies[51].

Coronaviruses are common pathogens in humans and animals. Some infect the upper respiratory tract causing mainly mild respiratory symptoms, while others can affect the lower respiratory tract to the point of manifesting even serious potentially fatal complications. To this category belong Sars-CoV, Mers-CoV and the more recent Sars-CoV-2[52], the responsible for the three most important Coronavirus outbreaks in recent decades.

Sars-CoV-2 is transmitted by exposure to aerosols and respiratory droplets, direct contact with contaminated surfaces, and some cases of orofecal transmission have also been reported[53]. The incubation period before the onset of symptoms is between 3 and 11 days with an average of 4-5 days[51]. Slightly different incubation periods have been observed for new variants of SarS-CoV-2[46].

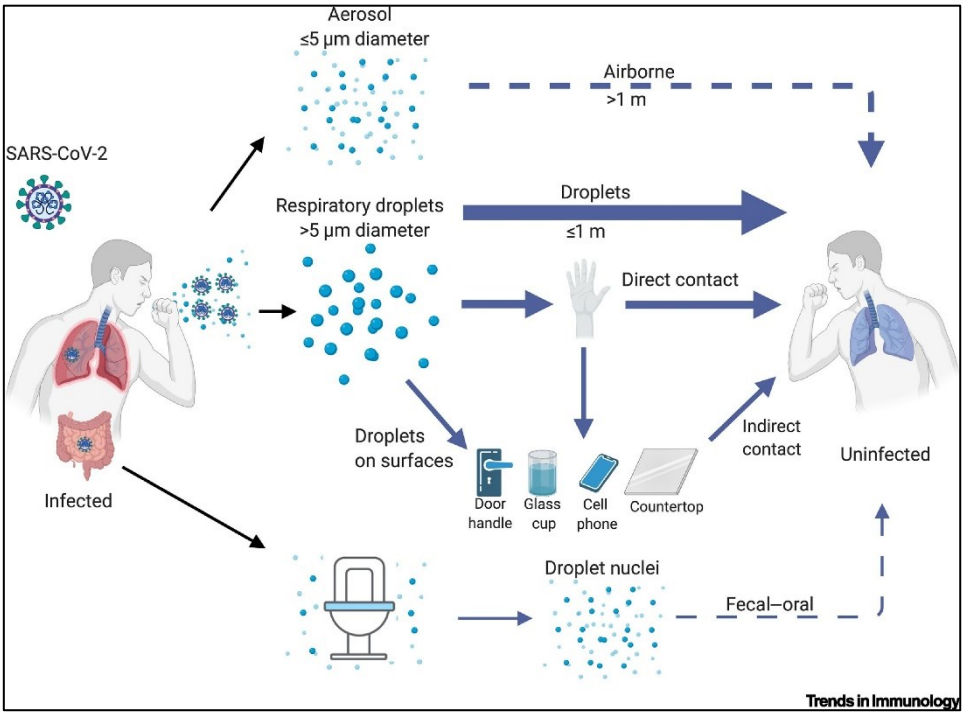


FIGURE 10: SARS-COV-2 TRANSMISSION[53]

Sometimes (about 20%) Sars-CoV-2 infects the host but the host is completely asymptomatic remaining completely free of COVID-19 symptoms. The reason why some individuals do not develop symptoms although infected and their role regarding virus transmission have been extensively studied in recent years[54]. Only recently, however, has it been estimated that the impact on the spread of the pandemic by asymptomatic subjects has been negligible; on average, an asymptomatic subject is 66% less likely to transmit the virus[55]. In addition, it has been observed that asymptomatic subjects often carry a gene variant in HLA (human leukocyte antigen) called HLA-B*15:01 that allows their immune system to recognize and counteract the virus in a timely manner[55].

In most cases, patients infected with Sars-CoV-2 develop mild to moderate symptoms typical of Covid-19.In general (in 97.5% of cases) symptomatic patients develop the disease within 8 days[56]. The main symptoms are dry cough and fever, these may progress to difficulty breathing, muscle and joint pain, severe headache, persistent diarrhoea and haemoptysis, causing the patient to be hospitalized[56]. After 5-6 days from the onset of symptoms, SARS-CoV-2 is in its peak replication and infectivity, and after 8-9 days, in severe cases, it is possible to progress to Acute Respiratory Distress Syndrome (ARDS), which is characterized by severe difficulty in breathing and low blood oxygen levels, in turn making patients more susceptible to bacterial and fungal infections[53].

The average age of infection with SARS-CoV-2 is about 50 years, and the manifestation of symptoms varies with age, despite this, individuals of all ages are susceptible. Generally, individuals over 60 years of age and with other ongoing debilitating conditions have a higher likelihood of developing ARDS or being hospitalized[53]. There is no study justifying an increased risk in pregnant women, although the possibility of transmission of the virus from mother to fetus has been observed instead[57].

Studies have been conducted on the clinical, radiological and molecular characteristics of infected patients. In general, fever (98%), cough (76%) and fatigue (44%) are the most common symptoms, while at the radiological level ground-glass opacities can be observed in the lungs[56]. Differences have also been observed at the molecular level in the plasma of hospitalized and non-hospitalized patients, in the former the levels of inflammatory and pro-inflammatory cytokines are very high, indicating an excessive inflammatory response that can lead to shock and death[52].

This process occurs because of the cytopathic of SARS-CoV-2, it in its infectious cycle is able to cause morphological-structural changes to the host cell leading to pyroptosis, a programmed cell death[50]. Destruction of respiratory cells triggers an initial local immune response, resulting in macrophage and monocyte recall, cytokine release, and activation of an initial adaptive T-cell and B response[52]. In many cases the immune system is able to resolve the infection, but in others it is possible to trigger this excessive immune response that leads to uncontrolled inflammation, damaging several organs, particularly the cardiac, renal, and hepatic systems[51]. Of all these, certainly the respiratory system is the one that is most damaged[52].

We can say that 70% of COVID-19 deaths are due to respiratory failure while the remaining 30% result from the strong immune response by the body[56].

4.1.8 Diagnosis and therapeutic approaches

Since 2019, the COVID-19 pandemic has had a huge impact on public health and the world economy[58]. Huge economic and scientific efforts have been deployed by the most important countries in the world to counter it. These efforts made it possible in a short time to have more and more weapons at the disposal of the medical community to counter the uncontrolled spread of the virus[59].

Initially, early diagnosis techniques were developed, which were fundamental, especially during the first phase of the pandemic, when the only weapons available to the scientific community were the identification and isolation of infected individuals[60].

Subsequently, enormous efforts to develop new therapeutic strategies resulted in multiple successful vaccines in an exceptionally short time, as well as the evaluation of a wide range of potential treatments in clinical trials, some of which have reached the market[61].

The gold standard for the diagnosis of infection is RT-PCR (Reverse Transcriptase PCR), a molecular technique that specifically detects the presence of SARS-CoV-2 nucleic acid [62].

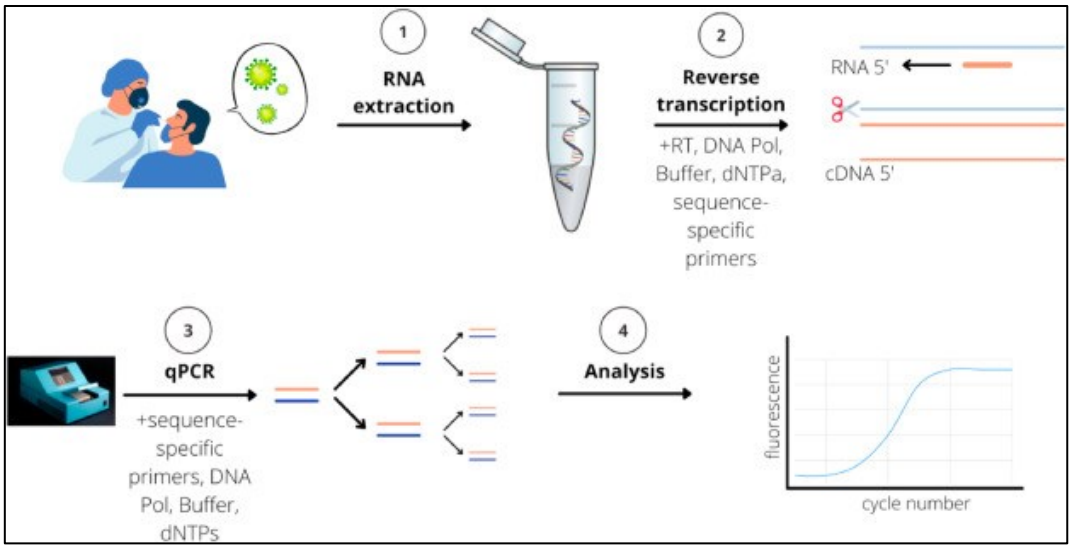


FIGURE 11: SARS-COV-2 TESTING AND DIAGNOSTIC METHODS[63]

The method involves reverse transcription of viral ssRNA (single strand RNA) into complementary DNA (cDNA), followed by amplification of certain cDNA regions. Different kit manufacturers use a variety of RNA gene targets, with most assays targeting one or more of the envelope (E), nucleocapsid (N), spike (S), RNA-dependent RNA polymerase (RdRp) and ORF1 genes[64,65].

Samples used for this test can be nasopharyngeal swabs or other upper respiratory tract samples, including throat swabs or saliva[66].

In order to avoid cross-reactions with other coronaviruses, at least two molecular targets must be included in the assay, and continuous oligonucleotide optimisation is necessary given the incessant evolution and accumulation of mutations in these genes[67].

This technique is very simple, high-throughput and very fast but has several factors that may interfere with the results related to the virus, the method itself or the different viral load present in the sample analysed[68].

Drugs

Since the start of the pandemic in the last two years, many therapeutic and immunotherapeutic molecules have been identified to control the spread of the infection[69]. Therapeutic agents considered in studies conducted during the pandemic include various approaches and mechanisms of action such as, antiviral therapies that inhibit viral replication directly, recombinant neutralising monoclonal antibodies that block viral entry into

host cells and adjunct therapies that target the host immune response, for example, anti-inflammatory and antithrombotic therapies[70]. The purpose of these studies was to be able to determine the efficacy and safety of new or repurposed drugs, so that the results could be submitted to the various regulatory agencies for marketing[69].

Among the first therapeutic strategies undertaken, the most widely accepted was therapies targeting inflammation. It was observed early on that hyperinflammation played an important role in the severe patho-physiology of COVID-19[71]. In support of this, high values of pro-inflammatory cytokines (e.g. IL-6)[72], coagulation and fibrinolytic markers (D-dimer)[73] and elevated circulating concentrations of acute phase reactants (ferritin) were observed in hospitalised patients with COVID-19 symptoms[74]. Some of the most used drugs are**:**

- Dexamethasone a synthetic glucocorticoid anti-inflammatory drug already exists and has been administered to patients with a history of heart failure. Mortality benefits have been observed when administering this drug in COVID-19 positive hospitalised patients undergoing invasive mechanical ventilation. On the other hand, it appears to be harmful when administered to patients not receiving oxygen therapy[75,76].
- Tocilizumab is a monoclonal antibody that interferes with the binding of IL-6 to its receptor[77]. Early studies showed an increase in survival rates in COVID-19 patients hospitalised and treated with tocilizumab provided the drug was administered during the early stages of infection[78].
- Baricitinib is an oral Janus kinase 1 (JAK1) and JAK2 inhibitor with anti-inflammatory properties[79]. This drug has proven to be an excellent adjuvant when administered in combination with Remdesivir[79].

Another therapeutic strategy undertaken was that of antiviral therapies. The target of these drugs is to actively interfere with the normal replication cycle of Sars-CoV-2. Immediately after the emergence of the new Sars-CoV-2 we had no effective antiviral drugs and many of the existing ones (including hydroxychloroquine, lopinavir or ritonavir, and ivermectin) proved to be ineffective[80–82]. We now have more reliable and effective antivirals. One of these is Remdesivir. Remdesivir is a prodrug nucleoside analogue, its active metabolite reduces genome replication by inhibiting RNA-dependent RNA polymerase and has antiviral activity against many RNA viruses in vitro, including SARS-CoV-2[83,84]. It has been shown to be effective when administered to patients at high risk of COVID-19 syndrome[85]. Other drugs that may be mentioned are certainly Nirmatrelvir-ritonavir, a combination therapy consisting of nirmatrelvir, an oral 3C-like protease inhibitor active against the main viral protease that

cleaves SARS-CoV-2 polyproteins during viral replication, and ritonavir, a strong cytochrome P450 3A4 (CYP3A4) inhibitor and a pharmacokinetic enhancing agent[86], and Molnupiravir, an oral β-D-N4-hydroxycytidine pro-drug that has broad-spectrum antiviral activity against SARS-CoV-2 and that in particular is incorporated into the new RNA strands of the SARS-CoV-2 genome during their synthesis, causing an accumulation of deleterious mutations that is referred to as lethal mutagenesis[87,88].

The last therapeutic approach to be analysed is neutralising antibodies. The administration of pathogen-specific polyclonal or monoclonal antibodies (mAbs) has been used in the past to control viral infections, with the aim of specifically neutralising a target virus either by eliminating it directly or by preventing its entry into host cells, thus preventing the associated disease from occurring[89]. Neutralising antibodies can be transferred from convalescent patients (i.e. plasma transfusions) or synthesised as recombinant neutralising mAbs through established molecular engineering techniques[90].

In the early stages of the pandemic, the use of plasma from convalescent patients was immediately used due to the absence of other viable options[91]. Subsequently, however, many studies showed that treatment with convalescent plasma did not bring substantial improvements in the clinical picture of hospitalised COVID-19 patients[92]. In addition, the spread of new Sars-CoV-2 variants further decreased the efficacy and thus the use of this therapeutic strategy. So much so that now the use of convalescent plasma collected prior to the Omicron (B1.1.529/BA1 and BA2) surge is not recommended by the FDA108 and has not been considered by the EMA[93].

Neutralising recombinant antibodies have been the subject of numerous studies[94]. These mAbs are designed to target the S-protein, thus hindering the binding between the virus and the ACE-2 receptor and consequently preventing the virus from entering host cells[95]. There are currently five mAbs available in the US under EUA licence: bamlanivimab plus etesevimab, casirivimab plus imdevimab, sotrovimab, bebtelovimab and tixagevimab plus cilgavimab[96].

Vaccines

Vaccinations have always played a key role in safeguarding global public health[97].Vaccination is the most effective method for stemming the spread of viruses and thus preventing symptoms and limiting deaths in the population. This was also evident during the recent pandemic[98].

Immediately after identifying Sars-CoV-2 in China, Chinese scientists isolated and sequenced the virus in January 2020. Immediately afterwards, a race against time began to produce an effective vaccine[13,99]. The first pharmaceutical companies to start clinical trials were Moderna, with the mRNA-1273 vaccine, and Pfizer/BioNTech with the BNT162b2 vaccine[100]. Both mRNA vaccines received approval from the major regulatory agencies USFDA and EMA in late 2020 early 2021[101]. These were only the first as by December 2022, as many as 50 COVID-19 vaccines had been approved worldwide. At the same time, the largest vaccination campaign in the history of mankind was carried out, allowing more than 201 countries worldwide to vaccinate their populations[102].

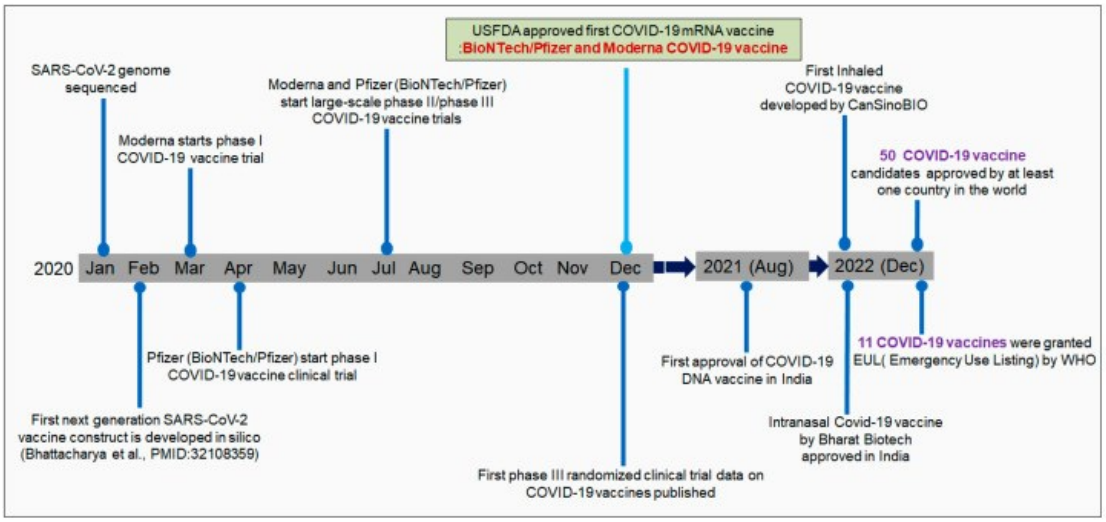


FIGURE 12: THE TIMELINE OF VACCINE DEVELOPMENT[103]

Still now 11 COVID-19 vaccines have been granted an Emergency Use Listing (EUL) by the WHO[102]. Several vaccine candidates have been developed that have entered clinical trials over time[104]. In total, 242 vaccine candidates are in clinical development. Among them, 66 are in the phase-I developmental phase, 72 vaccines are in phase-II, and 92 are in phase-III[102].

COVID-19 VACCINE COMPARISON [June 2022]

	TYPE	PROTECTION	APPROVAL	AGE GROUPS	DOSAGE	1st BOOSTER	2nd Booster
Pfizer / BioNTech	mRNA	95.6% (3 doses)	WHO, FDA, EMA	5 years and up	2 doses (21 days apart)	5 months, Pfizer or Moderna	4 months, Pfizer or Moderna
Moderna	mRNA	93%	WHO, FDA, EMA	Adults (FDA) 6 and up (EMA)	2 doses (28 days apart)	5 months, Moderna or Pfizer	4 months, Pfizer or Moderna
Oxford / AstraZeneca	Vector	76%	WHO, EMA	Adults	2 doses (8-12 weeks apart)	4-6 months, Pfizer or Moderna	4 months, Pfizer or Moderna
Sputnik V	Vector	92%	Other	Adults	2 doses (21 days apart)	3 months, Pfizer	4 months, Pfizer or Moderna
Johnson & Johnson	Vector	66%	WHO, FDA, EMA	Adults	1 dose	2 months, Pfizer or Moderna	4 months, Pfizer or Moderna
Sinopharm	Inactivated	79%	WHO	Adults (WHO) 3 and up (other)	2 doses (21 days apart)	3 months, Sinopharm or Pfizer	4 months, Pfizer or Moderna
Coronavac / Sinovac	Inactivated	51%	WHO	Adults (WHO) 3 and up (other)	2 doses (14 days apart)	Mix and Match	4 months, Pfizer or Moderna
Covishield	Vector	62%	WHO	Adults	2 doses (8-12 weeks apart)	3 months, Pfizer or Covishield	TBD
Covaxin	Inactivated	77.8%	WHO	Adults	2 doses (28 days apart)	Covaxin	TBD
Novavax	Subunit	90.4%	WHO, EMA	Adults	2 doses (3-4 weeks apart)	Novavax	TBD
CanSinoBio/ Convidecia	Vector	58%	WHO	Adults	1 dose	TBD	TBD

* All the information in this table is based on data available in the media. MYA Care 2022 – www.myacare.com

FIGURE 13: COVID-19 VACCINES APPROVED [105]

Considering all the vaccines developed in recent years, these can basically be divided into two categories: whole-virus and component-virus vaccines. In turn whole-virus can be divided into live attenuated and inactivated[106]. Component-virus vaccines can be divided into: DNA-based, RNA-based, protein subunits, virus-like particles (VLPs)-replicated viral vectors, and nonreplicated viral vectors[107].

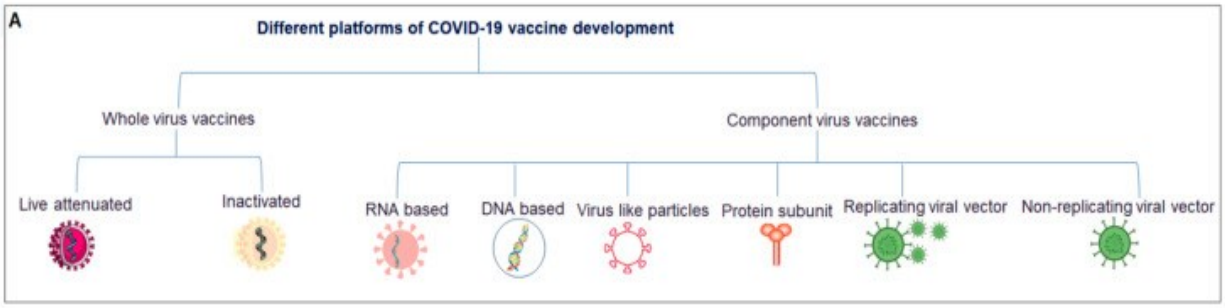


FIGURE 14: COVID-19 VACCINE'S PLATFORM[103]

There are also different targets that these vaccines interact with. Moderna and Pfizer/BioNTech mRNA vaccines express the COVID-19 spike glycoprotein[108]. Vaccines from Oxford-AstraZeneca express spike proteins using adenovirus vector platforms[109]. Sinopharm developed a whole inactivated virus vaccine (BBIBP-CorV) using aluminium hydroxide as an adjuvant[110]. Similarly, a whole-virion inactivated virus vaccine was

developed by BharatBiotech (Covaxin), and this vaccine was formulated with a TRL-7/TRL-8 agonist molecule that was adsorbed onto alum (AlgelorAlgel-IMDG)[111]. ZF2001 (RBD-Dimer) is a protein vaccine developed using the receptor binding domain (RBD) from the spike protein of the virus[108]. This vaccine uses aluminium as an adjuvant. EpiVacCoron is constituted with chemically synthesized epitopes conjugated to a recombinant protein carrier. This COVID-19 vaccine is adsorbed onto aluminium hydroxide[112]. Sputnik V is a viral-vector vaccine developed on a recombinant adenovirus platform using adenovirus 26 and adenovirus 5 (Ad26 and Ad5, respectively) vectors to express the spike protein of SARS-CoV-2[113,114]. Furthermore, many studies were able to evaluate the worldwide efficacy of these vaccines. During most phase III studies for COVID-19 vaccines, a high efficacy of these vaccines against Sars-CoV-2 infection and symptom development could be observed. For instance Pfizer-BioNTech's mRNA vaccine's VE was reported to be 95%; Moderna's mRNA-1273 vaccine, 94.1%; Oxford-AstraZeneca's ChAdOx1 nCoV-19 vaccine, 70.4%; and CoronaVac's absorbed inactivated vaccine, 50.7%[115,116].

Nevertheless, the emergence of new Sars-CoV-2 variants worldwide has led the scientific community to question whether the efficacy of these vaccines would remain unchanged or not[117]. Studies have shown that the efficacy of vaccines against COVID-19 is reduced in the presence of these new variants, leading to the inference that emerging variants may partially escape the preventive action of vaccines[117]. Several mutations were noted for immune escape and vaccine escape, and the vital mutations reported include D614G, P681R, E484K, N439K, K417N/T, K444R, and N501Y[118]. Furthermore, vaccines are less effective at protecting against infection from recently emerging viral variants, such as Omicron. Less effectiveness was noted even after the administration of a booster dose[118].

Some studies reported that VEs of the mRNA-based BioNTech, Pfizer vaccine, and mRNA-Moderna mRNA-1273 against alpha were similar to those against the previous variant[119]. However, most vaccines have reduced neutralization capacity against the Beta variant. The Sputnik V Ad26/Ad5, ChAdOx1 nCoV-19/AZD1222, CoronaVac, BNT162b2, mRNA-1273, and BBIBP-CorV vaccines showed reduced neutralization efficiency against Beta[120,121]. Similarly, the Omicron variant showed reduced neutralization capacity of immune sera elicited by vaccines, even after a booster[122].

All this can only indicate that attention must remain high and that research and development of new vaccines that keep pace with the virus mutations are and will be crucial to preserving global public health[117].

4.2 Monkeypox Virus

Over the centuries, there have been many viral epidemics that have occurred around the World and have had a great impact on public health, the economy, and global society. In addition to seasonal epidemics caused by the influenza virus, there have been many other viruses that can be held responsible for major epidemics such as HIV-1, Ebola, SARS,SARS-CoV-2, smallpox, and last in chronological order, monkeypox virus (MPXV)[123]. Smallpox was a virulent and deadly disease induced by the variola virus, which posed a serious threat to humanity[124]. Finally, a virus in the poxviridae family is monkeypox virus. Monkeypox is a zoonotic infectious disease caused by monkeypox virus. As mentioned above MPXV belongs to the poxviridae family and has double-stranded DNA. It was first identified in 1970 in rural villages in the rainforest areas of central and West Africa, when smallpox virus was in the final stages of eradication instead. It can be transmitted to humans through infected animals such as monkeys, rats and squirrels[125]. The virus is spread through direct contact with the body fluids of an infected person, such as saliva, mucus or skin lesions. The disease manifests with flu-like symptoms such as fever, headache, and muscle aches; it also causes as well a characteristic rash that begins with small lesions and bumps that later evolve into raised pustules filled with fluid[126]. Pustules caused by MPXV can appear on any part of the body, but are usually found on the face, hands, feet, and genitals. The aggravation of symptoms, in some cases can lead to complications such as pneumonia and sepsis, potentially fatal[127]. Currently, there are no specific therapeutic treatments against MPXV, so only through supportive care, such as drugs to reduce fever and pain, is an attempt to manage the patient's symptoms[126]. Smallpox vaccine, as we shall see later, seems to be able to provide some protection against MPXV. To counter the spread of the virus, in addition to the need for common sanitation, one must avoid the with infected animals and sick people[128].

4.2.1 Epidemiology

MPXV is a tropical pathogen endemic in various regions of sub-Saharan Africa and has a documented history of human infection[129]. MPXV can be distinguished into two different genetic categories, the Central African strain and the West African strain. The former has been shown to be more virulent and cause more severe symptoms in infected individuals than the west African strain[130]. The first few cases were observed in Nigeria between 1971

and 1978. Subsequently over the past three decades, the number of confirmed cases of MPXV has increased[131]. The largest recorded outbreak of the West African strain of MPXV occurred in Nigeria in 2017[132]. Between 1970 and 1979, other African countries also saw the first cases of MPXV in humans appear on their national soil. Among the 47 cases recorded in the region, 38 were concentrated in rural areas of the Democratic Republic of Congo. In the period between 1970 and 1971, Sierra Leone and Liberia, which had previously recorded no smallpox cases, saw the sudden appearance of six cases of monkeypox[132].

More recently, however, smallpox outbreaks are occurring in regions where the disease had not previously been found such as the United States and the United Kingdom in 2003 and Israel and Singapore in 2017[133]. However, all of these outbreaks were traced to travellers returning from endemic regions or nosocomial exposures[134].

Since May 2022, however, there has been a rapid upsurge in the epidemic curve suggesting an epidemiological picture that was not comparable with those observed up to that time. By August 5, 2022, at least 88 nations had reported human-to-human transmission of the virus, showing steadily increasing numbers[129].

The sudden surge of MPXV cases in countries where it was not considered endemic has caused the scientific community to fear the occurrence of a potential new pandemic. Moreover, as the diagnosis and treatment of non-existent MPXV is very complicated, all of this prompted the WHO to issue a public health emergency of international significance[135].

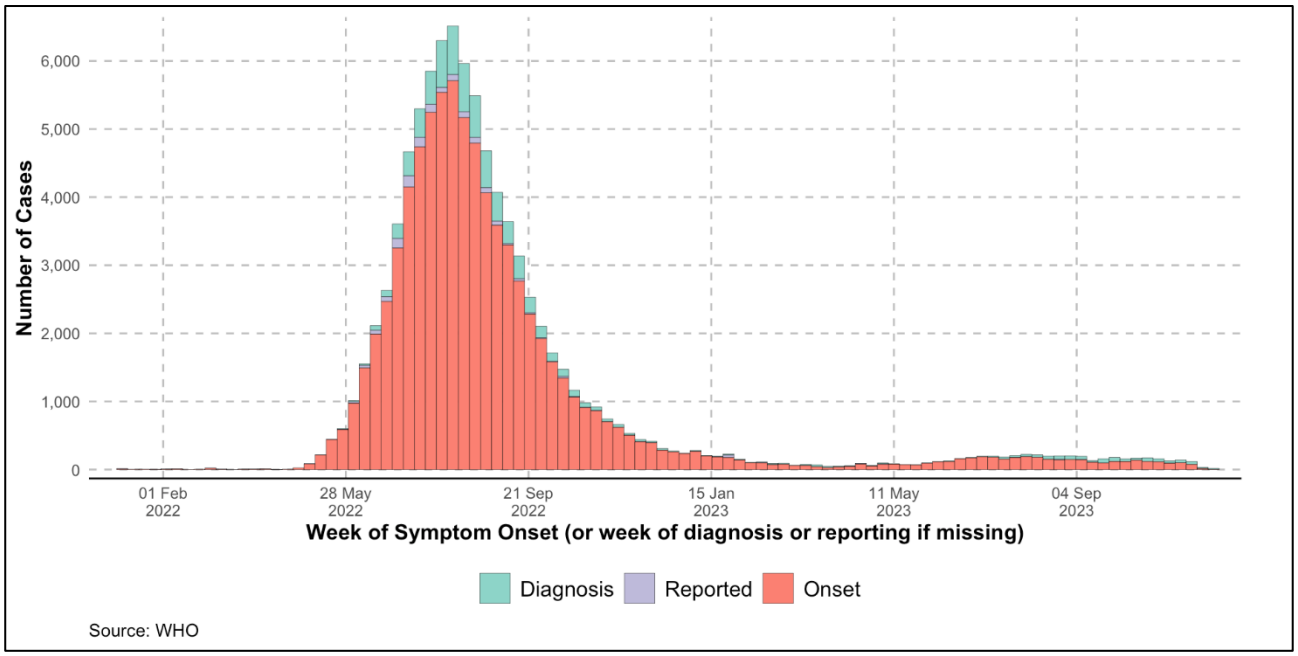


FIGURE 15 EVOLUTION OF MPXV OUTBREAK[136]

The first outbreak of monkeypox outside the African continent was reported in Europe in May 2022 and then quickly spread to other countries with a total of 28,220 confirmed cases in 88 countries and another 1685 suspected cases[137].

The first case in the United States was reported on May 18, and shortly afterward new infected patients were registered in Australia, Israel and Brazil[137]. The increase in the incidence of MPX cases continued in August 2022, with more than 5,000 cases reported in the first five days of the month. Deaths due to MPX were reported in three countries that were previously considered non-endemic (Brazil, Spain, and India)[129]. An established epidemiological link to regions in Central or West Africa could not be found.

As of November 2023, the confirmed cases are 92 783, 171 deaths, 116 countries reporting cases. The number of monthly reported new cases has increased by 25.7%, compared to the previous month. The majority of cases reported in the past month were notified from the Recent analysis of people affected by the ongoing global outbreak of monkeypox virus (MPXV) revealed that the majority of cases (97.4%) involved men, with an average age of 35 years and homosexuals[136].

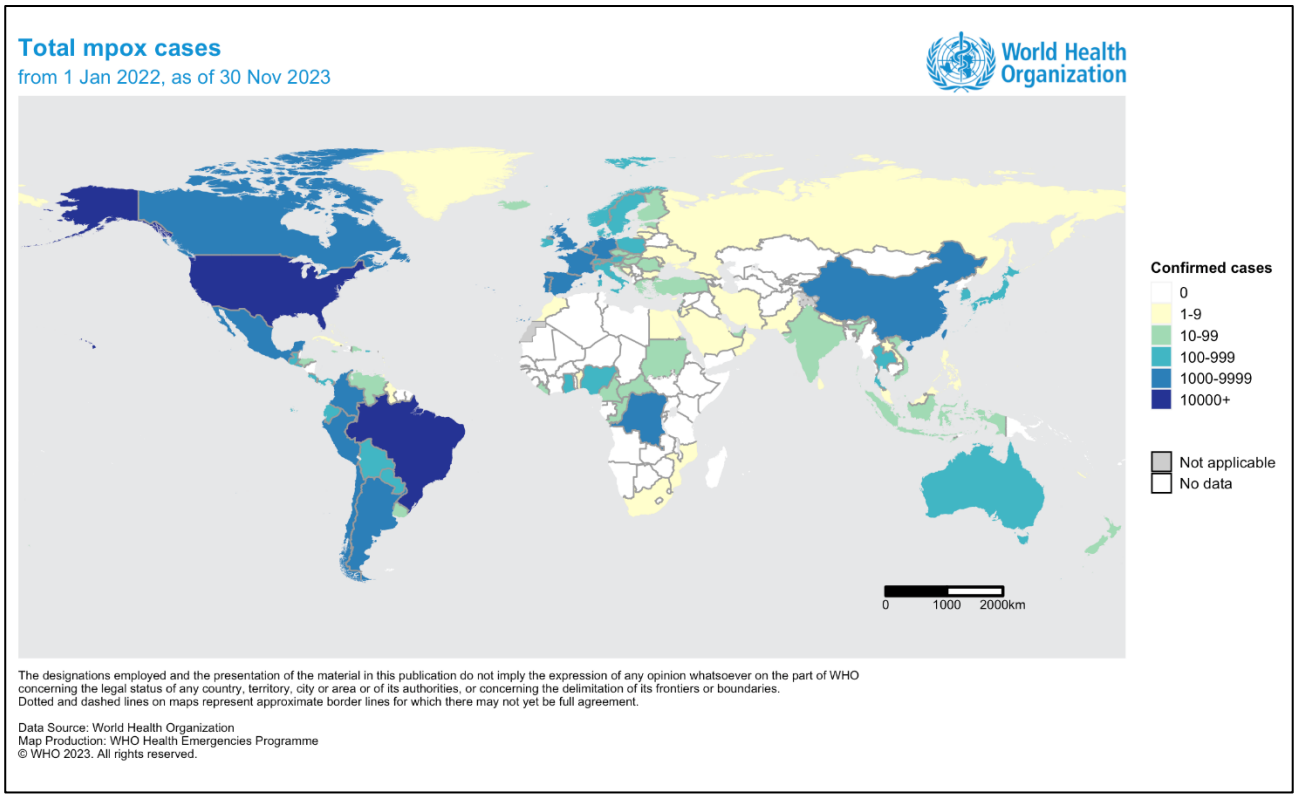


FIGURE 16 MPXV GLOBAL DISTRIBUTION[136]

Most confirmed cases of monkeypox (MPXV) have been reported in Europe and the Americas, with 14 countries (including the United Kingdom, Spain, Germany, France, Portugal, Italy, Switzerland, Belgium, the United States, Mexico, Canada, the Netherlands, Brazil, and Peru) accounting for more than 90 percent of all reported cases. In contrast, only 345 cases have been reported in seven African countries where MPXV has been endemic Asian countries and those in the Oceania region reported fewer cases of MPXV. Israel reported 160 cases, while countries in Southeast Asia and the Middle East, such as the United Arab Emirates, Singapore, India, Saudi Arabia, Thailand, Qatar, Taiwan, Japan, South Korea, and the Philippines reported a few MPXV cases. Australia reported 58 cases of MPXV, and some countries in Oceania reported a small number of cases[136].

On August 16, 2022, the Iranian Ministry of Health announced the discovery of the first human case of monkeypox, a 34-year-old woman from Khuzestan province[138]. Moreover, after detecting the presence of the disease in neighbouring countries such as Qatar, the United Arab Emirates, Saudi Arabia, Turkey, Pakistan, and Lebanon suggests that the virus may have entered Iran from one of these places[136].

According to the Nextstrain database, the strain of human monkeypox identified in Iran is part of the B.1 lineage, which originated in Europe then spread globally[139].

4.2.2 Virology

The family Poxviridae collects a large group of viruses that show common characteristics in morphology and biology. Indeed, they are typically large in size, have a membrane envelope, and double-stranded DNA. These viruses are usually found in rodent, rabbit and monkey populations[140]. The family Poxviridae is divided into two subfamilies: Entomopoxvirinae and Chordopoxvirinae. The subfamily Entomopoxvirinae includes viruses that infect insects, while the subfamily Chordopoxvirinae includes viruses that infect vertebrates. The subfamily Chordopoxvirinae in turn is subdivided into 18 genera, each of which comprises several viruses, most of them of zoonotic origin[141] MPXV is a virus that can be transmitted from animals to humans belonging to the genus Orthopoxvirus of the family Poxviridae[142].

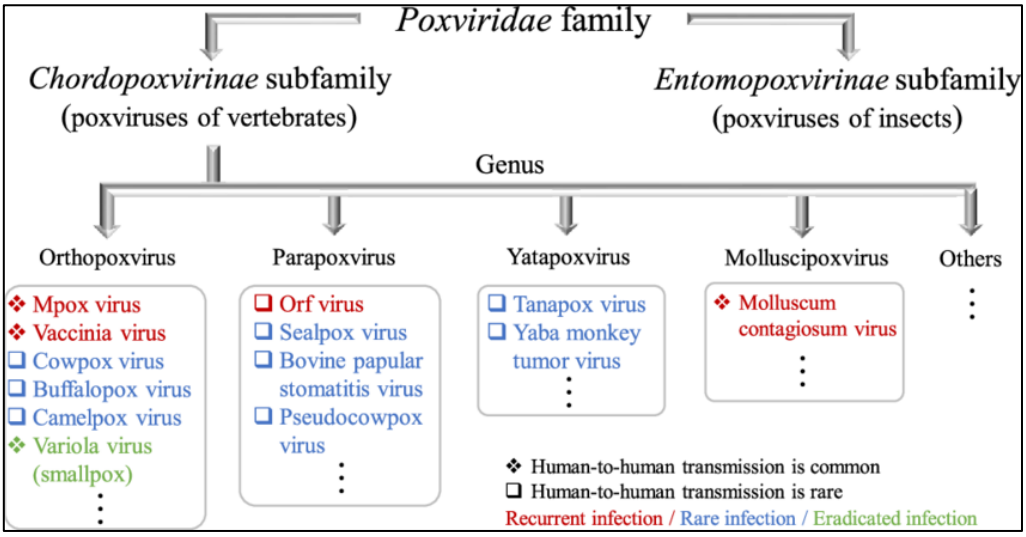


FIGURE 17: POXVIRIDAE FAMILY[143]

4.2.3 Structure and genomic organization

MPXV consists of almost 197,000 base pairs (bp) and has hairpin terms, as well as >190 ORFs, i.e. open reading frames[144]. The virus genome shows a conserved central coding region and is flanked by different ends, including inverted terminal repeats[144]. The morphogenesis and replication of poxviruses require a minimum of 90 ORFs, but numerous other open-reading frames have been identified whose functional role is currently not fully

understood[145]. It appears likely that these ORFs play a key role in variations in host tropism, pathogenesis and immunomodulation of poxviruses[146].

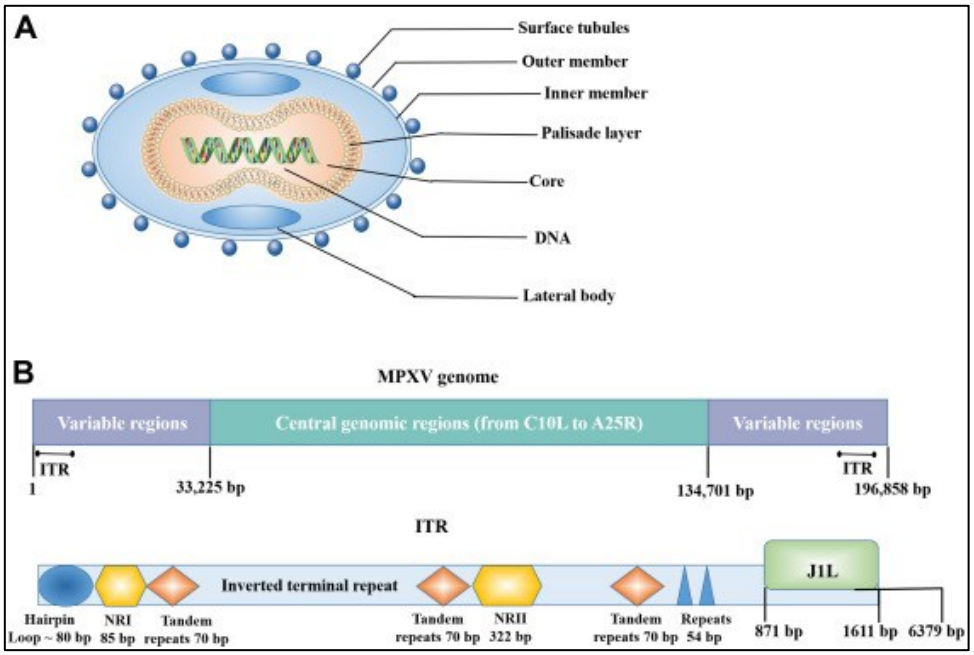


FIGURE 18 A MPXV STRUCTURE; B MPXV GENOME ORGANITATION[147]

MPXV virions are between 280 nm and 220 nm in size and can take on a barrel or oval shape[148]. The nucleocapsid, in mature poxvirus nanoparticles, has a typical dumbbell shape where the double-stranded DNA sequence is contained[24]. Like MPXV, virions contain a DNA-dependent RNA polymerase with related transcriptional enzymes and more than 30 structural and membrane viral proteins[149]. MPXV present two infectious forms of virus called intracellular mature virus (IMV) and extracellular enveloped virus (EEV) that are structurally and antigenically different[150]. IMVs are structurally distinct from EEVs in that they do not have an additional outer membrane.

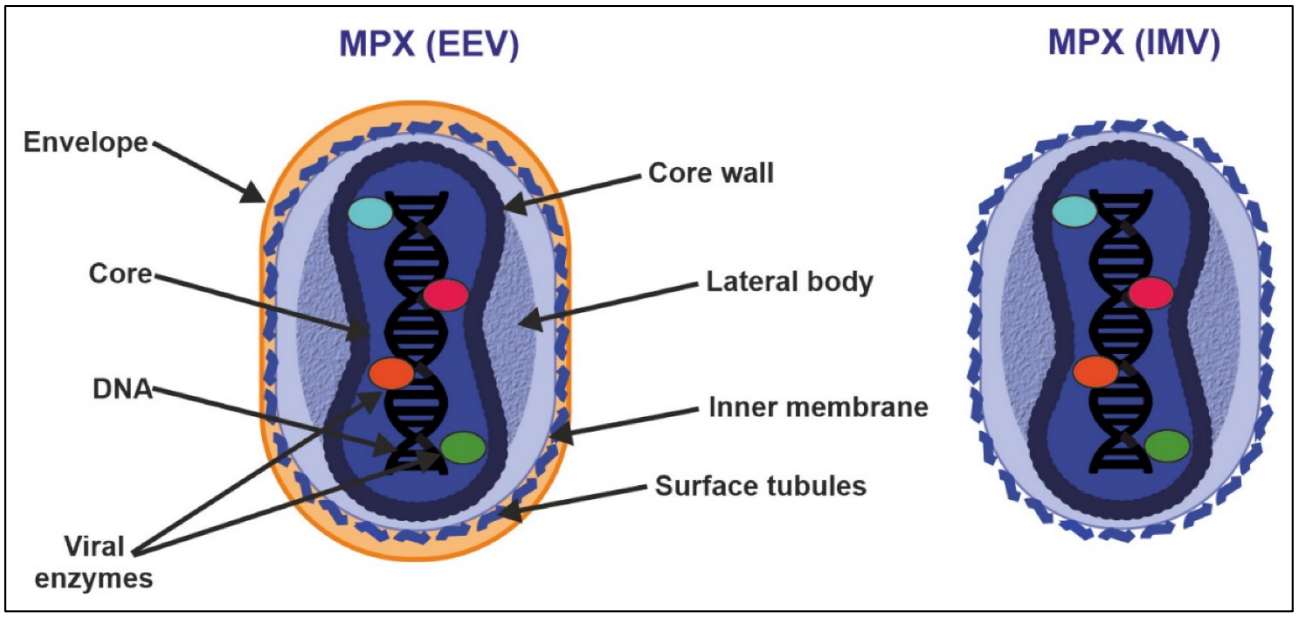


FIGURE 19 MPX VIRIONS INTRA AND EXTRA CELLULAR STRUCTURE[151]

However, the two forms of virions have different amounts of integrated viral proteins. The mechanisms by which IMV and EEV enter the cell are poorly understood[152].

4.2.4 Lifecycle of Monkeypox Virus

Poxviruses, including monkeypox, exploit modulator proteins to hide from the body's immune system and promote viral replication[153]. The replication process of these viruses is peculiar and takes place in several stages, beginning with the attachment to the host cell and culminating with the release of the virus[154] The process of fusion with the host cell membrane depends on several transmembrane proteins, and the stability of the IMV or EEV plays an important role in transmission between host animals and dissemination within the host[155].

MPXV has been shown to infect a large variety of mammalian cell lines in vitro[141,156]. Other poxviruses bind to target cells via laminin, heparin-sulphate and chondroitin sulphate, all common glycosaminoglycans[157]. Thus, it is likely that glycosaminoglycans and other extracellular matrix proteins, on the surface of the target cell, mediate MPX virion binding. Entry into human host cells occurs via the endosomal pathway at lower pH or through immediate fusion with the plasma membrane at neutral pH levels. Non-glycosylated viral

membrane protein complexes are required for fusion of IMVs, as well as EEVs, with the cell, after which the viral core is released into the cytoplasm[158].

Early and late proteins are translated by the host ribosomes after viral transcription is performed by the DNA-dependent RNA polymerase encoded by the virus as a multi-subunit [159]. The structures in the cytoplasm that synthesize viral DNA are called 'factories' and undergo a progressive transformation. From dense DNA-containing complexes enclosed by the extracellular environment, to crescent-shaped complexes in which virion assembly takes place[160]. While most mature virions remain inside the cell (IMV), some are transferred from the microtubules and acquire a double membrane from the endoplasmic reticulum. These double-membrane virions can either leave the cell by fusing with the cytoplasmic membrane, producing VMEs[160] , or induce actin polymerization, which pushes particles on an actin tail towards a neighboring cell[141].

4.2.5 Pathogenesis

Monkeypox is a zoonotic disease for which the animal reservoir is yet unknown[161]. The main route of transmission is by direct contact with diseased parts or body fluids from infected animals (i.e. squirrels, rodents, monkey, and sooty mangabey)[162].

On the other hand, human-to-human transmission of this virus is unusually frequent and happens mainly with close contact, this is demonstrated by the inefficient spread of MPXV in new countries via travel related infected individuals[163]. As a proof of this, the large number of cases in the outbreak of 2022 did not recently travel to the endemic regions of Africa, but the major part where men having sex with men[164]. This suggested to consider sexuality as another form of close contact and it was proven by the detection of viral particles in semen samples[165]. Nevertheless, further research is needed to determine whether these cases were determined by the close contact required in the “sexual behaviour” or by the sexual transmission instead.

Contact with respiratory secretions, skin lesions, genitals or bedding/clothing from infected individuals are also comprehended in the “close contact”. Even if there is a very limited data on infection during pregnancy, a study has demonstrated vertical transmission of MPXV[166].

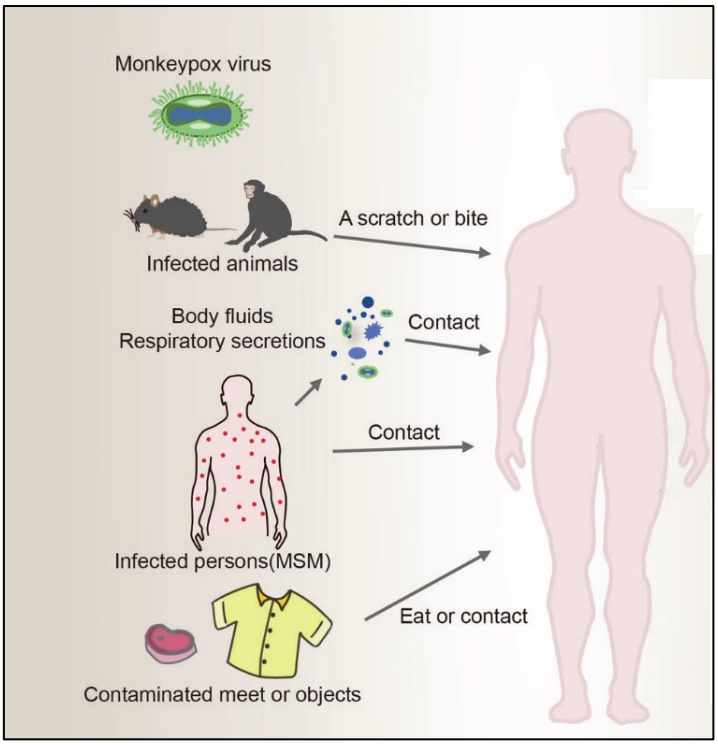


FIGURE 20 MPXV TRANSMISSION[126]

MPXV infection outcomes are usually similar to smallpox but with milder symptoms, that usually last from 2 to 4 weeks[167]. The disease caused by the infection of the virus is usually self-limiting and, in humans, can be divided into two phases: the pandrome and the rash[168,169]. Incubation time ranges from 5 to 21 days before clinical symptoms start to show[170]. During the pandrome phase, symptoms like headache, lack of energy, fever, chills and/or sweats, sore throat, muscle ache start to appear and 90% of infected patients suffer from lymphadenopathy[171]. Commonly, few days after lymph node enlargement, plaques start to appear, first in the face and then spreading in all parts of the body[132,148,172]. Rash phase conclusion takes about 2-4 weeks and it resolves by the substitution of plaques with papules, blisters, pustules, scabs and, finally, shedding[93]. For these clinical characteristics, monkeypox infection is usually confused with chickenpox, also because pandrome phase does not occur in all patients; for this reason, clinicians must remain vigilant to carry out the right diagnosis.

Shared trait between smallpox and monkeypox infection is a higher lethality in children than adults[173]. The fatality rates depends on patient's age, route of infection and clade of the virus (higher with CA clade rather than WA clade)[129,167].

Previous studies reported that the case fatality rate of monkeypox ranges from 1 to 11%[174].

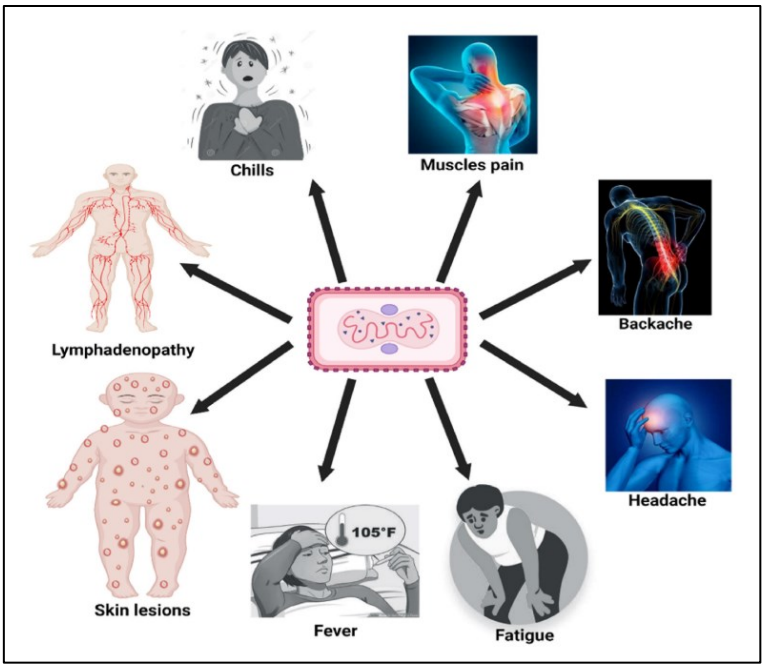


FIGURE 21 DISEASE CAUSED BY THE INFECTION OF MPXV[175]

4.2.6 Diagnosis

MPXV diagnosis is usually carried out by the correlation of clinical symptoms, epidemiological information and laboratory tests[126]. The latter, represent the "confirmed diagnosis", useful to rule out other similar disease like smallpox or chickenpox[176].

Laboratory tests can be divided into three categories: DNA assays, serological tests and electron microscopy observations. In order to conduct DNA assays, lesion exudate or crust must be collected on a swab, which will be analysed by real-time PCR (rt-PCR)[177] or recombinase polymerase amplification (RPA)[178]. These tests have different target sites on the genome and vary for sensitivity and limits of detection[179]. Due to the high sensitivity, high throughput and fast results, rt-PCR is the preferred method for WHO to diagnose MPXV during acute infection (Diagnostic testing for the monkeypox virus (MPXV): interim guidance, 9 November 2023).

Serological testing can be conducted performing a Western Blot (WB) assay, using MPXV proteins, immunohistochemistry (IHC) or by Enzyme Linked Immunosorbent Assay (ELISA) testing. The latter is the most frequently used due to its capacity to detect specific IgM and IgG antibodies. IgM antibodies usually appear and peak after 2 weeks from the rash phase and decline/disappear within 1 year; while IgG antibodies take more time to show up (~ 6 weeks) but then last for decades[180]. Drawback to take into consideration for this assay is the low specificity, due to the possible cross-reactivity with other orthopoxviruses[181,182].

Lastly, electron microscopy observations are considered as auxiliary methods due to the laborious and high-cost sample preparation[183,184].

4.2.7 Treatment and prevention

MPXV disease is usually self-limiting and induces mild symptoms, but for high-risk patients or individuals with severe disease, treatment is necessary. There is no specific therapy for monkeypox, but it is possible to take advantage of its genetic similarity with smallpox to use the antiviral drugs developed for the last virus[185].

Examples of antiviral medications that can be used to treat infected patients are:

- Cidofovir (Vistide): its role is to inhibit the activity of the DNA polymerase. Since there is a lack of clinical evidence and it can cause nephrotoxicity, its use is suggested only for severely ill patients[176]. One modified version of this drug is the CMX-001, lacks the side effect of the previous “version” and can be effective to treat various orthopoxvirus infections[186].
- Tecovirimat (ST-246): can block the release of intracellular virus from the cell. This is a promising antiviral for all orthopoxviruses and can be used during monkeypox outbreaks in paediatric and adult patients[187]. This drug targets the VP37 protein and prevents the viral particles to leave the infected cell; this is not achieved by inhibiting DNA/protein synthesis or the formation of mature virus, but simply forcing it to stay in the host cell until lysis occurs[188].
- Vaccinia Immune Globulin Intravenous (VIGIV) is used to treat side effects and complications derived from vaccinia vaccination and can be used during monkeypox outbreaks FDA. Vaccinia IMMUNE GLOBULIN INTRAVENOUS (HUMan)[189].
- Brincidofovir (Tembexa) is approved by the FDA to treat smallpox infections and is now under investigation for its use in monkeypox outbreaks[190].

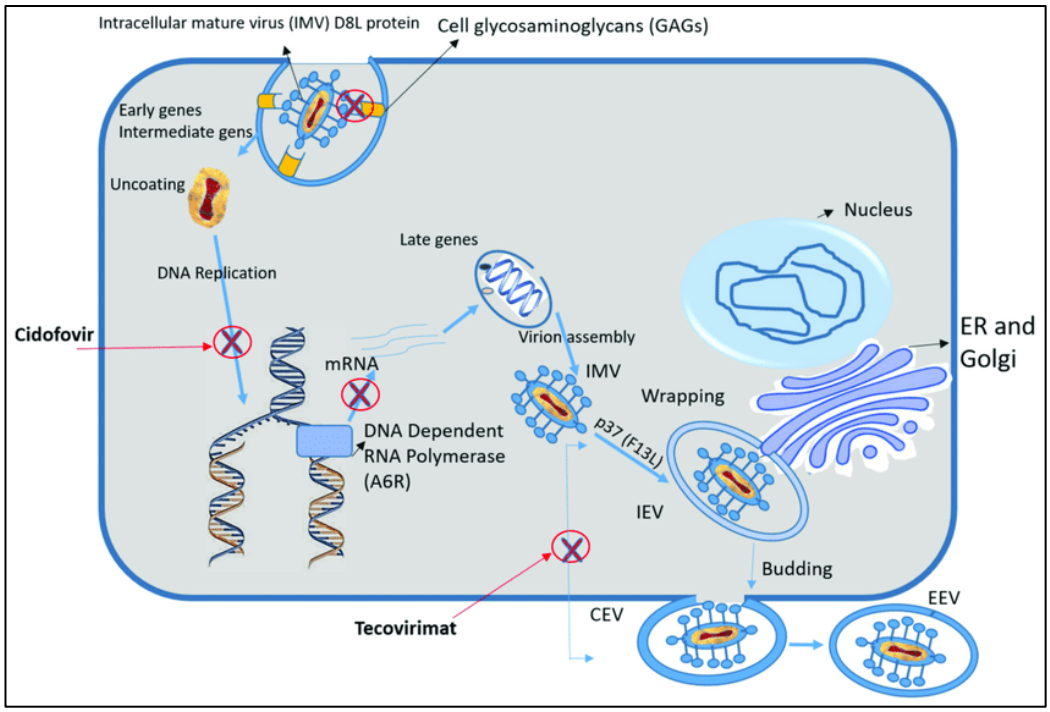


FIGURE 22 HOW ANTIVIRAL AGAINST MPOX WORKS[191]"

Even if there are multiple different treatment to fight monkeypox disease, the main strategy to avoid spreading of the virus is to prevent the infection. This can be achieved by applying better public health behaviours (cough and hand hygiene), isolating and euthanising suspected animal reservoir of the virus, isolating infected patients and suppling proper personal protective equipment (PPE) to front line workers[175].

As for other virus-depending disease, vaccines should be the main answer to prevent infection and spreading. Taking advantage of the genetic similarities between monkeypox and smallpox, the JYNNEOS vaccine is expected to provide protection when administered maximum 4 days after exposure to the virus[192]. This Modified Vaccinia virus Ankara (MVA) is a live vaccinia third-generation vaccine approved for smallpox in 2019[193], its use to prevent monkeypox disease can be useful for high-risk people but is not recommended for the general public but only[194]. This vaccine has been approved for prevention of monkeypox in Canada and the United States of America; while in the European Union, it can be used only under exceptional circumstances[195].

5. Aim of the study

Sars-CoV-2 was responsible for one of the largest pandemics recorded in recent centuries, seriously threatening public health, and causing millions of deaths worldwide. The emergence of the virus and the resulting COVID-19 syndrome affected many of the world's most important countries completely unprepared to deal with the pandemic; since initially there were not effective guidelines to control the spread of the infection, no therapeutic strategies and no vaccines.

Just as the COVID-19 crisis was beginning to subside, the emergence of a new human-borne virus such as Monkeypox forced many nations around the world to deal with numerous epidemic outbreaks.

Unlike in the past, the united global scientific community, supported by prior scientific knowledge and a high degree of technological advancement, was able to provide the most rapid and effective response possible in this situation. Worldwide studies and clinical trials have been undertaken that, with enormous economic efforts, have made it possible to develop new therapeutic strategies and safe and effective vaccines against the new viruses in unprecedented timescales, leading to the resolution of this enormous worldwide crisis.

All this has further highlighted the need for serological tests and assays capable of assessing the efficacy of new vaccines formulations, which remain the best weapon against the occurrence of new viral epidemics. Among the various serological tests, one of the most relevant is CPE-based Microneutralization assay (MN-CPE).

The aim of this thesis is to verify the reliability the serological test in object and its flexibility to study different pathogens. In order to do this, this project was divided into two tasks:

- Task 1: MN-CPE based was used to evaluate the efficacy of the BioNTech/Pfizer vaccine against Sars-CoV-2 Wild type and Delta VOC and to follow up the neutralization efficiency after 180 days in subjects that received two shots of vaccine.

- Task 2: MN-CPE based was customized to perform on MPVX convalescent and VACV vaccinated subjects, to discover a potential role of complement, with and without the addition of an external source of Baby Rabbit Complement.

6. Materials and methods

The MN-CPE (MicroNeutralization based on Cytopathic effect) method is a highly sensitive technique that can be used for the detection and quantitation of virus-specific neutralizing antibodies (nAbs) in human and animal samples (either plasma or serum), or to study the potency of monoclonal antibodies (mAbs).

6.1 Materials

Maintenance Medium

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplied with 10% (v/v) heat-inactivated FBS, 1% (v/v), L-glutamine and 1% (v/v) penicillin/streptomycin.

MN Medium

Medium to be used for the test is composed of Dulbecco's Modified Eagle Medium (DMEM) with 2% (v/v) of heat-inactivated Foetal Bovine Serum (FBS), 1% (v/v) of L-glutamine and 1% (v/v) of penicillin/streptomycin.

Viral working solution

The standard concentration of the virus to be used is usually 500 TCID50/ml.

The volume of the virus stock used to prepare the viral working solution must be ≥ 100 µl. If the volume used is lower the virus stock will be pre-diluited 1:10, 1:100 etc.

6.2 Operative method

Titration and back titration procedure

To perform Microneutralization assay, the virus titre (TCID50) must be calculated to apply the correct virus dilution. For each session performed, the titre obtained was checked by performing a back titration.

The virus titration is performed as described below:

1. Add 180 µl of MN Medium to each well from column 1 to 12.
2. Seed 20 µl of virus stock solution in column 1.
3. Perform a ten-fold serial dilution (Log_{10}) by transferring 20 µl from column 1 to column 2, up to column 11.
4. Remove the medium from the 96-wells plates containing confluent VERO E6 cells monolayer by using a vacuum suction system.
5. Transfer 100 µl from the dilution plate to the plate containing cells
6. Plates are incubated at 37°C, 5% CO2 for 3 days
7. After incubation, observe plate under an inverted microscope and score wells as positive for SARS-CoV-2 (i.e., CPE) or negative for SARS-CoV-2 (i.e., cells are alive and without CPE).

The back titration is performed as follows:

1. In a dilution plate add 200 µl of MN Medium to each well from column 2 to 12.
2. Add 292 µl of working viral working solution to column 1.
3. Perform a 16-fold serial dilution (0.5 Log_{10}) by transferring 92 µl from column 1 to column 2, and up to column 11.
4. Follow the steps 4-7 from the viral titration assay reported above.

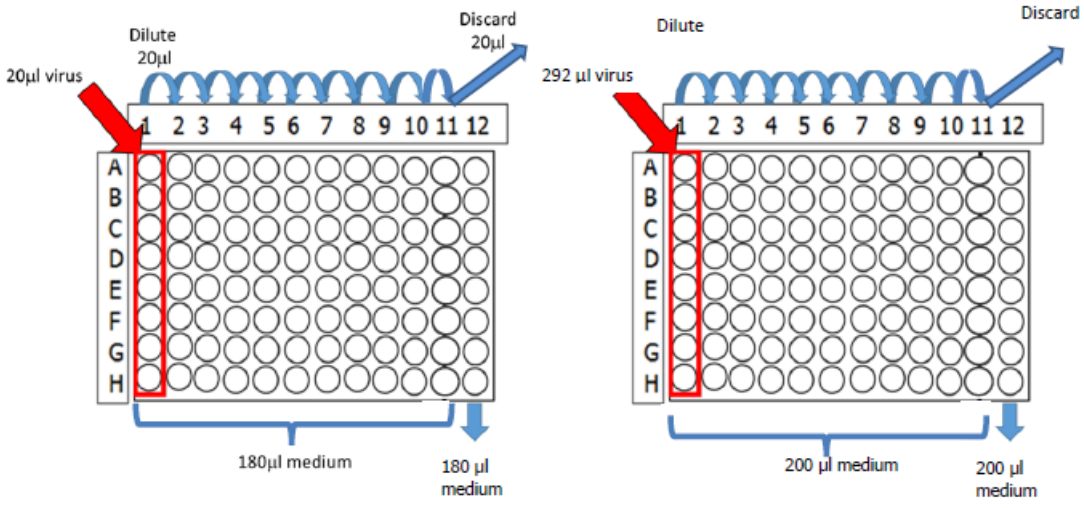


FIGURE 23:PLATE LAYOUT OF DILUTION PLATES FOR TITRATION AND BACK TITRATION OF THE VIRUS

To obtain the dilution factor, the virus stock titre is divided for the titre that the viral working solution must have 500 TCID50/ml or $10^{2.7}$ (i.e. 10^5 / $10^{3.3}$ = 5-3.3 = 1.7, so $10^{1.7}$ is the dilution factor, or 50.11).

Microneutralization Assay procedure

Before starting the test, all the samples and controls were heat inactivated for 30 minutes ± 10 minutes at 56°C ± 1°C.

Samples were tested in duplicate in two different flat-bottomed 96-well microtiter plates (Test 1A and Test 1B).

1. Fill all the wells of a dilution plate except column 1 and column 11 with 60µl of MN medium.
2. Fill with 108µl of MN medium column 1
3. Add to column 1 120µl of MN medium, this will correspond to the cell control (CC)
4. Seed 12µl of heat inactivated serum sample into each well of column 1 in order to achieve a 1:10 dilution.
5. 2-fold serial dilutions are performed by transferring 60µl progressively from column 1 to 10 and 60µl after the last dilution step are discarded.
6. 60µl of viral working solution are added to each well of the plate excepted for column 11, first point of dilution will now become 1:20.
7. Incubate 1 hour at 37 ± 1 °C and 5 ± 1% CO_2.
8. At the end of the incubation time, 100µl of virus-serum mixture is added to the 96-wells microtiter plate containing a healthy and sub confluent-to confluent cell lawn.
9. Incubate at 37 ± 1 °C and 5 ± 1% CO_2 for 3 days.
10. After incubation, observe the plate under an inverted microscope and score wells as “protected” for and “unprotected” wells, respectively without and with CPE effect.
11. Evaluate the Microneutralization titre (MNt) of each sample by using the reciprocal of the highest dilution that protects the cell monolayer form CPE. When no neutralization capacity is observed from the first well, the MNt corresponds to the reciprocal of the first dilution.

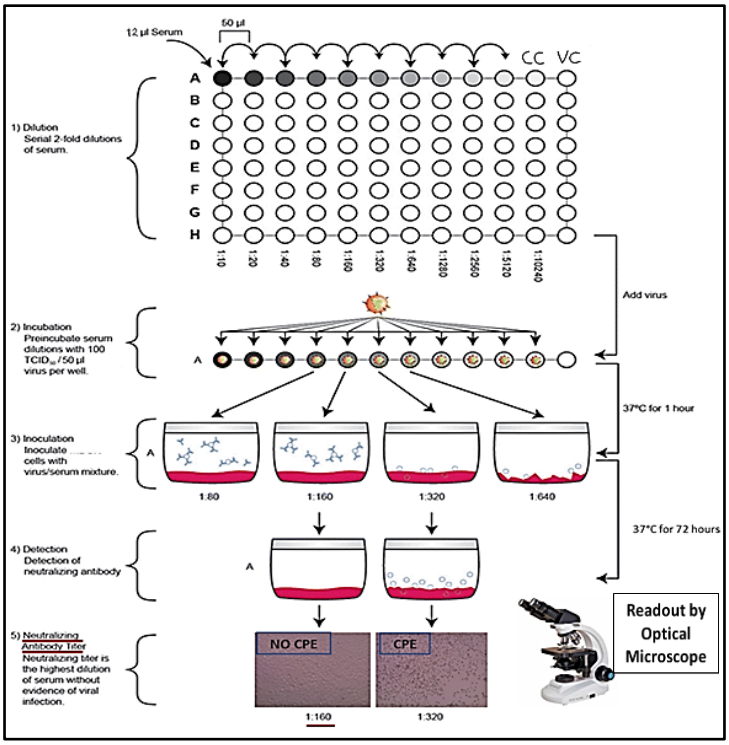


FIGURE 24: A SCHEME OF THE MN-CPE ASSAY PROCEDURE

The validity of the test is confirmed when:

- At least 7 wells of the cell control (CC) show a healthy cell monolayer and no evidence of 'CPE'.
- At least 7 wells of the virus control (VC) show a cytopathic effect in the cell monolayer.
- The back titration lies within the defined target range of 102.20 -103.20 TCID50/ml or according to viral load used in the test
- The single neutralization titre of serum sample from a duplicate determination lies within a range of ± 1 titre step.

6.3 Task 1 – Sars-CoV-2 vaccine efficiency study

It has been showed that immunity and clinical protection induced by mRNA vaccines against SARS-CoV-2 tend to decline overtime. To investigate this aspect, 392 HWs that were vaccinated with BioNTech/Pfizer vaccine starting from February 11th 2020, and ending on April 11th 2021 were enrolled to this study. The aim was to evaluate their IgG levels against S1 portion and the whole spike protein (EUROIMMUN, anti-SARS-CoV-2 QuantiVac enzyme-linked immunosorbent assay), the interferon-gamma (IFN-Y) secretion

(EUROIMMUN SARS-CoV-2 IGRA stimulation tube set, EUROIMMUN IFN-Ɣ ELISA) and the neutralizing antibodies.
The analysis was perfomed on the medical data gained from the multicentre longitudinal study (Covidiagnostix, funded by the Italian Ministry of Health).
All the subjects received two vaccine injections 21 days apart. The planned testing time for binding antibodies was day 0 (d0) (before the first dose), day 7 (d7), day 21 (d21), day 31 (d31) after the first shot, and day 90 (d90) 60 days after the second shot, day 180 (d180) after the second shot corresponding to 210 days after the first shot.

The micro-neutralization (MN) assay was performed following the procedure previously reported in paragraph 6.2
Briefly, serial 2-fold dilution of human serum samples, starting from 1:10 to 1: 5120, were incubated with an equal volume of SARS-CoV-2 (Wuhan Strain and Delta VOC) viral solution containing 25 tissue culture infective dose 50% (TCID50).
The plates were incubated for 3 days (Wuhan strain) and 4 days (Delta strain) at 37ºC and 5% CO2. At the end of incubation, the presence/absence of cytopathic effect (CPE) was evaluated. A CPE higher than 50% was indicative of infection.
The titer of 10 was considered as the lower limit of quantitation (LLOQ) and a titer equal to 5 was considered as negative.
All experiments with live SARS-CoV-2 viruses were performed inside the Biosecurity Level 3 laboratories of VisMederi Srl. Standardization and harmonisation of the obtained neutralizing titers were performed by using the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human) (NIBSC code: 20/136).

6.4 Task 2 – MN-CPE development against MPXV

For this study, a total of 50 human serum samples from general population (named population samples) were analysed and grouped by age. It was assumed that subjects born in or before 1975 had been routinely vaccinated against smallpox according to the Italian immunization schedule, while subjects born in 1979 or later didn't undergo this vaccination. These samples were anonymously collected in 2022 in the Apulia region (Southern Italy) as residual samples for unknown diagnostic purposes and stored at the University of Siena, Italy, in compliance with Italian ethics laws. To identify them, the age of individual was used.
Additionally, two convalescent human samples for MPXV were provided by the Department of Infectious Diseases (San Donato Hospital, Arezzo, Italy) 1 month after the onset of symptoms. These samples are identified ConvA 1 and ConvA 2.
Anti-monkeypox antibodies serum (NIBSC Code: 22/218) was used as positive sample , while NIBSC 2 is a negative human sample. These samples are identified in this study as NIBSC 1 and NIBSC 2, respectively.
As an additional negative control, a commercial human serum sample provided by Merck catalog N°S1-M, defined as "normal", was used.

The MN assay was performed as previously reported in paragraph 6.2, with minor modifications.
Two-fold serial dilutions, from 1:8 up to 1:4096, were mixed with an equal volume of MPXV and VACV viral solutions containing 25 Tissue Culture Infectious Dose 50% (TCID50) and incubated follow the original protocol.
After the incubation period, 100µL of the serum-mixture was transferred to a Vero E6 cell-seeded plate. Plates were incubated for 5 days (MPXV) or for 4 days (VACV) at 37°C in a humidified atmosphere containing 5% CO_2, then inspected by means of an inverted optical microscope to evaluate the presence/absence of CPE at each dilution point.

The same assay method was performed by adding Baby rabbit complement (BRC) or Guinea pig complement (GPC) 5% (v/v) to the virus solution, for a final concentration of 2.5% after the addition of the diluted sample.

7. Results and discussion

7.1 Task 1 - Results

Of 392 enrolled subjects, 352 were analyzed, as 40 (10.2%) had to be excluded because they did not complete the planned sample collection. 271 subjects had no experience of the previous infection and were defined as *naive*. Subjects infected before or immediately after the first vaccine dose (n = 81) were classified as experienced. The statistical analysis were performed by Dr Allessandra Mangia.

Prior COVID-19 experience		
	Yes (n=81)	***No (n=271)***
Age,mean (SD),years	**49,71 (12,32)**	**47,55 (11,85)**
Median (IQR)	**51 (40,75-59,25)**	**47 (39,0 - 57,0)**
Male	**38 (46,9)**	**113 (41,7)**
Female	**43 (53,1)**	**158 (58,3)**
Baseline SARS-CoV-2 IgG No(%)	**79 (97,31)**	**0**
Day 180 SARS-CoV-2 IgG No(%)	**81 (100)**	**271 (100)**
Day 180 SARS-CoV-2 IgG level Mean	**418,81 ± 415,01**	**212,93 ± 182,98**
Day 180 SARS-CoV-2 IgG BAU/mL Median	**248,96 (140,48-610,0)**	**179,79 (90,0-287,19)**
Day 180 SARS-CoV-2 IgG >384 BAU/mL Mean	**778,04 ± 40,15**	**630,50 ± 361,46**
Day 180 SARS-CoV-2 IgG >384 BAU/mL Median	**630,41 (584,32-895,72)**	**489,93 (398,31-666,08)**
Day 180 Neut. Ab >10 No(%)	**81 (100)**	**178 (65,89)**
Day 180 Neut. Ab >10 Mean	**419,08 ± 430,75**	**229,27 ± 213,92**
Day 180 Neut. Ab >10 Median	**231,52 (138,46-612,16)**	**200 (90,0-310,72)**
Day 180 Neut. Ab >320 Mean	**740,24 ± 588,37**	**246,09 ± 65,17**
Day 180 Neut. Ab >320 Median	**663,36 (209,04 - 921,54)**	**246,09 (200,0 - 292,17)**
Day 180 IFN-ϒ >100 mLU/mL No(%)	**81 (100)**	**267 (98,52)**
Day 180 IFN-ϒ >200 mLU/mL No(%)	**81 (100)**	**254 (93,72)**
Day 180 IFN-ϒ >100 mLU/mL Mean	**2299,97 ± 491,25**	**1201,24 ± 846,24**
Day 180 IFN-ϒ >100 mLU/mL Median	**2499,0 (2400,0-2500,0)**	**926,0 (463,0-2272,0)**

FIGURE 25: BASELINE CHARACTERISTICS, ANTIBODY LEVELS, NEUTRALIZING ANTIBODY TITERS, AND IFN-ϒ CONCENTRATION OF VACCINATED SUBJECTS

The mean values of IgG antibodies were 212.93 ± 182.98 BAU/ml. None had results below the 35.2 BAU/ml positivity assay threshold. Overall, 22 individuals (8.1%) had antibody

values above the highest threshold. Their mean values were 630.50 ± 361.46 BAU/ml. No difference was observed between genders.

Among 81 experienced, the female was 53.1%. The mean age was 49.71 ± 12.32. At d180 after the second dose (210 days after the first vaccination), the mean values were 418.81 BAU/ml ± 415.01. None had results below the assay's threshold. Overall, 41.03% had results above the 384.0 BAU/ml. Their mean values were 778.04 ± 40.15 BAU/ml. Values for men and women were not different regardless of the threshold used.

When the neutralizing titres were analysed, 100% of previously infected patients and 178 (65.89%) of naive showed a titre of ≥10 (LLOQ). Individuals associated with stronger neutralizing capacity (titre > 320) were 2 (0.73%) among naive and 25 (31.2%) among experienced. Median neutralizing titre of 200 (90.0–310.72) was observed among 271 naive. The corresponding value among experienced was 231.52 (138.46–612.16) (Figure 26).

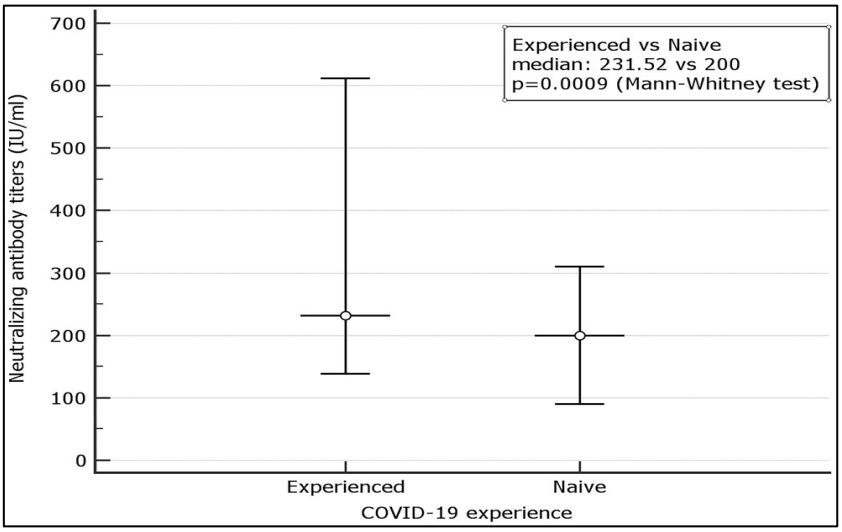


FIGURE 26: COMPARISON BETWEEN MICRONEUTRALIZATION RESULTS IN NAÏVE AND EXPERIENCED

When only subjects with strong neutralizing titres (>320) were analysed, the median titres were 246.09 (200.0–292.17) for naive and 663.36 (209.04–921.54) for experienced.

Following the predictive model of protection suggested by Khoury et al.[196] and using the standard IU/ml results suggested by WHO as a reference to normalize the different neutralizing testing (https://www.nibsc.org/documents/ifu/20-136.pdf), we transformed the neutralizing titers in IU/ml (SerumTitre(IU/mL)=[(10(serumlogD50value))×theoretical titer of Positive Ref serum0.5IU/mL](10(theoretical logD50of Pos Ref serum)) and used a 54 IU/ml threshold to identify subjects with 50%protective humoral immunity. Overall,32.78% of naive

and 91.89% of previously infected showed protective neutralizing activity. No correlation was observed between neutralizing antibody titers and IgG levels for naive (r= 0.06; p = 0.321), at d180.

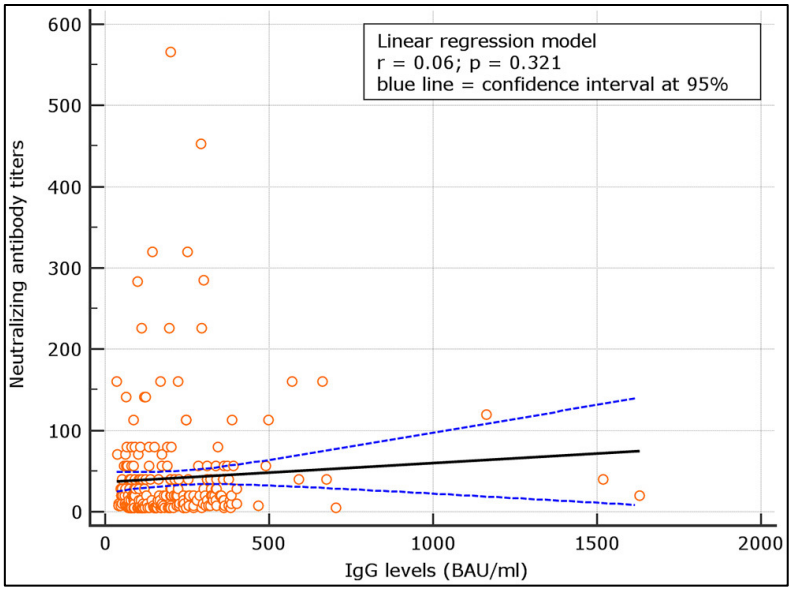


FIGURE 27: CORRELATION BETWEEN NEUTRALIZING ANTIBODY TITERS AND IGG LEVELS AMONG NAÏVE

At variance, for experienced, the correlation was significant (p = 0.48; p < 0.001). Despite the analysis of neutralizing antibody, IU/ml ≥ 54 conversions, we failed to observe correlation with binding antibody.

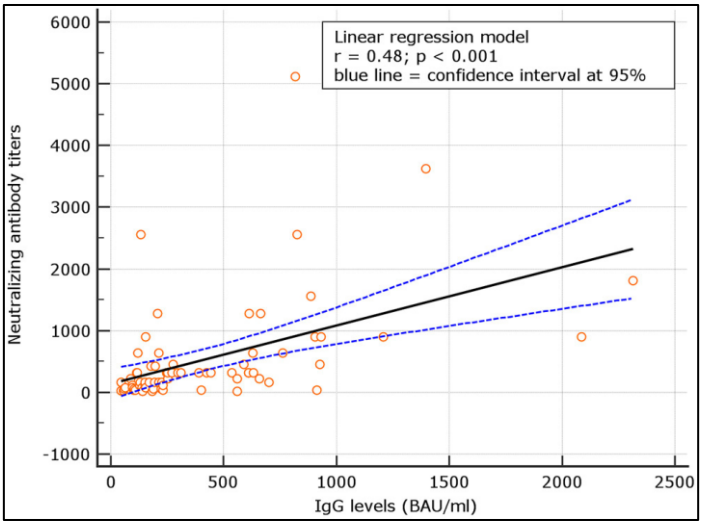


FIGURE 28: CORRELATION BETWEEN NEUTRALIZING ANTIBODY TITERS AND IGG LEVELS AMONG EXPERIENCED

An interesting correlation between neutralizing titers and IGRA levels was found for both naive and experienced. The results showed r = 0.26; p = 0.001 for naive and r = 0.18 p = 0.134, respectively.

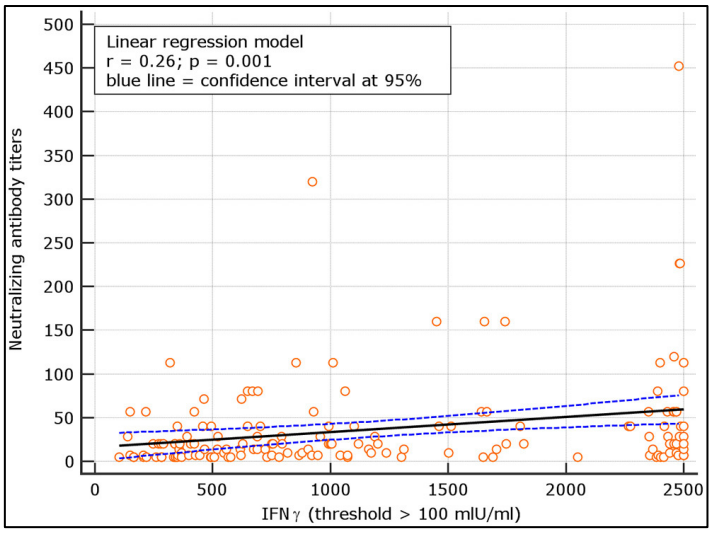


FIGURE 29: LINEAR REGRESSION MODEL BETWEEN NEUTRALIZING ANTIBODY TITERS AND IFN-Ɣ CONCENTRATION IN NAÏVE WITH IFN-Ɣ THRESHOLD > 100 MIU/ML

The significance of the correlation increased for naive when the IFN-positive cut-off of 200 was used (r = 0.25; p = 0.003).

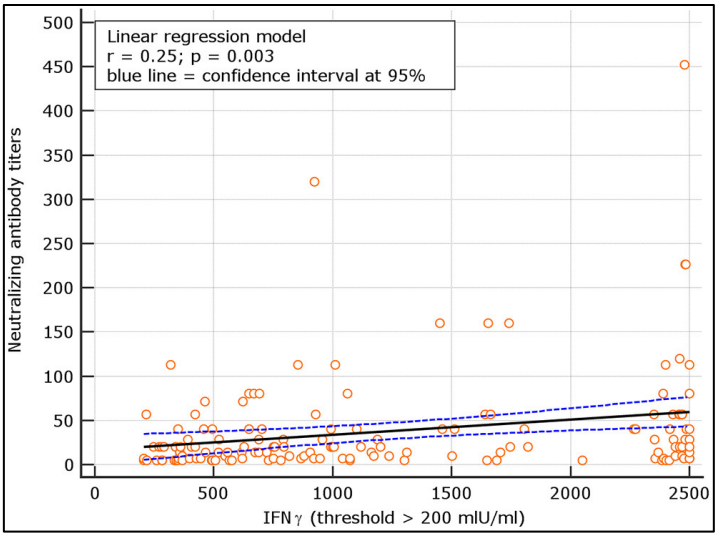


FIGURE 30: LINEAR REGRESSION MODEL BETWEEN NEUTRALIZING ANTIBODY TITERS AND IFN-Y CONCENTRATION IN NAÏVE WITH IFN-Y THRESHOLD > 200 MIU/ML

It did not change for experience given the identical number of subjects with IFN-concentration >100 and >200 thresholds in this group.

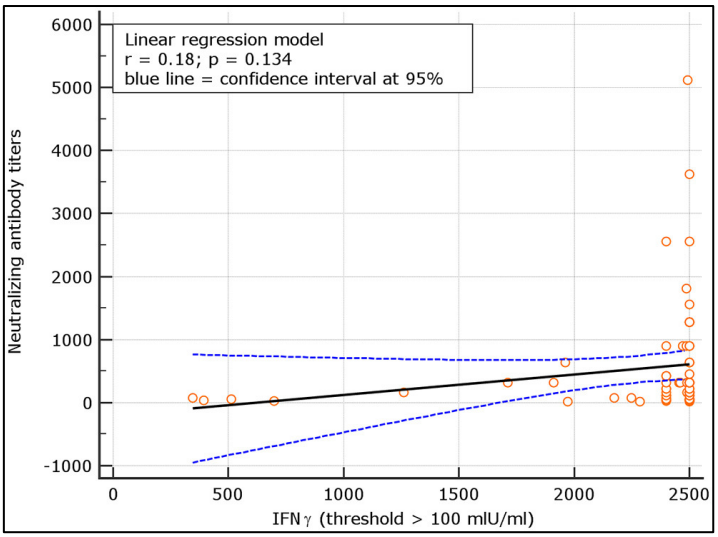


FIGURE 31: LINEAR REGRESSION MODEL FOR CORRELATION BETWEEN NEUTRALIZING ANTIBODY TITERS AND IFN-Ɣ CONCENTRATION IN EXPERIENCE THRESHOLD OF 100 AND 200 MIU/ML

During the study breakthrough infections were observed in only 6 cases among naïve fully vaccinated subjects (2.2%). In all the cases, the infection was mild, none of the subjects required hospitalization. For 4 out of 6, a common unvaccinated index case was identified. The remaining two cases came from the same household, where one of the individuals, a healthcare worker, was exposed and subsequently infected the second individual within the household.

Pt initials	Gender	Age	IgG level BAU/ml	IGRA titers mIU/ml	Neutralizing antibody dilution
RF	M	35	311.14	905.1	14.1
VV	F	67	172.39	750	7.1
D'AG	M	57	105.53	420	5
VA	F	57	160	360	10
RG	F	59	200	620	20
CM	M	70	80.6	100	5

FIGURE 32: CHARACTERISTICS OF PATIENTS WITH BREAKTHROUGH INFECTION

Demographic, virologic, and immunologic characteristics of these subjects were compared with those of the remaining not infected naive subjects. Our small group of subjects with breakthrough infection showed simultaneous neutralizing antibody titers below 20, binding antibody levels below 200 BAU/ml and IFN- < 1,000. Similar results in subjects older than 58 years may be considered an alarming condition.

7.2 Task 1 - Discussion

The protection given by mRNA vaccines against Sars-CoV-2 infection resulted immediately significant for both convalescent and uninfected subjects, with a consequent reduction of severe COVID-19 cases. On the other hand, this immunity and clinical protection decreased over time, allowing the virus to infect double-dose vaccinated subjects.
Our study aimed to investigate this aspect, by collecting data to understand when the immunological picture can be a reliable indicator for preventing severe COVID-19 syndrome and subsequent hospitalization.
Our study showed that at d180, all HWs had IgG levels above the cut-off of 35.2 BAU/ml, although only 8.1% showed results above 384.0 BAU/ml. Significantly higher IgG values appeared for experienced.

When analyzing neutralizing antibodies, it was shown than 1/3 of the subjects had titers below LLoQ, while titers ≥ 320, usually associated with protection, were observed in few cases (1.2%). Converting MN-titre to international units (IU/ml), we observed that only 32.78% of our patients had 50% neutralizing antibodies.

In addition, there was an interesting correlation observed between INF-Ɣ and neutralizing antibodies after 180 days for high positive sera. This suggested that the combination of these two techniques may provide information on the specific humoral protective capabilities against Sars-CoV-2.

Finally, results observed from the analysis of symptomatic positive samples suggested that when titers of neutralizing antibodies < 20 are coupled with IgG levels < 200 BAU/ml and IFN-Ɣ levels < 1000 mIU/ml in patients older than 58 years, even the administration of the two doses of vaccine could not prevent the developing of COVID-19.

The results of this study appear to be in line with two other studies conducted in Israel on a much larger population. The first demonstrated a high efficacy of vaccines in disease prevention and in infection transmission up to 42 days after the first vaccination[197]. The second one was characterized by longer follow ups: it showed that 39 (2.6%) out of 1,497 fully vaccinated HWs were infected in the 14 weeks after their second dose of the BNT162b vaccine. All the infected subjects had lower neutralizing antibody levels than their uninfected colleagues during the peri-infection period[198].

7.3 Task 2 - Results

Plates were inspected for 6 days post-infection, to evaluate if the virus titer increased over time, in order to set read-out days of the method. No substantial increase in the TCID50 of MPXV and VACV was observed after 5 and 4 days, respectively. An example is reported on Figure 33 , where the gradual progression of CPE at 1-2 (A, D), 3 (B, E), and 4 days(C, F) post-infection is reported for both MPXV (A–C) and VACV (D–F), .

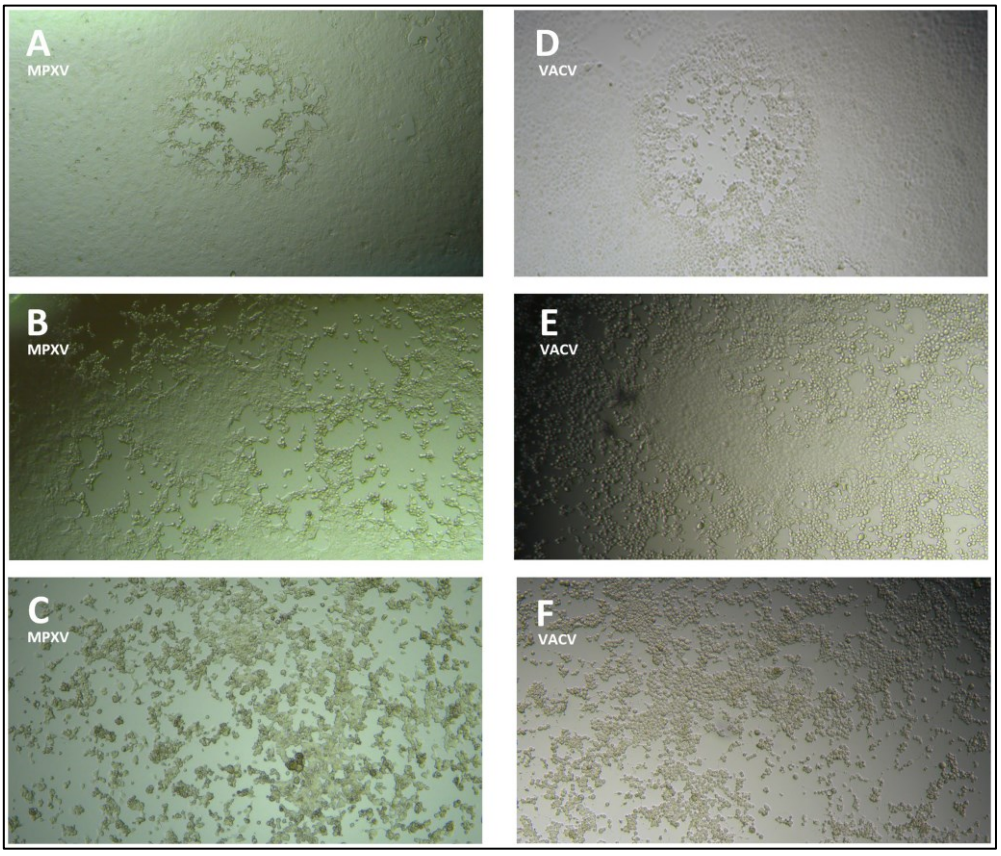


FIGURE 33: MONKEYPOX VIRUS (MPXV) AND VACCINIA VIRUS (VACV) CYTOPATHIC EFFECT (CPE) PROGRESSION ON VERO E6 CELL MONOLAYER

To select which sources of exogenous complement to use (BRC or GPC), several concentrations between 2 and 5% of these reagents were tested on 4 heat-inactivated serum samples:

- ConvA 1 and ConvA 2: expected positive
- 1 sample from a 60-year-old subject
- 1 negative control.

Along with the serum samples, virus back-titration was performed in order to determine whether the complement concentration could interfere by reducing viral infectivity.

Concentrations above 4% were discarded, since the high BRC and GPC concentrations interfered negatively with viral infectivity, reducing the titers of the back-titration below the acceptability threshold.

Figure 34 shows that 2.5 and 3% were the best concentrations for both complement sources, BRC and GPC.

Since a higher sensitivity was registered by using the BRC, it was decided to use this reagent with a final concentration of 2.5%, representative of an optimal balance between sensitivity and virus titer robustness.

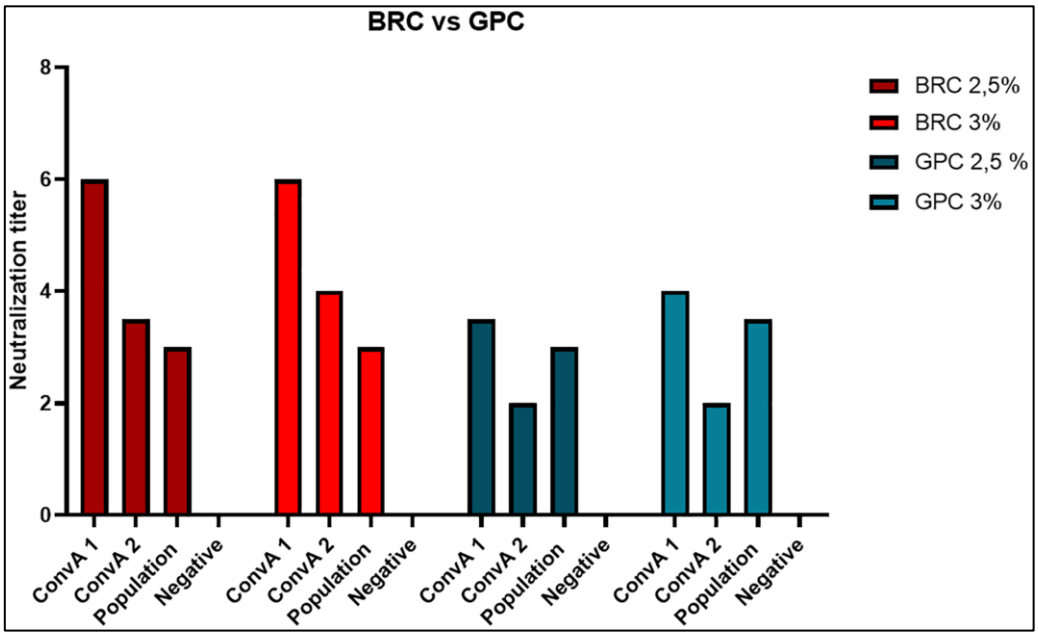


FIGURE 34: NEUTRALIZATION TITER ACHIEVED ON USING TWO DIFFERENT SOURCES OF EXOGENOUS COMPLEMENT, BABY RABBIT COMPLEMENT (BRC) AND GUINEA PIG COMPLEMENT (GPC)

All serum samples were tested with and without the external source of BRC. Samples NIBSC 1 and ConvA 1 showed detectable neutralizing titers against MPXV, with (Figures 35A, 36A, respectively) and without complement (Figure 35A for NIBSC 1, data not shown for ConvA 1).

Sample ConvA 2 showed neutralization only in the presence of 2.5% complement (Figure 36A, data without complement are not shown); as expected, no neutralization was detected in NIBSC 2 or the normal human serum sample.

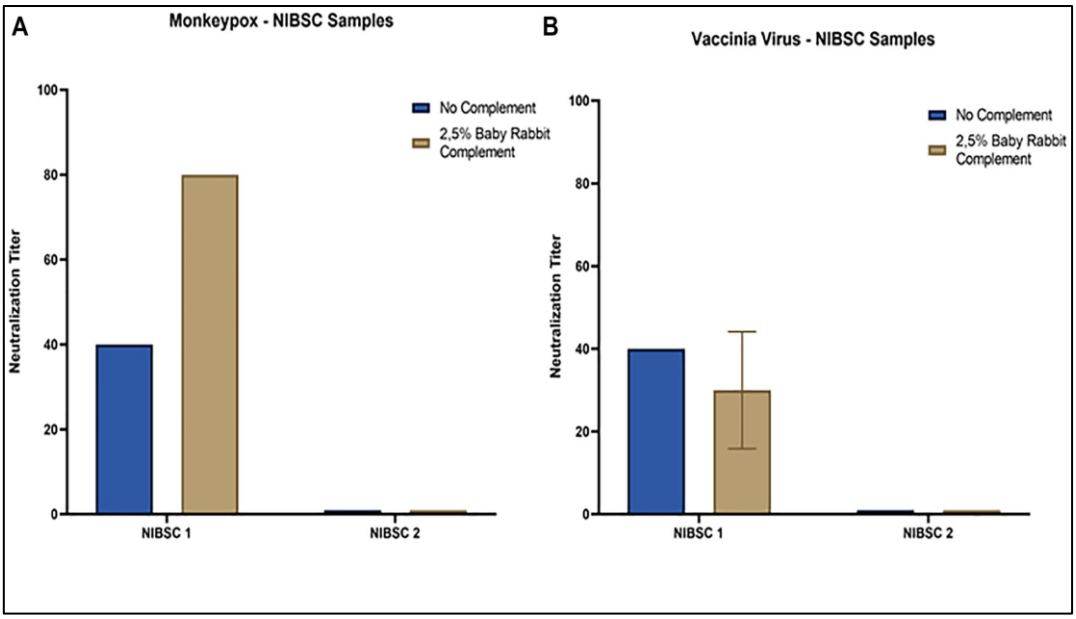


FIGURE 35: NEUTRALIZATION RESULTS FOR NIBSC 1–2 SAMPLES

Samples analysed belonged to people who were either non-vaccinated or probably vaccinated against smallpox, but with no records of the vaccine type and number of doses. All yielded negative results when tested whit the assay in object without BRC (data not shown).

However, the majority of samples from people who should have been vaccinated against smallpox according to their age, showed variable and detectable neutralizing antibodies in the presence of BRC (Figure 36A). Specifically, subjects aged 52, 62, 69, 77, 79, and 85b, the complement-based neutralization titers observed were particularly high, indicating high antibody cross-reactions.

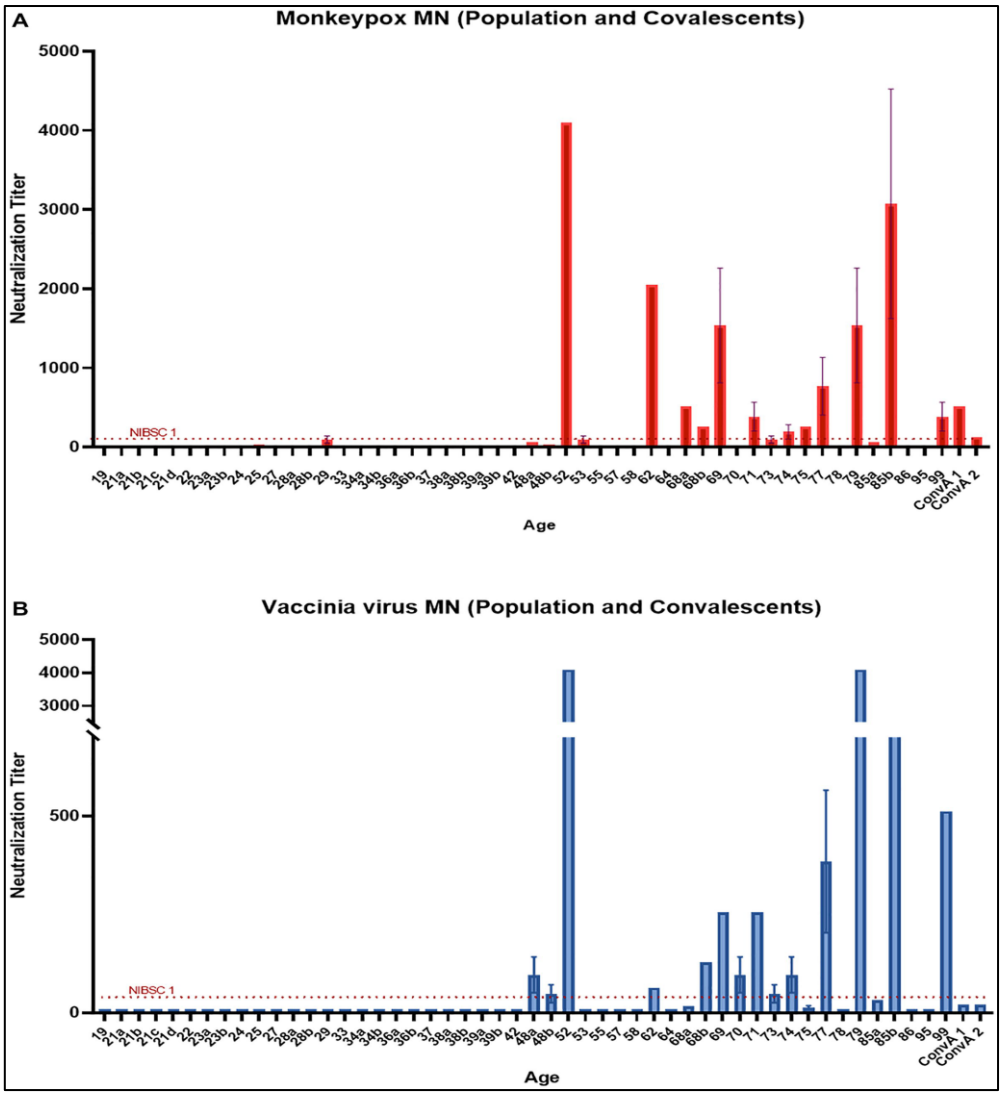


FIGURE 36: MONKEYPOX VIRUS (MPXV) AND VACCINIA VIRUS (VACV) NEUTRALIZATION RESULTS IN THE PRESENCE OF 2.5% BABY RABBIT COMPLEMENT

Samples tested against MPXV were also tested against VACV, with and without complement. Neutralisation properties were observed only with the complement-based neutralization assay variant (Figure 36B). In accordance with the MPXV MN results, high neutralization titers were measured in samples from subjects aged 52, 62, 69, 77, 79 and 85b. In addition, high MN titers were also observed in subjects aged 68b, 70 and 74. Interestingly, only samples from MPXV convalescent donors (NIBSC 1 and ConvA 1–2) (Figures 35B, 36B) proved positive on VACV MN assay without BRC, although the titers were quite low.

Overall, a good correlation was seen in the complement-based neutralization assay against MPXV and VACV.

7.4 Task 2 - Discussion

The complete eradication of smallpox declared by WHO in 1980 was one of the greatest achievements in human medical history. After this, the scientific community raised the question on whether another Orthopoxvirus closely related to human variola virus (smallpox), such as MPXV, could fill the void left by occupying the vacant ecological niche. A risk factor is the established decline in the immune protection against other zoonotic Orthopoxvirus infections, since of smallpox vaccination was stopped after 1980. These concerns found reason when in 2022 an unprecedented number of human MPXV infections occurred outside the endemic areas of Africa, forcing WHO to declare MPXV a global health emergency in July 2022.

Thus, further research on vaccines homologous to MPXV and dedicated serological testing are needed, as well as diligent investigation of MPXV transmission and epidemiology.

In the present study, it was tested the performance of the MN assay based on CPE inhibition after incubation of several serial dilutions of human serum samples with a standardized dose of live MPXV and VACV. Since it has been reported in the literature that many antibodies directed against MPXV surface antigens are able to neutralize the virus in a complement-dependent manner, we decided to apply the MN assay with and without an external source of complement in order to evaluate the different performance of the method.

We evaluated the performance of the assay on a panel of samples including MPXV convalescent serum samples, historical smallpox-vaccinated serum samples, and unvaccinated, uninfected human serum samples. Previous studies have shown that antibodies directed against VACV show some degree of cross-reaction with MPXV and orthopoxviruses, thus providing some degree of protection[199,200].

Results demonstrate that the presence of an external complement source increases neutralization titers in samples from MPXV-infected convalescent donors and it also permits to detect positive responses in samples from vaccinated subjects who had previously tested negative for both MPXV and VACV by the classic BRC-free MN test. Thus, the use of the complement source increases the sensitivity of the test and offers a better correlation of protection by mimicking the host immune response [201].

All samples from people who had presumably received the VACV vaccine (based on the age) were negative in the MN test without complement; this could indicate that the amount

of "fully self-neutralizing" antibodies was low because the vaccine was administered many years earlier.

In conclusion, our results are in line with previously published data[199,202] confirming that historical smallpox vaccination is able to generate antibodies that cross-react with MPXV. However, the presence of an external source of complement can potentially increase the sensitivity of the test in detecting neutralizing antibodies.

In addition, antibodies elicited directly by MPXV infection can neutralize MPXV and cross-neutralize VACV, and antibodies induced by smallpox vaccination resulted long-lasting and cross-reactive against MPXV.

8. Conclusions

Although nowadays there are many viable therapeutic strategies to control or limit the spread of viral infections, the best option still lies on vaccines. As vaccination is a non-invasive and safe technique, very specific and guarantees a long immune coverage over time, it is essential to protect the part of the population more at risk. It is therefore essential to have the most up-to-date technology available for both the production and the evaluation of the efficacy and safety of vaccine formulations, to be prepared for possible future health emergencies.

SARS-CoV-2 pandemic and Monkeypox outbreak have shown us that we must always be prepared for potential new public health emergencies. This requires a comprehensive set of countermeasures, including vaccines, antiviral drugs, serological tests, diagnostic tools, public health policies, preventive practices, political awareness and global collaborations. Serological tests such as Microneutralization, can play a very important role both by helping us to understand in advance the proportion of different population groups susceptible to emerging virus infections and by assessing the efficiency of the immune response induced by the various vaccine therapeutic strategies, subsequently allowing us to determine potential correlates of protection after natural infection and/or vaccination.

Micro-neutralization test is a highly sensitive and specific test to detect virus-specific neutralizing antibodies serum samples. Measuring antibody titers against specific viruses is essential for virus research. Virus neutralization gives the most accurate answer to whether an individual has antibodies that can neutralize the infectivity of a particular virus strain.

The aim of this thesis was to evaluate the performance of a new serological assay (based on live virus) capable of successfully assessing the immune response induced by vaccination and natural infection.
In the first part of this thesis, MN-CPE assay was used to assess the decay of serum neutralising antibody levels against Sars-CoV-2 and to determine an increased susceptibility to infection. The ability to monitor serum neutralising antibody levels, highlighted the need to complete vaccination cycles with an additional booster dose of vaccine and to establish a time interval between doses to avoid antibody decay that could affect subjects' protection.

In the second part, it wase developed an MN-CPE protocol by including a source of exogenous complement, in order to obtain a reliable serological assay capable of detecting neutralising antibodies specific for MPXV and VACV. We evaluated the performance of MN assay on a panel of sera from MPXV convalescent subjects, smallpox vaccinated subjects and international standard anti-MPXV. The assay proved to be effective and reliable, with all the potential to be applied in future phase II and III human clinical trials for the evaluation of specific serum neutralising antibodies against MPXV.

In conclusion, even though the need for Bio Safety Level 3 containment may be considered a limiting factor, these types of assays are currently the only ones capable of generating data on neutralising antibodies and should be further evaluated to understand the correlates of protection against diseases induced by viruses such as Sars-CoV-2 and MPXV.

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