Preliminary rapport on SARS-CoV-2 spike mutations arising in Danish mink, their spread to humans and neutralization data.

SARS-CoV-2 spike mutations arising in Danish mink and their spread to humans

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Background

Despite control measures, SARS-CoV-2 continued to spread among mink farms across northern Denmark, with more than 200 farms infected by November 2020. SARS-CoV-2 genome sequences obtained from infected mink and humans living on the farms provided evidence of SARS-CoV-2 spread between mink and human in zoonotic events. This study investigates the amino acid changes in the spike surface glycoprotein that appeared during this outbreak and their effect on the antigenicity of the SARS-CoV-2 virus.

Spike mutations

Within the infected mink, the SARS-CoV-2 virus mutated, giving rise to several amino acid changes in the spike protein. The first was a tyrosine to phenylalanine at amino acid 453 (Y453F), a mutation that also appeared during the Dutch mink farm outbreaks. It is a conservative amino acid substitution in the receptor binding domain that directly contacts the host ACE2 receptor at amino acid 34 (Wang et al). This ACE2 contact position differs between human and mink (histidine [34H] in humans and tyrosine [34Y] in mink and other mustelids (Damas et al)), which suggests that Y453F is an adaptation mutation to mink ACE2. Importantly, 453F increases affinity for human ACE2, which may explain its successful introduction and establishment in humans.

Following the appearance of 453F, additional spike mutations were observed in minks and the humans epidemiologically linked to the infected mink farms (Fig. 1). These include: i) 69-70deltaHV - a deletion of a histidine and valine at amino acid positions 69 and 70 in the N-terminal domain of the S1 subunit; ii) I692V – a conservative substitution at position 692 that is located seven amino acids downstream of the furin cleavage site; iii) S1147L – a non-conservative substitution at position 1147 in the S2 subunit; and iv) M1229I – a conservative substitution located within the transmembrane domain.

Clinical isolates

Efforts are underway to isolate each mink-associated SARS-CoV-2 spike mutant strain that occurs in people residing in Denmark. To date, Statens Serum Institut in Denmark has isolated two strains of mink-associated SARS-CoV-2 viruses. These include an isolate with the 453F spike mutation (F-spike) from cluster 1 and an isolate with a 69-70deltaHV, 453F, 692V, and 1229I mutation combination from Cluster 5 (hereafter referred to as ΔFVI-spike). To ensure that subculturing of SARS-CoV-2 clinical isolates on VeroE6 cells did not induce additional spike mutations, each isolate was sequenced. The spike protein of the cultured virus was identical to that of the SARS-CoV-2 virus in the original clinical sample.

A)

Spike mutation combinations*	Abbreviation	Number of positive clinical samples**
453F	F	N = 142
69-70delHV, 453F	ΔF	N = 162
69-70delHV, 453F, 1147L	ΔFL	N = 18
69-70delHV, 453F, 692V, 1229I	ΔFVI	N = 12

* All SARS-CoV-2 mink-associated sequences also contained the D614G
** For sequenced samples up until 31 October 2020. May include duplicate samples taken from the same person and is therefore not necessarily representive of the number of infected persons.

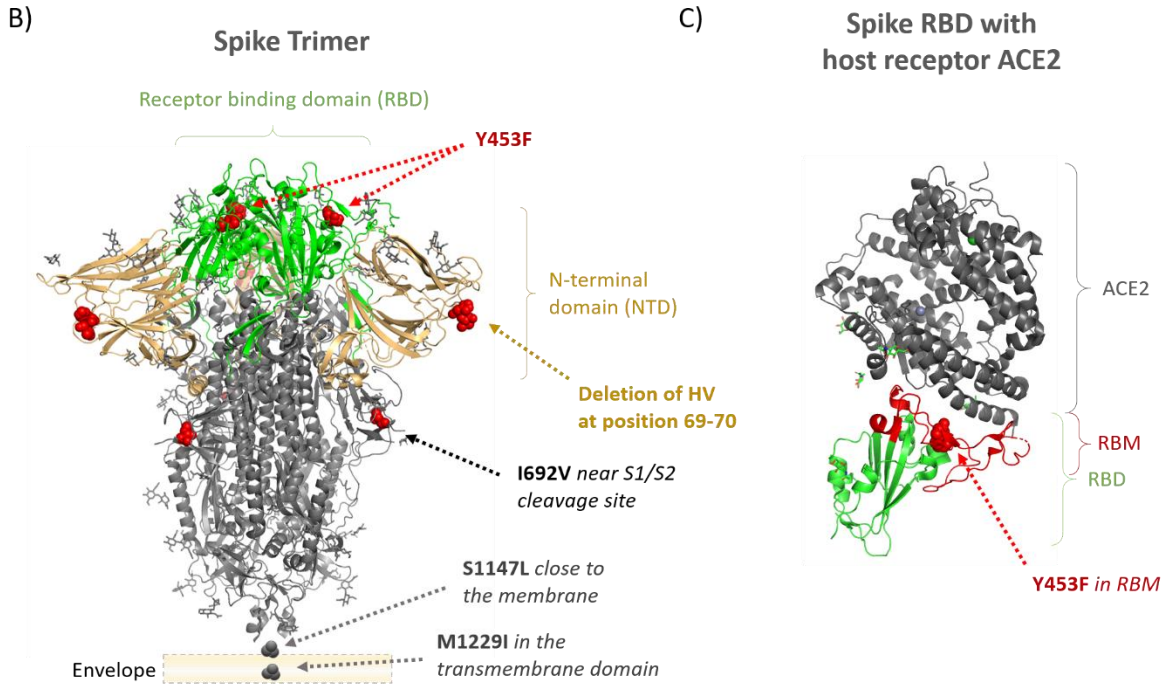

Figure 1. The mink-associated mutations in the SARS-CoV-2 spike protein. A) The combination and frequency of mink-associated spike mutations detected in SARS-CoV-2 infected humans B) The crystal structure of a closed prefusion spike trimer [PDB: 6ZGE] with the position of the Y453F variant in the receptor binding motif, the position of two amino acids deleted in the N-terminal domain, and the position of the I692V variant. The regions encompassing the S1147L and M1229I mutations are not within the crystal structure; however, their relative positions are indicated. C) The position the Y453F variant in a receptor binding domain complexed with a host ACE2 receptor [PBD: 6LZG].

The clinical isolates bearing the Y453F spike mutation replicated as efficiently as the unmutated/wildtype SARS-CoV-2 virus that predominates in Denmark (data not shown). Conversely, the SARS-CoV-2 virus with four mutations grew slower than both the wildtype virus and other SARS-CoV-2 virus isolates (Fig. 2). The cytopathic effect (CPE) induced by the ΔFVI-spike mutant virus appeared later and was less pronounced and had an approximate 10-fold lower titer 24 hours post-inoculation compared to human SARS-CoV-2 isolates prepared under the same conditions (Fig. 2A). At 96 hours post-inoculation the ΔFVI-spike mutant virus titer was comparable to that of the wildtype

virus and exceeded other SARS-CoV-2 viruses isolated and subcultured under the same conditions (Fig. 2B). The ΔFVI-spike mutant virus titer increased 54.7-fold from 24 to 96 hours post-inoculation, compared to an average of 4-fold (range: 2.6 to 5.7-fold) over the same time for other SARS-CoV-2 isolates. The ability to replicate to high viral titers is consistent with high levels of the ΔFVI-spike mutant virus detected in throat swab samples of infected persons, as indicated by an average qPCR assay (E-Sarbeco) cycle threshold of 24.7 (range: 20-35). Further evaluation of the SARS-CoV-2 ΔFVI-spike strain growth kinetics in other cells systems are warranted.

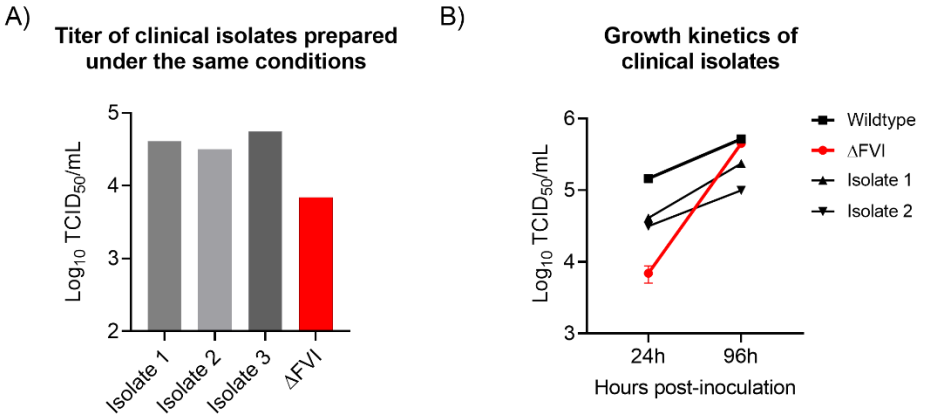

Figure 2. Growth kinetics of the SARS-CoV-2 ΔFVI-spike mutant virus. A) Virus titers 24h post-inoculation for SARS-CoV-2 viruses isolated from clinical samples under the exact same conditions. Isolate 1-3 each have different spike mutations unrelated to mink outbreaks, these include N439K (isolate 1), N439K+69-70delHV (isolate 2), and S477N (isolate 3). B) The growth kinetics of the ΔFVI-spike mutant virus relative to other clinical isolates, including the nonmutated virus (wildtype) that predominates in Denmark and spike mutant viruses (isolate 1 and 2 as for [A]).

Virus neutralization

The introduction of SARS-CoV-2 spike mutant viruses raises concerns about a potential reduced recognition of the protein by antibodies induced after SARS-CoV-2 infection or vaccination that may have implications for re-infections and vaccine efficacy, respectively. To evaluate the effect of the mink-associated SARS-CoV-2 spike mutant viruses on antigenicity, neutralizing activity of convalescent plasma from persons who recovered from a SARS-CoV-2 infection and sera from immunized rabbits were compared between the ΔFVI-spike mutant virus and an unmutated wildtype virus.

The neutralization activity was tested using a micro-neutralization assay that was adapted from the World Health Organization protocol for influenza virus neutralization. The assay was developed at Statens Serum Institut and validated on >300 convalescent plasma/serum samples as well as sera from vaccinated mice and rabbits. In brief, 2-fold serial dilutions of plasma/sera were pre-incubated with

SARS-CoV-2 virus for 1 hour before addition to a monolayer of VeroE6 cells prepared in 96-well plates. After a 24 hour incubation, the cells were fixed to the plates and the level of virus determined using a standard ELISA targeting the SARS-CoV-2 nucleocapsid protein. To determine the amount of virus to add to the assay, clinical isolates are usually titrated at 24 hours and from these titers 100× $TCID_{50}$ virus used in the neutralization assay. This equates to approximately 300× $TCID_{50}$ from titers calculated 96 hours post-inoculation. Due to the difference in growth kinetics of the ΔFVI-spike mutant virus, the $TCID_{50}$ titer calculated at 96 hours was deemed to reflect the amount of infectious particles in the virus stock more accurately than that measured at 24 hours post-inoculation. Thus, each serum samples were tested in duplicated with 300× $TCID_{50}$ as calculated from 96 hours post-inoculation titers.

The convalescent plasma was selected from persons living in the South of Denmark, geographically separated from the mink outbreaks in the North of Denmark, and had a documented SARS-CoV-2 infection at the beginning of the Danish epidemic before the mink outbreaks occurred. Since the effect of the spike mutations on different levels of neutralizing antibodies is unknown, sera with known low (N=4), intermediate (N=3) and high (N=2) neutralization titers were tested. Each plasma sample represents a different donor and was tested in duplicate.

The different convalescent plasma were not equally affected by the ΔFVI-spike mutant virus. The two plasma samples with high neutralization titers were largely unaffected, while plasma with low and intermediate titers were more likely to experience a loss in neutralization activity (Fig. 3a). In these preliminary data from 9 convalescent plasma, an average 3.58-fold (range: 0 to 13.5) reduction was observed. Only two plasma samples had a greater than 4-fold reduction, a threshold set for neutralization resistance by Li et al. who evaluated other spike mutants presented on pseudovirus particles. It is important to note that the findings are preliminary and warrant further investigation in other SARS-CoV-2 neutralization assays.

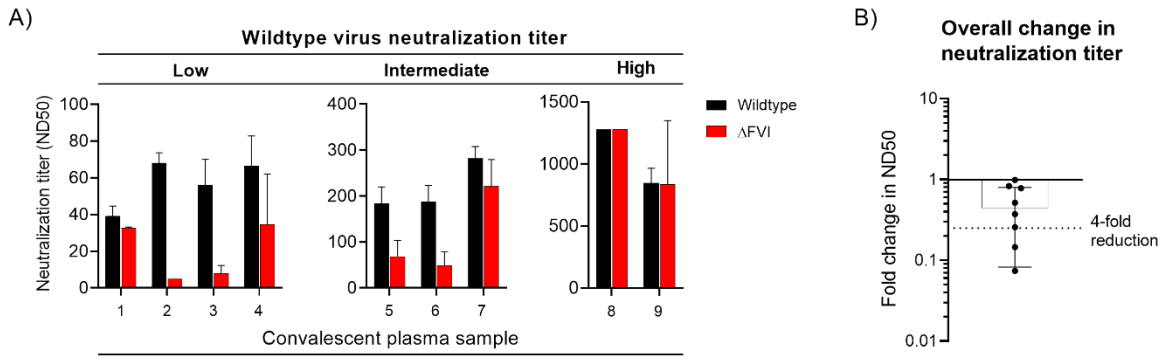

Figure 3. Neutralization of the SARS-CoV-2 ΔFVI-spike mutant virus relative to an unmutated SARS-CoV-2 virus. A) Convalescent plasma from nine individuals with known low, intermediate, or high neutralizing titers were used to assess the effect of the spike mutations on neutralization activity of antibodies induced following infection with an unmutated SARS-CoV-2 virus. The neutralization titer was determined as follows: a 50% cut-off value was calculated using quadruplicate virus controls (prepared for each virus) and cell controls included on each plate. The titer was calculated as the interpolation of a 5-parameter titration curve with the 50% cut-off value. The reciprocal serum dilution is reported as the 50% neutralization antibody titre. B) The fold-change in neutralization titer for the SARS-CoV-2 ΔFVI-spike mutant virus relative to an unmutated SARS-CoV-2 virus. The horizontal dotted line indicates a 4-fold reduction. The bars represent the mean of duplicate measurements with the standard deviation.

PRELIMINARY References

Wang et al (2020) Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2

Damas et al (2020) Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates

Li et al (2020) The impact of mutations in SARS-CoV-2 spike on Viral Infectivity and Antigenicity. Cell 182, 1284-1294