Severe acute respiratory syndrome-coronavirus and human coronavirus-NL63: an updated overview

Vanessa Ditt[a] and Oliver Schildgen[a,b]

Severe acute respiratory syndrome-coronavirus and human coronavirus-NL63, though both belonging to different groups of the same genus, are interesting representatives of their kind; the first one is of zoonotic origin, accounted for the pandemic in 2002/2003 that was distributed worldwide and had a mortality rate of about 10%, the other one was identified later and probably has been circulating within the population for centuries and belongs to those viruses that cause the 'common cold'. A lot of effort has been made to investigate both viruses and to understand their differences and their similarities with regard to further zoonotic events. This review gives an overview of severe acute respiratory syndrome-coronavirus and human coronavirus-NL63, their history and the current state of research.

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Introduction

Since the first human pathogenic coronaviruses (HCoV)-229E and HCoV-OC43 were identified in the 1960s and associated with mild respiratory diseases [1,2], only little new information had emerged in this field until the beginning of this century. Suddenly the focus was directed towards coronaviruses again as a special, meanwhile well known member of this group appeared in 2002/2003 [3–5] in the province of Guang Dong (China) and caused 'severe acute respiratory syndrome (SARS)'; fatal symptoms that were very unusual for coronaviruses as they were considered to be nonhazardous at this time. Strenuous efforts were made to identify and characterize this new pandemic threat and to curb it.

Amongst the heterogeneous family of coronaviridae coronavirus NL63 (HCoV-NL63) was identified in 2004 [6] and coronavirus HKU1 (HCoV-HKU1) in 2005 [7]. Compared with SARS-coronavirus (CoV), these newly detected coronaviruses led to clinical symptoms more similar to those caused by HCoV-229E and HCoV-OC43. A lot of research work has been undertaken since then to learn more about these viruses, their differences and similarities and their characteristics. This review will mainly focus on SARS-CoV and HCoV-NL63.

Identification of severe acute respiratory syndrome-coronavirus and human coronavirus-NL63

Several techniques were employed to characterize and identify the causative agent of SARS. Patient material was inoculated onto rhesus monkey kidney cells (fRhk4) to observe for cytopathic effects; tests for other known respiratory viruses were negative. Electron microscopy revealed the morphology and led to characterization of the virus family. Histopathological examination displayed mild interstitial inflammation with scattered alveolar pneumocytes. In an immunofluorescence antibody assay, sera from patients had high titres of antibodies against the infected cells. Random reverse transcriptase-PCR assay generated DNA fragments of unknown origin but with homology to viruses of the family of coronaviridae and confirmed the results of electron microscopy [5]. A few

[a]Institute for Virology, University Hospital Bonn, Bonn, and [b]Kliniken der Stadt Köln gGmbH, Institut für Pathologie, Cologne, Germany.

Correspondence to PD Dr rer. nat. Oliver Schildgen, c/o Kliniken der Stadt Köln gGmbH, Institut für Pathologie, Ostmerheimer Str. 200, D-51009 Cologne, Germany.

E-mail: schildgeno@kliniken-koeln.de

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days later, these results were confirmed by two other groups [3,4].

Sequence of events

SARS and its causative agent appeared quite unexpectedly and spread quite explosively. The infection was transmitted from palm civets to humans, although it has since been confirmed that bats are the natural reservoir of SARS-CoV [8,9]. Because of the fast mutation rate of RNA viruses and their resulting genotypic markers, the course of infection could be reconstituted very exactly.

In the early phase of the SARS pandemic, the very first index patient fulfilling the subsequent WHO definition of SARS appeared in Foshan near Guangzhou on 16 November 2002. One month later, the second case occurred in Shenzhen; a man who had regular contact with wild animals was affected and infected his family and several staff members from the hospital to which he was admitted. Similar cases were reported nearby.

In January 2003, the second phase of the SARS outbreak started in Guangzhou. Several affected people became fatally ill and were transferred to the major hospitals where they accounted for nosocomial spread to other patients and healthcare workers.

The next and final phase started in mid-February and heralded the big pandemic, when a doctor was infected in Guangdong province and took the disease to Hong Kong where he stayed in a hotel ('hotel M'). Seventeen other people were infected by him and were admitted to different hospitals where further nosocomial infections occurred. Some of the infected people transferred the virus via air travel to Vietnam, Singapore and Toronto where new sources of infection emerged.

On 21 March 2003, a novel coronavirus was identified [5] and a few days later confirmed [3,4]. The first strain (Tor2) was fully sequenced on 12 April 2003 and SARS-CoV was proven to be the causative agent for SARS [10]. In July 2003, the epidemic ended after no further human-to-human transmissions were reported. In September 2003, a new case was reported from a laboratory in Singapore and during the next 2 years other accidents in laboratories occurred.

HCoV-NL63 was discovered in a 7-month-old child with bronchiolitis. Diagnostic tests for all known respiratory viruses were negative. The sample with the unknown agent was inoculated on cell culture and a cytopathic effect could be observed on tertiary monkey kidney (tMK and LLC-MK2) cells. In the supernatant of the latter, a new virus could be identified by using the Virus discovery based on cDNA-AFLP (VIDISCA) method [11,12]. Sequence comparison displayed that the virus is most closely related to HCoV-229E and belongs to the subgroup 1b coronaviruses. Two further research groups were able to identify the same information soon afterwards [13,14].

There are some hints in the previously published literature about coronaviruses that HCoV-NL63 was detected much earlier. Some viruses were described that did not totally correspond with HCoV-229E and HCoV-OC43. Unfortunately, these isolates were lost so it cannot be checked whether one or more were identical to HCoV-NL63 [6].

Taxonomy and genome structure

Both SARS-CoV and HCoV-NL63 are members of the genus *Coronavirus*, belonging to the family of *Coronaviridae* within the order *Nidovirales*. Both viruses are positive single-strand RNA viruses with a large genome of about 30 kb in size. Virus particles are enveloped and possess peculiar spike proteins on their surface leading to their crown-like appearance. Electron microscopy revealed particles of 80–140 nm located either inside infected cells at the rough endoplasmic reticulum in double-membrane vesicles or outside the cell attached to the plasma membrane.

The genome of both SARS-CoV and HCoV-NL63 can be roughly divided into two parts. The 5′ two-thirds consist of one large polyprotein (ORF1ab) including several domains with autocatalytic activities, thus producing nonstructural proteins (NSPs) involved in replication and immune evasion. ORF1ab encodes 16 NSPs *in toto* in both SARS-CoV and HCoV-NL63.

The last third at the 3′ end of the genome contains the ORFs coding for the functional proteins: spike (S), envelope (E), membrane (M), nucleocapsid (N) and further accessory proteins genes varying in number and position from species to species.

Traditionally, coronaviruses are classified – due to their antigenic cross reactivity – into three different groups, which were mainly confirmed later by performing sequence analysis. Group I and II viruses afflict mammals, whereas viruses from group III infect exclusively birds. While HCoV-NL63 belongs to the group 1b, SARS-CoV as well as bat coronaviruses are considered group 2b viruses, although the latter rather represent a new group of coronaviruses.

HCoV-NL63 is most closely related to HCoV-229E, and phylogenetic analysis supports the fact that HCoV-NL63 diverged from HCoV229E in the 11th century [15]. Furthermore, there seem to be two main genetic clusters of HCoV-NL63 [16], and there is evidence that the genome of HCoV-NL63 is arranged in a mosaic-like manner [15].

Disease/virology

Patients infected with HCoV-NL63 generally suffer from only mild symptoms, including cough, rhinitis, rhinorrhoea and pharyngitis, often together with fever [17]. In rare cases, pneumonia can occur. Mostly children from zero to 3 years and older people as well as immunocompromised individuals are afflicted.

In children suffering from severe lower respiratory tract infections, a substantial number had croup compared with a control group [18–21]. Croup or laryngotracheobronchitis is characterized by a loud barking cough, inspiratory stridor and hoarseness. Also, an association with Kawasaki disease has been postulated [22] but could not be confirmed by a number of research groups [23–25].

After its detection in the Netherlands and later on in New Haven, USA, the presence of HCoV-NL63 could be proven in a number of countries, suggesting a worldwide distribution [17,18,21,26–36]. Out with subtropical regions, HCoV-NL63 was mainly detected in winter months and often turned up with other copathogens such as influenza, respiratory syncitial virus, parainfluenza and human metapneumovirus (hMPV) [13,18,20,26]. The viral load of HCoV-NL63 is attenuated when accompanied by another pathogen [18]. However, and not surprising, the infection itself seems to be stronger when a challenge with two pathogens had occurred [16]. Like SARS-CoV, HCoV-NL63 is detectable up to 2 months after the recovery from the disease [30,37]. Seroprevalence studies showed that virtually every adult encounters HCoV-NL63 infection at least once in a lifetime. Antibodies specific for the S protein are present and even display a neutralizing effect [6].

People infected with SARS-CoV suffered from fever, chills, myalgia, rigor and a nonproductive cough. Clinical symptoms such as rhinorrhoea and sore throat could be detected less often. In contrast to HCoV-NL63, children were not affected [38–40] but normal and healthy adults as well as old people were susceptible.

Of 8096 infected people, 774 died, which accounts for a mortality rate of almost 10%. SARS-CoV was spread worldwide in more than 30 countries [3–5,10,39,41–46]. The first outbreak occurred in late 2002/early 2003. Seasonality of SARS-CoV infection is not known as the pandemic occurred just once, with its peak occurring in winter.

It is meanwhile confirmed that SARS-CoV can be ascribed to a zoonotic origin primarily from bats [47,48]. Although SARS-CoV was initially spread form civets to humans [41], the actual transmission route was from human to human most likely by droplets and occurred most likely within healthcare facilities, workplaces and public transportation. The virus could be detected not only in the respiratory tract but also in the gastrointestinal tract, liver, kidney and brain as well as other tissues [49].

The seroprevalence was quite low amongst the general population, ranging from zero to 1.81% depending on the performed study and slightly higher in asymptomatic healthcare workers. In contrast, a much higher rate was found in asymptomatic animal handlers (up to 40%), which is not surprising, as they have probably acquired immunity by less pathogenic SARS-CoV-like strains, which also emerged by zoonotic recombinations [50]. An infection with SARS-CoV can be accompanied by copathogens such as other respiratory viruses, hMPV, or other coronaviruses [38].

Viral life cycle

Viral entry

It is amazing that both viruses use the same receptor for cell entry as SARS-CoV and HCoV-NL63 are only distantly related and belong to different groups. Angiotensin I converting enzyme 2 (ACE2), a cellular metallopeptidase, negatively regulates the conversion of angiotensin I to angiotensin II by ACE and is part of the renin–angiotensin–aldosterone system that contributes to the regulation of the cardiovascular system.

Especially for HCoV-NL63, the use of another receptor would have been more likely as it is closest related to HCoV-229E, which enters the cell via CD13 [51]. Despite of this similarity, there are some different features concerning entry and replication as both SARS-CoV and HCoV-NL63 cause harm to such a different extent.

It could be demonstrated that both viruses engage the cellular receptor in a different way as mutations in ACE2 lead to an impairment of SARS-CoV binding, but do not change binding properties between HCoV-NL63 and ACE2 [52]. The interaction of HCoV-NL63 S-protein turned out to be generally weaker than binding of SARS-CoV S-protein as the binding sites are indeed similar but not identical [53].

Entry of HCoV-NL63 is less dependent on low pH than it is for SARS-CoV and does not need the cathepsin L function [54]. Furthermore, it is known for SARS-CoV that after binding to ACE2, both the virus particle as well as the bound receptors are internalized, thus reducing the amount of ACE2 on the cell surface. ACE2 is essential for cleaving of ACE, a molecule that positively affects lung injury during respiratory disease; therefore, downregulation of ACE2 could account for the exacerbation of the lung's state during SARS [55].

Nonstructural proteins

For most of the 16 NSPs, the structure could be identified and a putative function could be predicted or even confirmed. NSP1 is known to play an important role in immune system evasion [56,57]. The function of NSP2 and NSP11 has yet to be determined. NSP3 contains two papain-like proteases (PLPs) that – together with the main protease (Mpro) encoded in NSP5 – are responsible for processing the pp1a and pp1ab. Furthermore, NSP3 is important for virion assembly and immune evasion.

NSP4 and NSP6 – together with NSP3 - are thought to function in double-membrane vesicle (DMV) formation but this assumption has yet to be confirmed. NSP 7–NSP10 all seem to be essential in the replication process, and in contrast to NSP1 and NSP3, they are highly conserved. NSP8 could probably function as a primase [58]. Together with NSP7, it forms a structure with RNA-binding properties [59]. NSP9 is supposed to play a certain role in RNA binding as well [60]. NSP12 is a RNA-dependent RNA polymerase and NSP13 is a helicase. NSP8 seems to play a key role in the process of replication as it could be demonstrated that there are interactions with almost every NSP involved in replication [61]. NSP14 has a 3′–5′ exonuclease activity, and like NSP16, it functions as a methyl transferase for RNA cap formation [62,63]. At last, there is NSP15, a unique protein amongst all nidovirales and which is essential for replication. It is an urydilate-specific endoribonuclease (NendoU), but its role for viral replication remains unclear to date [58,64].

Structural proteins

Viral RNA is located within a bilayer containing the structural proteins S, E and M. The last one is the most abundant protein and plays an important role in the assembly of new emerging virus particles and the incorporation of other viral components [65]. The S-protein is a glycosylated surface protein that associates with the host cell receptor, thus allowing the entry of the virion by fusion with ACE2. It is not important for virus assembly or budding. The envelope protein (E) is an integral membrane protein that does not appear abundantly on the cell surface and forms ion-like channels on membranes of infected cells [66]. It has a crucial role in virus morphology and budding [67,68] and – for yet unknown reasons – is essential for group I coronaviruses such as HCoV-NL63 but not for group II coronaviruses such as SARS-CoV, although lack of the E protein could lead to attenuation of replication *in vivo* as it is confirmed with mouse hepatitis virus (MHV) [69,70].

The N protein is the only structural protein that is not present on the cell surface. By self-association and interaction with M, it is needed for the encapsidation of the RNA genome. Furthermore, it is known for being a strong interferon (IFN) antagonist [71–73].

Accessory proteins

The presence of accessory proteins varies to a big extent in SARS-CoV and HCoV-NL63. Whereas SARS-CoV contains ORF3a and b, 6, 7a and b, 8, 9b and an additional ORF within the N gene, there is only ORF3 existing in HCoV-NL63. It is supposed that they somehow play a role in replication, but currently, only accessory proteins from SARS-CoV are known to interfere with the IFN defence mechanism [74–76].

Interferon and innate and immune response

The expression of IFN is a basic reaction of the immune system to initiate a response against pathogens infiltrating host cells [77–79]. IFN-secreting cells alert their neighbouring cells to produce a subset of antiviral proteins to inhibit viral amplification. This is accomplished by components of the innate immune system that are able to sense typical pathogen patterns inside or outside of a cell. Once viruses succeed in entering a host cell, there are molecules such as RIG-I and MyD88 that are able to recognize uncapped double-stranded (ds) RNA. By initiating a signalling cascade via the membrane-bound mitochondrial antiviral signalling (MAVS) protein, via TANK-binding kinase 1 (TBK1) and IκB kinase ε, interferon regulatory factor 3 (IRF3) becomes phosphorylated and dimerizes. After translocation into the nucleus, expression of IFNβ is activated and thereafter secreted where it stimulates nearby cells and enhances the cells' own activation. Toll-like receptors (TLRs) recognize pathogen structures outside of the cell. Depending on the TLR, different pathways are activated to stimulate IFNβ expression.

After binding of IFNβ to its appropriate receptor on a host cell, the expression of more than 100 genes encoding antiviral proteins is implemented by signalling via the Janus kinase 1/tyrosine kinase 2 kinases. After activation of both kinases by IFNβ, signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) are activated leading to the formation of the interferon-stimulated gene factor 3 complex, which in turn has the ability to activate expression of those antiviral proteins mentioned above.

Coronaviruses have developed strategies to undermine the immune response at distinct sites of these signalling cascades. During the replication phase, a huge amount of dsRNA is produced, but does not activate the IFN system [80,81].

Cells make use of both an active and passive evasion system. On one side, they are able to hide their dsRNA from the cell, probably in an endoplasmic reticulum–Golgi intermediate compartment as coronaviruses are supposed to replicate in those DMVs [82]. On the other side, there are several proteins involved in blocking the induction of IFN, namely N, the replicase proteins NSP1 and NSP3 and the two accessory proteins ORF6 and

ORF3b. To date, only accessory proteins of SARS-CoV could be associated with antagonizing IFN pathways [74,76].

In expression assays, it could be demonstrated that N of SARS-CoV inhibits nuclear factor kappa B (NFκB) signalling. It is very likely that other components are affected by N as well, but studies carried out so far were mainly performed with MHV. NSP1 intervenes in more than one way to block IFN induction. It is able to degrade not only IFN mRNA but also host mRNA as well, which seems to be important for inhibiting IFN expression [56]. In other studies, it could be observed that NSP1 affects the signalling pathways by blocking STAT1 phosphorylation and IRF3 dimerization [57].

NSP3 is another IFN antagonist. The PLP seems to carry over much of IFN inhibitory function [83]; PLP may block the NFκB pathway or affect IRF3 [75,83]. ORF6 makes use of a totally different effect namely by affecting karyopherin, thus preventing nuclear translocation of IFN-inducing factors such as STAT1 [84].

Animal models

In-vivo models that mimic the natural course of infection in humans are necessary and essential for carrying out fundamental research and assessing efficacy of antiviral drugs and immunization reagents. Unfortunately, there is currently no usable animal model available for studying HCoV-NL63 infection.

In contrast, a lot of animals were tested for their susceptibility to SARS-CoV. Several nonhuman primates were infected with SARS-CoV; all were able to replicate SARS-CoV within their lungs and display clinical symptoms to a varying extent depending on the species.

Infected macaques demonstrated clinical and pathological features, diffuse alveolar damage, formation of hyaline membranes and pneumocytic type II hyperplasia similar to those found in humans [42]. Furthermore, it was observed that these animals displayed a similar pattern of upregulated chemokines and cytokines compared with infected humans [85].

On the contrary, African green monkeys did not display any significant lung disorder, although the virus was able to replicate in the respiratory tract [86]. Considering the lack of consistency, the cost and handling of primates, small animal models are preferred for studying SARS-CoV infection. It could be demonstrated that golden Syrian hamsters can replicate different SARS-CoV strains to high titres accompanied by pathological changes after infection with different SARS-CoV strains as Urbani, Frankfurt (FFM) and Hong Kong (HK) [87,88]. Unfortunately, they do not demonstrate clinical signs of illness; but in the last few years, they were successfully used as a model for evaluation of vaccines and antiviral therapies [89,90].

The possible use of ferrets as an optimal model for studying SARS has been controversially discussed [91,92], but recent results demonstrate that they constitute a good model as they display virus replication in the upper and lower respiratory tract and clinical signs such as fever, sneezing, lung damage and a similar blood count compared with humans and, therefore, provide a possibility for studying antiviral therapeutics [93–95].

Different inbred mouse strains have been tested so far with different results. SARS-CoV is not able to cause comparable respiratory symptoms of illness in young BALB/C or C57/BL6 mice, although virus replication peaks at days 2–3 later and slightly elevated levels of tumour necrosis factor alpha (TNFα) and moderate interstitial pneumonitis at day 3 could be demonstrated [96–98]. Pronounced signs of clinical illness can be seen in 129S6 mice [88,99].

In aged BALB/C mice, the infection with SARS-CoV leads to a more severe disease than in young BALB/C mice, mimicking the age-related course of infection in humans [100]. This makes the aged BALB/C mouse an excellent animal model for studying SARS-CoV pathogenesis as they not only support viral replication but also display clinical signs of illness as well as several histopathological findings [88].

A similar effect was also observed for C57/BL6 and 129S6 mice but with less prolonged viral shedding within the lungs of the 129S6 mice. Early histopathological changes in the lungs of 129S6 and C57/BL6 mice displayed similar results.

From all tested mouse models with specific gene deletions (knockout mice), only the STAT1−/− mice with 129S6 background displayed a significantly different outcome from wild type strains [88,99].

Ageing and pathogenesis

As seen with many infections and diseases, there is a clear tendency for enhanced suffering and more severe clinical symptoms in children and older people, with an increased mortality rate for the latter group. Although children do not possess a sufficiently developed immune system, the problem for the elderly is certainly that their immune system has somehow lost its ability to fight pathogens properly. So the elderly represent a high-risk group for both viruses. Not much is known on a molecular level about this phenomenon termed 'immunosenescense'.

What has been found out so far is that the effect of vaccination with SARS-CoV decreases in mice with increasing age. After challenging with SARS-CoV, the adaptive immune system of old mice does not display the ability to generate an appropriate response against the virus as is seen in young mice [101]. So far studies with other viruses have been performed with controversial results, but further studies on SARS-CoV and HCoV-NL63 are still outstanding.

Antiviral agents

Currently, there is no effective antiviral treatment against HCoV-NL63 and SARS-CoV available. Several agents have been tested for their antiviral effect *in vitro*, affecting the replication cycle at different stages. Particularly those that affect replication at the early stages of infection appeared to be candidates with good prospects. Amongst those promising agents against HCoV-NL63 are immunoglobulins – obtained from healthy volunteers – that are administered intravenously [102]. In the past, they displayed good results in treating immune deficiencies, autoimmune neuromuscular disorders, respiratory diseases and Kawasaki disease [103,104]. Good results were demonstrated with neutralizing antibodies, as well from human as from nonhuman origin in different animal models. Equine antibodies limited infection in aged mice, Syrian and Chinese hamsters, in rats and even in macaques [105–108].

Antibodies of human origin have the advantage of not being degraded so rapidly and to provide a higher efficacy than those of nonhuman origins. Apart from the possibility to generate human antibodies by immunizing and by exploiting the hybridoma cell line technique, phage display is now available. A self-constructed or commercially available human antibody library can be screened for antibody fragments that specifically bind to an antigen of choice. With this technique, an antibody against the S protein of SARS-CoV was identified [109], which turned out to be very successful in cell culture. The efficacy of a neutralizing antibody can be further enhanced by using several antibodies in parallel, all of them targeting different epitopes [110].

For SARS-CoV, other immunomodulators corticosteroids, pentaglobulin, thymosin, thalidomide and anti-TNF were used for the first time during the epidemic [111,112]. Another possibility is to disturb the fusion process of the viral spike protein and the ACE2 host receptor with artificial peptides. The heptad repeat regions 1 and 2 (HR1 and HR2) of the spike S2 domain undergo a conformational change when binding to the ACE2 receptor, thus allowing fusion of the viral and cellular membrane. Applied HR2 derivatives are able to reduce viral replication, presumably by competing with the natural HR2 for the HR1-binding positions and thus preventing fusion and virus entry [102,113].

Another possibility is the use of specific antibodies directed against the HR regions of the S protein. These antibodies were able to inhibit viral replication *in vitro* [114]. The RNA interference technology is also applicable via inhibition of viral replication at the transcriptional level. Small interfering RNAs (siRNAs) directed against conserved regions of the spike protein were tested for their antiviral effects and displayed a strong inhibition of virus replication in cell culture. Especially, the combination of several siRNAs at the same time displayed good results with both HCoV-NL63 and SARS-CoV [102,115].

The usage of nucleoside analogues can also inhibit viral replication of HCV-NL63 in cell culture, even though the molecular mechanism for this effect is unclear [102]. The purine nucleoside analogue ribavirin was given to patients during the SARS outbreak. The dosages and mode of administration were not standardized and was often accompanied by adverse effects such as haemolytic anaemia, hypomagnesaemia, calcaemia or all [116]. Animal studies with ribavirin have not been encouraging [117], and when tested *in vitro*, the effect of ribavirin was highly dependent on the cell types used. But when ribavirin was combined with type I IFNs, promising results could be demonstrated *in vitro* [118].

Protease inhibitors turned out to be quite powerful in suppressing viral replication at a posttranslational level. Replication of HCoV-NL63 and SARS-CoV requires proteolytic processing of pp1a and pp1ab by the Mpro and a PLP. Recently, several agents have been identified to inhibit Mpro, mostly by direct targeting of the catalytic site of the enzyme [119–125].

Conclusion

It is now 6 years since the big SARS pandemic occurred. A lot of time and effort has already been invested and will be in the future. So the question arises whether this effort is worth making? Is there really a chance that SARS-CoV will come back?

Although SARS-CoV may not reemerge, there is a good chance that other zoonotic coronaviruses will appear with similar devastating potential. Coronaviruses do recombine and have the ability to cross species barriers, so it may be just a question of time when the next new coronavirus turns up.

So efforts that are made in this field so far are not in vain, and there remains a lot of work to be done. Although much has been found out about the appearance and

functions of most viral structural and NSPs, there is as yet no effective treatment available either for SARS-CoV or for HCoV-NL63. Another question still unsolved is why SARS-CoV is so much more pathogenic than HCoV-NL63? At first glance, they both use ACE2 for entry, a receptor that was never used before by a coronavirus to gain access into a host cell. So both viruses share an important feature, although they are genetically very different. With the development of infectious clones, now available for both viruses, there has been created a powerful tool to study the function of every single protein. It remains to be seen when this knowledge has to be used again.

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