Identification of new human coronaviruses

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To date, there are still a variety of human infections with unknown etiology. Identification of previously unrecognized viral agents in patient samples is of great medical interest but remains a major technical challenge. Acute respiratory tract infections are responsible for considerable morbidity and mortality in humans and animals. A variety of viruses, bacteria and fungi are associated with respiratory tract illness. Most of the respiratory viruses belong to the *Paramyxoviridae*, *Orthomyxoviridae*, *Picornaviridae*, *Adenoviridae* and *Coronaviridae* families. No pathogens can be detected in a relatively large proportion of patients with respiratory disease, partially owing to limitations of current diagnostic assays but also since some infections are caused by as yet unknown pathogens. This review will focus on human coronaviruses. In the mid 1960s, two human coronaviruses were identified that cause the common cold: human coronaviruses (HCoV)-229E and HCoV-OC43. The recent outbreak of severe acute respiratory syndrome-CoV and subsequent identification of two additional human coronaviruses (HCoV-NL63 and HCoV-HKU1) has drawn attention to this virus family. This review summarizes the knowledge of current methodologies for identifying novel human coronavirus species. Furthermore, information on the discovery of known human coronaviruses will be presented.

Expert Rev. Anti Infect. Ther. 5(2), 245–253 (2007)

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KEYWORDS:
children, coronavirus, respiratory infection, virus discovery

Human coronaviruses

Coronaviruses are positive-strand RNA viruses with one of the largest viral genomes among the RNA viruses (27–33 kb). The virus particles are enveloped and carry extended spike proteins on the membrane surface. Currently, several coronaviral species are known to infect mammals and birds. These species were first divided into three groups based on their serological relationship [1,2]. As the number of species increased and molecular biology tools became available, the serological groups were converted into three phylogenetic clusters based on genome sequence analysis. The group III viruses are found exclusively in birds, whereas members of groups I and II can infect mammals.

The genome organization of coronaviruses is conserved among species, with the 5´ two-thirds of the genome encompassing the large 1a and 1b open-reading frames (ORFs) encoding nonstructural, replicase proteins and the 3´ terminal part encoding structural proteins. Accessory protein genes are located between the structural genes but they differ in number and size among viral species [3]. The coronaviruses can cause a variety of diseases in animals, including gastroenteritis and respiratory tract disease. In humans, currently identified coronaviruses are exclusively associated with respiratory tract illnesses. At present, there are five known human coronaviruses (HCoV): HCoV-229E, HCoV-OC43, severe acute respiratory syndrome (SARS)-CoV, HCoV-NL63 and HCoV-HKU1 (FIGURE 1; TABLE 1).

HCoV-229E and HCoV-OC43 were first described in the mid-1960s and, for over 40 years, they were believed to be the only representatives of human coronaviruses. Inoculation of healthy adult volunteers revealed that infection causes common cold symptoms. Coryza often occurs in individuals infected with HCoV-229E, whereas HCoV-OC43-positive patients frequently have sore throat manifestations [4]. The SARS epidemic began in 2003 in the Guandong province of China.

10.1586/14787210.5.2.245

ISSN 1478-7210

At first, it was suspected that known viral pathogens were involved but soon SARS-CoV was identified as the responsible pathogen [5–7]. The SARS-CoV probably originated from a wild animal reservoir, likely bats, and was transmitted in a zoonotic event to humans (e.g., via civet cats that are traded as food in China) [8,9]. The exploding epidemic was controlled in early July 2003, mostly by quarantine measures; however, there were also some sporadic cases later on in the 2003–2004 season [10]. HCoV-NL63 and HCoV-HKU1 were identified in 2004 and 2005, respectively. Further research showed that HCoV-NL63 was not introduced recently in humans but had been previously unrecognized [11]. Also, the genetic variability among HCoV-HKU1 strains suggests that this virus was introduced into the human population some time ago [12,13]. Both viruses have spread worldwide and display a spectrum of disease similar to those described for HCoV-229E and HCoV-OC43. The four currently circulating human coronaviruses can probably all be classified as common cold viruses but a more severe lower respiratory tract infection is frequently observed in young children, patients with underlying disease and the elderly [14–22].

Current techniques for coronavirus discovery

Currently, there are several techniques for the discovery of novel coronaviruses. Here, we provide an overview of the methods that have been used successfully for coronavirus detection and identification (e.g., electron microscopy, consensus primer reverse transcriptase [RT]-PCR amplification, virus discovery based on cDNA amplified fragment length polymorphism [AFLP] [VIDISCA], random RT-PCR or microarrays).

Electron microscopy

Coronaviruses are named after their shape under the electron microscope. The virus particles (50–150 nm diameter) are easily recognizable because of their pleomorphic shape, surrounded by the 'crown' consisting of the extended surface spike proteins (FIGURE 2). The first identification of a virus occurs frequently by electron microscopy. This technique was used for the identification of SARS-CoV, HCoV-229E, HCoV-OC43 and HCoV-NL63 [5,6,23–25]. The main disadvantage of this method is the need for a high-titer virus stock. Obviously, this physical technique does not itself provide detailed information regarding the virus type but, combined with serology and molecular biology methods, it remains a powerful instrument for virus discovery.

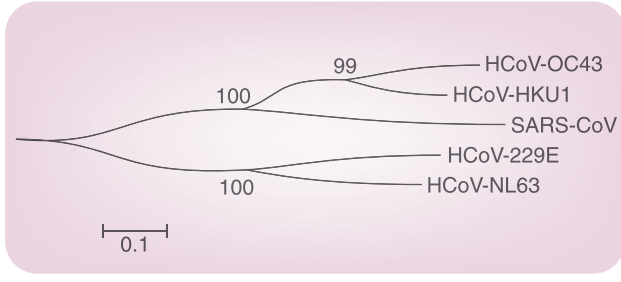

Figure 1. Phylogenetic relationship of human coronaviruses.
Phylogenetic tree was constructed with MEGA 3.1 software using a Neighbor-Joining algorithm. The scale bar unit is equivalent to 0.1 substitution per site. The tree was rooted with the sequence of the Torovirus (Breda virus; NC_007447).
HCoV: Human coronavirus; SARS: Severe acute respiratory syndrome.

RT-PCR amplification based on universal coronavirus primers

The most specific method for the identification of coronaviruses in clinical samples is a RT-PCR amplification-based method that uses primers that can amplify any member of the coronavirus family. Besides quick screening for several pathogens in a single assay, it provides the opportunity to identify previously unknown coronaviruses. The ideal target for primers is a conserved gene, which is preferably identical in all known coronaviral species. If such a 'perfect target' is not available, degenerate primers that mimic the natural sequence diversity are an alternative. For coronaviruses, conserved regions of the 1a/1b ORFs constitute a convenient target for the design of universal coronavirus primers. The encoded RNA-dependent RNA polymerase is the most conserved gene and several broad RT-PCR assays have been described based on this region (TABLE 2) [5,6,26–28]. The simplicity and time effectiveness of the method are counterbalanced by some limitations since assays designed before the identification of SARS-CoV, HCoV-NL63 and HCoV-HKU1 were later demonstrated to be unable to efficiently amplify these coronaviruses. Therefore, optimized universal primers should not only target all sequences of known members of the coronavirus family but, ideally, should have broader specificity. Such an effect can be obtained by including outgroup sequences (e.g., other virus family members from the order of *Nidovirales*) in the multiple sequence alignment used for primer design. Unfortunately, this approach often results in highly degenerate primers that lack specificity and, therefore, produce many false-positive signals.

Serology

The coronavirus family was initially divided into three distinct groups based on serology [1,2]. The shared immunogenic epitopes might be employed for broad detection of coronaviral proteins, even from species not previously identified. Coronaviruses from one serological group are generally recognized by the sera raised by any member of that serogroup [29–31]. In fact, coronaviruses that belong to different serological groups also possess common epitopes that can give cross-reactivity in an immunoassay [32,33]. Thus, we suggest that the coronavirus-specific, broadly reactive sera, as known for *Picornaviridae* species, might be used in future as an introductory assay for the detection of a coronavirus in cell culture [34].

Sequence-independent amplification

There are several techniques that allow the amplification of a viral genome without prior sequence information.

VIDISCA

The VIDISCA method is based on the cDNA–AFLP [35,36]. The main advantage of the method is the reproducible amplification pattern that allows the comparison of the virus-infected sample

Table 1. Human coronaviruses.

Study	Coronavirus	Year of identification	Detection method	Ref.
Hamre and Procknow (1966)	HCoV-229E	1966	Tissue cultures, inoculation of healthy adult volunteers	[24]
Tyrrell and Bynoe (1965) McIntosh *et al*. (1967)	HCoV-OC43 (-like viruses)	1965/1967	Organ cultures, inoculation of healthy adult volunteers, electron microscopy	[44] [47]
Peiris *et al*. (2003) Drosten *et al*.2003) Ksiazek *et al*. (2003)	SARS-CoV	2003	Cell culture, electron microscopy, consensus primers, random RT-PCR	[7] [5] [6]
van der Hoek *et al*. (2004) Fouchier *et al*. (2004)	HCoV-NL63	2004	Cell culture, VIDISCA, electron microscopy, RAP-PCR	[36] [23]
Woo *et al*. (2005)	HCoV-HKU1	2005	Consensus primers	[51]

HCoV: Human coronavirus; RAP: RNA arbitrarily primed; RT: Reverse transcriptase; SARS: Severe acute respiratory syndrome; VIDISCA: Virus discovery based on cDNA amplified fragment length polymorphism.

with the mock-infected sample. PCR fragments present exclusively in the virus-infected sample are likely to represent viral RNA. In this technique, double-stranded cDNA generated from viral RNA by reverse transcription and second-strand synthesis, both primed with random hexamers, is digested with two frequently cutting restriction enzymes, of which the recognition sequence is likely to be present in every viral target. Subsequently, oligonucleotide anchors are ligated to the digested DNA termini and provide the primer template for subsequent PCR amplification. The second round of amplification is performed in a nested format to improve sensitivity and selectivity of the method.

Random RT-PCR

The random RT-PCR protocol uses primers with a random 3′ hexanucleotide sequence that can anneal to nearly any RNA or single strand (ss)DNA. The 5′ 20 nucleotides of the primer (tail) contain a unique sequence that serves as a template for subsequent PCR primer annealing. The primer that is annealed to the RNA template is extended by reverse transcriptase with an RNase H activity that allows the reattachment of the enzyme and insertion of the tailed random primer on both 3′ and 5′ sides. The cDNA product is PCR amplified using the unique region of the initial primer.

A similar approach is RNA arbitrarily primed RT-PCR (RAP-PCR), in which arbitrarily chosen oligonucleotides are used for priming. Competition between the annealing events during the initial low-stringency cycles results in the reproducible and semiquantitative amplification of many discrete DNA fragments during the subsequent high-stringency cycles [23].

Differential display

Differential display is a method that has been developed primarily to identify and isolate genes expressed differentially in various cells or under altered conditions [37]. The method can also be used for the identification of RNA viruses with a poly-A tail, which is used as a primer template during reverse transcription. The subsequent PCR amplification uses the specific tail sequence of the oligo-dT reverse transcription primer for annealing, together with a random oligonucleotide as a 5′ primer. The addition of radiolabeled deoxyribonucleotide triphosphates (dNTPs) to the reaction allows the precise gel visualization of the products. With multiple primer sets, reproducible patterns of amplified cDNA fragments can be obtained that can be compared with the mock-infected control samples [38].

Microarrays

The use of microarrays for virus discovery is a relatively new application [39,40]. A virus-broad microarray system, consisting of 70-mer nucleotides that represent the conserved regions of

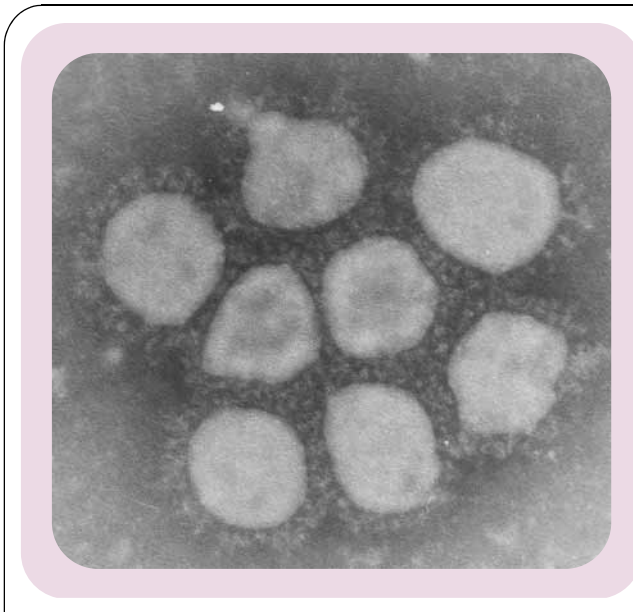

Figure 2. Negatively stained electron micrograph of human coronavirus NL63. Courtesy of Bermingham and Hoschler and the electron microscopy unit of the Health Protection Agency, Colindale, London, UK.

Table 2. Consensus primers developed for coronaviruses.

Study	Primer sequence	Targeted gene	Species included for primer design‡	Virus identified	Ref.
Escutenaire *et al.* (2006)	5´-TGATGATGS*NGTTGTNTGYTAYAA-3´ and 5´-GCATWGTRTGYTGNGARCARAATTC-3´	1b gene	SARS-CoV, HCoV-229E and HCoV-OC43, HCoV-NL63 and HCoV-HKU1	-	[53]
Moes *et al.* (2005)	5´-ACWCARHTVAAYYTNAARTAYGC-3´ and 5´-TCRCAYTTDGGRTARTCCCA-3´	1b gene	SARS-CoV, HCoV-229E, HCoV-OC43 and HCoV-NL63	-	[26]
Esper *et al.* (2005)	5´-SGCAAAATAATGAATTAATGCC -3´ and 5´-GACGCACCACCATATGAATCCTG -3´	1a gene	SARS-CoV and HCoV-229E	-	[50]
Adachi *et al.* (2004)	5´-TGATGGGTTGGGACTATCCTAAATGTGA-3´ and 5´-GTAGTTGCATCACCGGAAGTTGTGCCACC-3´	1b gene	SARS-CoV, HCoV-229E and HCoV-OC43	-	[27]
Drosten *et al.* (2003)	5′-GGTTGGGACTATCCTAAGTGTGA-3′ and 5′-CCATCATCAGATAGAATCATCATA-3′	1b gene	SARS-CoV, HCoV-229E, HCoV-OC43 and HCoV-NL63	HCoV-HKU1	[5]
Ksiazek *et al.* (2003)	5'-GGGTT-GGGACTATCCTAAGTGTGA-3' and 5'-TAACACACAACICCATCATCA-3'	1b gene	HCoV-229E and HCoV-OC43	SARS-CoV	[6]
Stephensen *et al.* (1999)	5´-ACTCARWTRAATYTNAAATAYGC-3´ and 5´-TCACAYTTWGGATARTCCCA-3´	1b gene	HCoV-229E and HCoV-OC43	-	[28]

*The degenerated positions are underlined.
‡The human coronaviruses included in the multiple alignment used for design of universal primers.
HCoV: Human coronavirus; SARS: Severe acute respiratory syndrome.

virtually all known viral species (>1000 viruses represented on a single array), was described recently [39]. Key features of this approach are the cross-hybridization of viral material to highly conserved sequence motifs and direct recovery of hybridized material from the microarray. For SARS-CoV, Wang and colleagues retrospectively stated that they were able to ascertain whether a novel coronavirus was present in the unknown sample within 24 h. They also showed that novel viruses with limited homology to known viruses can be detected successfully by this method [39]. In light of the continuous threat of emerging infectious diseases, this powerful approach will greatly help in the rapid identification and characterization of novel viruses.

Enrichment of viral nucleic acids

Cell culture

Most viruses are discovered upon culturing in susceptible cells, rather than directly in clinical specimens, owing to the increased viral RNA content and decreased number of interfering cellular nucleic acids. Unfortunately, not all viruses replicate in cell culture; for example, HCoV-HKU1 does not replicate *in vitro*. Organ cultures or airway epithelium cultures may provide a powerful *ex vivo* extension to the standard cell line-based *in vitro* cultures.

Removal of cells & mitochondria

All samples derived from patients or from cell culture contain cells, mitochondria, cell debris, mucus and other contaminants. All these components might contain cellular DNA or RNA. A centrifugation step is a very simple, but potent, purification method. Only 10 min centrifugation with 14,000 × G significantly improves the sample quality and purity [36]. Another option is sample filtration through a 0.22 µm filter [41]. Although the latter method is also very simple, the limiting factor is the large volume of sample that is needed. While centrifugation of a 110 µl specimen results in the loss of 10 µl, which is discarded together with the pellet, relatively large sample losses are encountered upon filtration.

DNase & RNase pretreatment

As mentioned above, contaminating RNA or DNA can interfere with the virus detection process. The cellular nucleic acids originate mostly from cells killed by the virus infection or from cells that are lysed during sample collection and preparation. To overcome this problem, a sample can be treated with nucleases before extraction of the RNA genome from virus particles. The general assumption is that the viral genetic material is protected inside the virus particle and that the DNase/RNase treatment will affect only the free, unprotected molecules. Although this seems to be true for DNase, RNase treatment can also decrease the viral RNA yield [VAN DER HOEK, UNPUBLISHED DATA] [36,41].

Other methods & possible applications

Apart from the methods for enrichment of viral nucleic acids that have already been used for coronaviruses, there are several alternative methods to increase the concentration of viral nucleic acid. One basic technique is ultracentrifugation, which allows the concentration of virus particles [42]. Endoh and colleagues suggested an additional interesting approach for selective amplification of viral nucleic acids. The enrichment takes place during the reverse transcription step, by using

specific random hexamers that rarely prime ribosomal RNAs but that can anneal to all known mammalian viruses listed in public databases [43]. Alternatively, especially for samples with a very low virus yield, a sequence-independent preamplification step may be included (e.g., with the random RT-PCR technique described previously). Inclusion of this assay before the universal coronavirus primer RT-PCR amplification may significantly increase the sensitivity of a virus search.

Five members of the coronavirus family: identification

HCoV-229E & HCoV-OC43

The first report about coronaviral infection in humans was presented by Tyrrell and Bynoe in 1965 [44]. The infectious material was recovered from the nasal wash of a boy with a typical common cold. The sample was obtained at the peak of disease symptoms and was subsequently inoculated in healthy volunteers who developed colds afterwards. The material obtained in these subsequent infections of volunteers was negative for the human pathogens known in the 1960s (influenza A, B and C, para-influenza 1, 2, 3, 4, respiratory syncytial virus [RSV], herpes simplex virus, enteroviruses, rhinoviruses, mycoplasma and adenoviruses). Laboratory experiments indicated that the pathogen was sensitive to ether but resistant to antibiotics. Furthermore, the pathogen could cross a bacteria-tight filter. These tests indicated that the isolated pathogen was an enveloped virus. The virus did not grow in cell culture using different cell lines and primary cells but it replicated on human trachea organ cultures. Replication was measured by the ability of the culture supernatant to induce common cold in volunteers. The initially described strain was called B814 [44].

A year later, Hamre and Procknow described the isolation and propagation in cell culture of another unknown respiratory virus. The infectious material was obtained from students at the University of Chicago with respiratory illness of unknown origin. The virus was propagated on primary human kidney cells and subsequently inoculated onto diploid human embryonic lung (HEL) cells and human fetal lung-derived, fibroblast-like (WI-38) cells. The infection resulted in slow development of cytophatic effects (CPE) first detected after 6 days. The pathogen (HCoV-229E) was shown to be ether-labile, to consist of particles approximately 89 nm in diameter and to contain RNA as genetic material [24]. The 229E virus isolate from Chicago became the prototype strain for the HCoV-229E species. Electron microscopy revealed that both B814 and 229E are similar in morphology to infectious bronchitis virus (IBV), an avian coronavirus [45,46]. A subsequent study from McIntosh and colleagues describes the isolation of several human viruses with an IBV-like morphology, among which was HCoV-OC43 [25,47]. The virus samples were obtained from individuals with common cold and inoculated in human embryonic trachea organ cultures (OC). None of these patients developed a significant antibody response to the 229E strain, providing the first evidence that OC-strains, including the initial B814 isolate, are serologically unrelated to HCoV-229E [47]. Unfortunately, the B814 sample and most of the OC isolates (except OC43) were lost over time. Therefore, it is impossible to determine whether these strains represent one of the known coronaviruses. The OC43 isolate was further propagated and is now the prototype strain for the HCoV-OC43 species.

The full genome sequences of HCoV-229E and HCoV-OC43 were obtained recently, in 2000 and 2004, respectively [48,49]. The sequence analysis confirms that HCoV-229E and HCoV-OC43 belong to separate coronavirus groups, I and II, respectively.

SARS-CoV

After the identification of SARS as a new infectious human disease, several groups made an effort to identify the responsible pathogen. It was suspected initially that the disease might be caused by *Chlamydia*, rhinoviruses or paramyxoviruses but these results could not be confirmed by other groups [5–7]. A new coronavirus was identified as the infectious agent linked to SARS by three independent research groups [5–7]. All three groups started their search by cell culture analysis, inoculating several cell lines that are used in routine diagnostics with patient specimens. A group from Hong Kong was the first to observe CPE after inoculating a lung biopsy specimen and a nasopharyngeal aspirate sample on fetal rhesus kidney cells (FRhK-4) [7]. The initial CPE of rounded refractile cells appeared 2–4 days after sample inoculation but reappeared within 24 h after subsequent passage. Furthermore, the infected cells stained positive in an immunostaining assay with sera derived from patients with SARS but not with healthy blood donor sera. The infected cells did not react with the routine panel of immunological reagents used to identify virus isolates (influenza A, B, parainfluenza types 1, 2 and 3, adenovirus and RSV) nor in RT-PCR assays (influenza A and human metapneumovirus, mycoplasma). The virus was ether-sensitive and electron microscopy showed the presence of pleomorphic enveloped virus particles of approximately 80–90 nm in diameter, with the surface morphology characteristic of coronaviruses [7].

Two other groups obtained similar results with Vero cells, including the description of coronavirus-like particles by electron microscopy [6,5]. Based on that finding, Ksiazek and colleagues designed universal coronavirus primers based on the sequence of the RNA-dependent RNA polymerase gene of several coronaviruses [6].

At the same time, Drosten and colleagues and Peiris and colleagues amplified the virus from their cell culture supernatants with random RT-PCR techniques [5,7]. Peiris and colleagues applied differential display primers and cloned the PCR fragments [7], whereas Drosten and colleagues utilized degenerated primers under low-stringency conditions [5]. All studies revealed that a novel viral agent was present, constituting a new species within the genus *Coronaviridae*. Full genome sequencing showed that the virus is not a recent recombinant of known coronavirus species but a distinct member of the group II coronaviruses [5–7].

HCoV-NL63

In January 2003, a 7-month-old child was admitted to a hospital in Amsterdam with coryza, conjunctivitis, bronchiolitis and fever [36]. A nasopharyngeal aspirate sample was collected 5 days after the onset of disease and subsequently tested for known respiratory pathogens. Diagnostic tests for RSV, adenovirus, influenza viruses A and B, rhinovirus, enterovirus, HCoV-229E and HCoV-OC43 were negative. The clinical sample (NL63) was inoculated in cell culture (human fetal lung fibroblasts, tertiary monkey kidney cells [tMK; Cynomolgus monkey] and HeLa cells). CPE was detected on tMK cells at day 8 after inoculation. The observed CPE was diffuse, with a refractive appearance followed by cell detachment. CPE was more pronounced when the virus was passaged onto a monkey kidney cell line (LLC-MK2). Acid lability and chloroform sensitivity tests indicated that the virus was probably enveloped. The sample was analyzed with the VIDISCA method, followed by full-length genome sequencing, which revealed that HCoV-NL63 is a previously unknown group I coronavirus.

Soon after the first publication, another group from The Netherlands reported on the same human coronavirus [23]. This study described the isolation of an unidentifiable virus from a nose swab sample collected from an 8-month-old boy suffering from pneumonia in The Netherlands in April 1988. The virus was inoculated on tMK cells, in which it caused CPE after 7 days, affecting approximately 50% of the cells after 13 days. The virus was subsequently passaged, which resulted in CPE development on tMK and Vero cells. Supernatants of infected tMK and Vero E6 cells were used for negative contrast electron microscopy analysis, revealing the presence of coronavirus-like particles with an average diameter of 140 nm and average envelope projections of 20 nm. The cell cultures appeared negative for HCoV-OC43 and HCoV-229E in diagnostic assays. Subsequently, the sample was analyzed with RAP-PCR and RT-PCR with primers specific for the coronavirus family.

Remarkably, 1 year after the publications of both Dutch groups, Kahn and colleagues described the identification of essentially the same virus, which was given a novel name: New Haven (NH) coronavirus [50]. Clinical respiratory specimens were screened for coronaviruses using universal primers based on the conserved regions of the 1a replicase gene of groups I, II and III. The screening was performed on pooled samples (5–10 specimens per pool) and 17 out of 80 pools yielded an expected amplicon of approximately 550 bp. Most samples (15 out of 17) contained the known coronaviruses HCoV-OC43 or HCoV-229E or human DNA but two pool samples contained the NH coronavirus. The sequences of these 550 bp fragments are very similar to the isolates from The Netherlands (94–100% at nucleotide level) and represent the same species. Considering that the publication appeared 10 months after the first HCoV-NL63 report, this study cannot be regarded as the one discovering this viral species.

In 2005, the coronavirus study group of the International Committee on Taxonomy of Viruses (ICTV) executive committee advised the use of HCoV-NL63 as the proper species name for all related viruses.

HCoV-HKU1

The index patient for HCoV-HKU1 was a 71-year-old Chinese man who was admitted to hospital in January 2004 owing to fever and productive cough with purulent sputum for 2 days [51]. A chest radiograph showed patchy infiltrates over the left lower zone. A nasopharyngeal aspirate was used for direct antigen detection of several respiratory viruses and RT-PCR assays for influenza A virus, human metapneumovirus and SARS-CoV. All tests were negative. Additionally, the ability of the virus to grow in cell culture was assayed. The nasopharyngeal aspirate was inoculated on several cell lines: RD (human rhabdomyosarcoma), I13.35 (murine macrophage), L929 (murine fibroblast), HRT-18 (colorectal adenocarcinoma) and B95a (marmoset B-lymblastoid), and on a mixed neuron-glia culture. No CPE was observed. Since no known microbiological agent could be identified, research was initiated to identify novel agents. An RT-PCR amplification with universal coronavirus primers resulted in a 440 bp product, of which the sequence clusters with the *Coronaviridae* in phylogenetic analysis. Full-length sequencing revealed that HCoV-HKU1 is a previously unknown group II coronavirus [51].

Summary

The main technical problem during the search for novel coronaviruses is the low virus yield in clinical samples. This precludes the selective but sequence nonspecific amplification of viral RNA. Most human coronaviruses have been identified after cell culture enrichment [5–7,23,36]. Once the virus can be propagated efficiently in cell culture, any of the molecular biology tools developed for virus discovery can be used to identify the pathogen. This is nicely illustrated by the discovery of SARS-CoV. Once the virus was cultured, it was identified by a variety of methods: random RT-PCR, differential display random amplification and universal primers [5–7]. However, the identification of a new coronavirus directly from patient material is significantly more difficult. The only approach that resulted in successful identification of a novel coronavirus directly from the clinical specimens was the universal coronavirus primer RT-PCR [51]. Enrichment of viral nucleic acids is a prerequisite for the use of sequence-independent amplification strategies, such as VIDISCA and random RT-PCR. This can be achieved by selective purification (e.g., by ultracentrifugation or centrifugation/filtration) or by selective amplification of viral RNA (e.g., by using nonribosomal random hexamer primers) [36,41,43].

Expert commentary

Given the recent explosion in the number of newly identified human coronaviruses (2003: SARS-CoV, 2004: HCoV-NL63, 2005: HCoV-HKU1), one wonders whether we currently know the complete arsenal of coronaviral pathogens. The SARS case demonstrated that new invaders can come from the animal kingdom via a zoonotic transfer. There are 25 known animal coronavirus species, including mammals and birds, of which ten were identified in the last year. This means that the count is

not finished yet and we should be aware of new introductions. A curious aspect of coronavirus pathology is the almost exclusive link to respiratory diseases in humans, whereas coronaviruses can also cause enteric, cardiovascular and neurological disorders in animals [2,52]. Thus, one should keep an eye open for such symptoms and disease correlations in humans. Finally, coronaviruses are commonly addressed as common cold viruses, except for SARS-CoV. This neglects the disease course frequently seen in young children, which can be much more serious, although usually not life threatening. This necessitates the further improvement of diagnostic tools and warrants the development of antiviral drugs.

Five-year view

Significant progress in the field of human coronaviruses has been made within the last 3 years. This includes the identification of three novel coronaviruses, of which two (HCoV-NL63 and HCoV-HKU1) have been circulating in humans for many years, whereas the third one (SARS-CoV) was introduced recently from an animal reservoir. As the search is in progress, we might expect the identification of more previously unknown human coronaviruses in the coming years. It will be important to perform a broad virus search of clinical samples derived from different tissues; for example, the gastrointestinal tract or the CNS. The further improvement of nucleic acid purification and amplification methods will accelerate virus discovery programs and may result in the identification of new viral species that are characterized by a low virus load (e.g., in chronically infected patients).

Several reports have described broad anticoronaviral drugs but at present there are no commercially available wide-spectrum agents. To prepare for a possible zoonotic transfer of animal coronaviruses, emphasis should be placed on the identification and commercialization of such compounds.

Key issues

- No pathogens can be identified in a relatively large proportion of patients with respiratory disease.
- There are 25 known animal coronavirus (CoV) species of which ten were identified in the last year.
- There are five known human CoVs, of which three were identified in the last 4 years.
- CoVs are known to infect mammals and birds and cause a variety of diseases, including gastroenteritis and respiratory tract disease.
- In humans, currently identified CoVs are exclusively associated with respiratory tract illnesses.
- The severe acute respiratory syndrome (SARS) epidemic began in 2003 and has affected approximately 8000 individuals, 10% of whom have died.
- The example of SARS-CoV shows that new coronavirus invaders can come from the animal kingdom via a zoonotic transfer.
- The improvement of molecular tools should lead to the identification of more CoV pathogens.
- There is a need for further improvement of diagnostic tools and antiviral drugs.

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