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Epidemiology and Clinical Presentations of the Four Human Coronaviruses 229E, HKU1, NL63, and OC43 Detected over 3 Years Using a Novel Multiplex Real-Time PCR Method

E. R. Gaunt,1 A. Hardie,2 E. C. J. Claas,3 P. Simmonds,1 and K. E. Templeton2*

Centre for Infectious Diseases, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, United Kingdom;1 Department of Virology, Royal Infirmary of Edinburgh, 51 Little France Crescent, Old Dalkeith Road, Edinburgh EH16 4SA, United Kingdom;2 and Department of Medical Microbiology, Center for Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands3

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Four human coronaviruses (HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43) are associated with a range of respiratory outcomes, including bronchiolitis and pneumonia. Their epidemiologies and clinical characteristics are poorly described and are often relied on case reports. To address these problems, we conducted a large-scale comprehensive screening for all four coronaviruses by analysis of 11,661 diagnostic respiratory samples collected in Edinburgh, United Kingdom, over 3 years between July 2006 and June 2009 using a novel four-way multiplex real-time reverse transcription-PCR (RT-PCR) assay. Coronaviruses were detected in 0.3 to 0.85% of samples in all age groups. Generally, coronaviruses displayed marked winter seasonality between the months of December and April and were not detected in summer months, which is comparable to the pattern seen with influenza viruses. HCoV-229E was the exception; detection was confined to the winter of 2008 and was sporadic in the following year. There were additional longer-term differences in detection frequencies between seasons, with HCoV-OC43 predominant in the first and third seasons and HCoV-HKU1 dominating in the second (see Results for definitions of seasons). A total of 11 to 41% of coronaviruses detected were in samples testing positive for other respiratory viruses, although clinical presentations of coronavirus mono-infections were comparable to those of viruses which have an established role in respiratory disease, such as respiratory syncytial virus, influenza virus, and parainfluenza viruses. The novel multiplex assay for real-time pan-coronavirus detection enhances respiratory virus diagnosis, overcomes potential diagnostic problems arising through seasonal variation in coronavirus frequency, and provides novel insights into the epidemiology and clinical implications of coronaviruses.

Four human coronaviruses (human coronavirus 229E [HCoV-229E], HCoV-HKU1, HCoV-NL63, and HCoV-OC43) are associated with a range of respiratory symptoms, including high-morbidity outcomes such as pneumonia and bronchiolitis (26, 31, 35). Specifically, HCoV-NL63 has been associated with group (33) and HCoV-HKU1 with febrile convulsion (18). Coronaviruses are frequently codetected with other respiratory viruses, particularly with human respiratory syncytial virus (HRSV) (17). Whether coronaviruses contribute to disease severity in such coinfections is currently unclear. Other coronaviruses infecting humans include human enteric coronavirus, which is closely related to HCoV-OC43 and is associated with necrotizing enterocolitis and gastroenteritis (10, 27).

Coronaviruses are globally distributed (7, 32, 34, 38), although there are differences in the frequency of detection of the four viruses in different parts of the world at different times (6, 11, 15, 16, 22, 28, 29). Longitudinal studies of coronavirus epidemiology are lacking in the literature and are restricted to descriptions representing a maximum of 1 year for all four respiratory coronaviruses or 2 years for three coronaviruses (9, 17, 18). This inevitably makes direct comparisons of the coronavirus difficult with respect to their epidemiologies, clinical presentations, and etiological roles in respiratory disease.

To directly address this situation, we conducted a large-scale survey of the incidences and epidemiological and clinical correlations of the four coronaviruses detected among 11,661 respiratory samples collected over a 3-year study period in Edinburgh, United Kingdom. Clinical presentations of study subjects infected with each of the coronaviruses were directly compared, and their roles in pediatric and adult respiratory disease were assessed.

MATERIALS AND METHODS

Sample collection. Respiratory samples collected at hospital and primary care settings in southeast Scotland are referred to the Royal Infirmary of Edinburgh (RIE) Specialist Virology Centre (SVC) for routine respiratory virus screening. Referral of samples to the RIE SVC is done by patient assessment following established clinical protocols. Samples were labeled with anonymous identifiers and deposited in the SVC sample archive prior to testing. Samples were stored at −80°C. A range of sample types are referred, including bronchial, nasal, nasopharyngeal (the most common category), oral, and tracheal samples.

A total of 11,661 respiratory samples from 7,383 patients referred to the RIE SVC between July 2006 and June 2009 were labeled with anonymous identifiers and archived with approval from the Lothian Regional Ethics Committee (08/ S11/02/2). Stored data included age band, recorded clinical information, referral source, month of sample collection, and results of routine virological testing of the sample. As part of routine virological screening, RNA and DNA were extracted using a Qiagen BioRobot chips and screened for HRSV, adenovirus (Adv), parainfluenza viruses 1, 2, and 3 (PIV-1 to PIV-3) and influenza A and B viruses by the use of multiplex one-step real-time reverse transcription-PCR (RT-PCR) (14, 30). Extracted nucleic acids were stored at −20°C.
Clinical data interpretation. Clinical symptoms recorded on sample referral forms were categorized into seven groups—inflammocompromised, chronic respiratory condition, lower respiratory tract infection (LRTI), upper respiratory tract infection (URTI), other, none, and no data. Inevitably, some samples were assignable to two or more categories, and so categories were treated in a hierarchical manner, whereby samples were preferentially assigned to categories in the order listed above. The immunocompromised group included patients with carcinoma and transplant patients. Coryza, cough, and sore throat were classified as URTIs. Bronchitis, bronchiolitis, pneumonia, influenza-like illness, and suspected HRV infection were classified as LRTIs. Samples for which no clinical data were available were excluded from the clinical data analysis. Chronic respiratory conditions included asthma and cystic fibrosis. The “other” group included symptoms which may be associated with but are not restricted to infection with respiratory pathogens, e.g., pyrexia, neurological and gastrointestinal symptoms, and requirement for ventilation. The “none” group included symptoms or data not associated with respiratory tract infection, e.g., rash or urinary tract infection.

Sensitivity of real-time multiplex PCR for coronavirus detection. RNA transcripts of the four coronaviruses were used as standards to determine assay sensitivity. HCoV-229E, HCoV-NL63, and HCoV-OC43 cDNAs (processed using nuclear acid extraction and reverse transcription as described below) were subjected to single-round real-time PCR as described below. PCR products were cloned into PCR2.1 T/A cloning vector (Invitrogen, Paisley, United Kingdom) and were transcribed in vitro using a Promega (Southampton, United Kingdom) T7 RiboMAX large-scale RNA production system. HCoV-HKU1-positive cDNA underwent PCR, using a sense primer incorporating a T7 promoter site for DNA amplification. DNA was cleaned using a Qiagen (Crawley, United Kingdom) QIAquick PCR purification kit and was transformed into BL21(DE3) competent Escherichia coli. Cultures were plated on agar containing lactose and kanamycin (50 μg/ml) and grown overnight at 37°C. Blue colonies were picked, and successful transformation was confirmed by sequence analysis. Products were purified using a Qiagen (Crawley, United Kingdom) Qiaquick spin miniprep kit and were transcribed in vitro using a Promega (Southampton, United Kingdom) T7 RiboMAX large-scale RNA production system. Reactions were terminated and RNAs purified using a Qiagen (Crawley, United Kingdom) RNaseasy mini-kit. Coronavirus RNA transcript purity and concentration were established using a nanodrop spectrophotometer (Nanodrop Technologies). The sensitivity of the multiplex assay was established for each coronavirus by testing transcripts of known concentrations serially diluted in Tris-E2TA (TE) buffer. Cycle threshold (Ct) values for each set of transcripts were established using multiplex and monoplex assays to detect any loss of sensitivity resulting from multiplexing. Specificity of real-time multiplex PCR for coronavirus detection. A panel of respiratory viruses and bacteria commonly found in the respiratory tract was used to determine the specificity of the multiplex PCR. Nucleic acids were extracted from samples positive for influenza A virus, influenza B virus, HRV, PIV-1 to PIV-4, AdV type 5, human metapneumovirus, rhinoviruses 1b and 16, echovirus 7, mumps virus, measles virus, Bordetella bronchiseptica, Bordetella parapertussis, Bordetella pertussis, Burkholderia cepacia, Chlamydia pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus pneumoniae and tested by multiplex PCR under the conditions described below.

Real-time multiplex PCR for coronavirus detection. Nucleic acids were pooled in groups of 10. Pools were screened for HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43 by one-step multiplex RT-PCR using a Qiagen (Crawley, United Kingdom) one-step RT-PCR kit for amplification of the membrane glycoprotein (HCoV-229E and HCoV-OC43) or the nucleocapsid phosphoprotein (HCoV-HKU1 and HCoV-NL63). Each 20-μl reaction mixture contained 5 μl of 5× reaction buffer, 1 μl of deoxynucleoside triphosphate (dNTP) (10 μM), 2 μl of MgCl2 (25 mM), 0.3 μl of each of the inner primers for HCoV-HKU1, HCoV-NL63, and HCoV-OC43 (10 nM). Each 0.4 μl of each inner primer for HCoV-229E, 0.25 μl of each fluorescent probe for each coronavirus (Table 2) (50 nM), and 1 μl of enzyme mix. Cycling conditions were 95°C for 30 s, 95°C for 15 min, and then 50 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 90 s. Amplified nucleic acid was detected using a Bioluminescence ABI machine.

Conventional nested PCR for coronavirus detection. Component nucleic acids from coronavirus-positive pools were screened using reverse transcription and nested PCR for their respective coronaviruses. This method was shown to be as sensitive as the real-time PCR (data not shown) and was undertaken (instead of repeated real-time PCRs) to reduce diversion of resources, a strategy that was of particular relevance during the influenza pandemic, and also served to reduce the cost of the reagents required to screen so many samples. Reverse transcription was conducted using a Promega (Southampton, United Kingdom) A3500 reverse transcription system. Each 20-μl reaction mixture included 4 μl of MgCl2 (25 mM), 2 μl of 2× reaction buffer, 2 μl of dNTPs (10 μM), 1 μl of random primers (10 μM), 0.5 μl of RNAsin, and 15 units of reverse transcriptase. Reactions were conducted according to the manufacturer’s instructions, with an extended elongation step of 5 min. Each 20-μl PCR mixture contained 4 μl of MgCl2 (25 mM), 0.2 μl of dNTP (3 mM), 1 μl of each species-specific sense and antisense primer (10 nM) (Table 1), and 0.4 units of Taq polymerase. The first-round reaction mixture contained 2 μl cDNA, and the second-round mixture contained 1 μl of the first-round product. Cycling conditions for first- and second-round PCRs were 94°C for 21 s, the virus-specific annealing temperature (Table 2) for 18 s, and 72°C for 90 s, and cycle numbers were also virus specific (Table 2) and were followed by a hold at 72°C for 5 min. PCR products were detected by agarose gel electrophoresis.

Coronavirus Ct values. Respiratory RNA was tested for coronaviruses by pooling RNAs in groups of 10 and screening using four-way multiplex RT-PCR. Identification of coronavirus-positive components of coronavirus-positive RNA pools was done using reverse transcription, nested PCR, and gel electrophoresis. Coronavirus-positive RNA identified by the nested PCR was then retested using the real-time RT-PCR to establish Ct values for individual samples.

Statistical analyses. Statistical analyses were conducted (unless otherwise stated) using a two-tailed Fisher’s exact test with a threshold for significance of P < 0.05, using population parameters defined by the sample set.

RESULTS

Sensitivity and specificity of four-way multiplex RT-PCR for coronavirus detection. Serial dilutions of coronavirus RNA transcripts were tested using the multiplex PCR. No differences in Ct values were found when transcripts were tested using single primers and probes compared to using multiplexed primers and probes (results not shown). The fluorescent signal observed at various dilutions for each virus type corresponded to calculated minimal amounts of detectable RNA copy numbers of 66 for HCoV-229E, 9 for HCoV-HKU1, 69 for HCoV-
NL63, and 18 for HCoV-OC43 in 10 μl of RNA. No nonspecific reactions were observed. As RNAs were pooled in groups of 10, 1 μl of each RNA was tested in the initial screening process. Consequently, the minimum theoretical concentration of viral RNA required for detection was likely a 10-fold increase compared to the calculated values.

Detection of coronaviruses in Edinburgh and assay sensitivity. All coronavirus-positive pools detected by real-time PCR were found to include at least one coronavirus-positive component by nested PCR. Some pools comprised samples positive for different coronaviruses, some contained more than one sample positive for the same coronavirus, and two samples were found to be positive for both HCoV-OC43 and HCoV-NL63. Of the 11,661 samples, 267 (2.30%) were positive for at least one coronavirus (Table 2); of all virus detections, 8.15% were of coronaviruses.

Coronavirus-positive samples detected by the nested PCR were rescreened using real-time RT-PCR to establish $C_T$ values; of these, 86.2% (232/269) tested positive for coronavirus by the real-time RT-PCR. In some cases, a sample that tested as negative on rescreening represented the only coronavirus-positive sample in its pool; therefore, these results were not attributed to false positives in the nested PCR screening.

**Patient parameters.** Coronaviruses were detected in all age groups, most frequently (4.86%) in the 7- to 12-month age category (Fig. 1). Significantly more males (64.6%; $P = 0.0332$) were infected with HCoV-OC43 than females. More males than females were infected with HCoV-NL63, but this result

![FIG. 1. Percentages of detection frequencies of the four coronaviruses HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43 by age group. The total number of samples within each age band is indicated at head of each column.](image-url)
did not reach significance (62.7%; $P = 0.132$). HCoV-HKU1 and HCoV-229E infected approximately equal numbers of males and females (the proportions of those infected who were male were 50.8% and 51.4%, respectively).

**Circulation trends.** Coronaviruses displayed the marked seasonality typical of other respiratory viruses, with high detection frequencies in winter months but few or no detections in the summer (Fig. 2). The exception was HCoV-229E, which was detectable primarily in the winter of the second year of the study and sporadically through the third year of study.

HCoV-OC43 displayed biennial peaks in detection frequency; it was present in 2.96% of samples collected in the first season (December 2006 to April 2007), in only January of the second season (November 2007 to March 2008) (0.83%), and in 3.17% of samples collected in the third season (November 2008 to January 2009) ($P < 0.0001$ for the first and third seasons compared to the second).

For HCoV-HKU1, in contrast to the pattern described for HCoV-OC43, the peak detection frequency occurred in the second season (1.92% of samples collected in November 2007 to March 2008). Conversely, when HCoV-OC43 was circulating at the highest frequency, detection frequencies of HCoV-HKU1 did not exceed 1% ($P < 0.0001$ for the first and third seasons compared to the second).

HCoV-NL63 was detected later than other coronaviruses in all three seasons (in December or January), and detection frequencies peaked in February to April. Detection frequencies between seasons were more consistent than for the other coronaviruses, although a significantly higher HCoV-NL63 detection frequency was observed in the first year of study than in the second and third years ($P < 0.001$ and $P = 0.0200$, respectively).

**Clinical associations of coronavirus infections.** Clinical data were available for 6,068/11,661 (52.0%) of samples included in the study. Although high rates of coinfection with other respiratory viruses were detected (11 to 41% of coronavirus-positive samples tested positive for at least one other respiratory virus), single infections associated with respiratory outcomes were observed for three coronaviruses (HCoV-HKU1, HCoV-NL63, and HCoV-OC43). HCoV-OC43, HCoV-NL63, and HCoV-HKU1 had clinical profiles similar to those of respiratory viruses currently included in routine diagnostic screening (Fig. 3A), with HCoV-OC43 associated with LRTI in over 40% of single infections for which clinical data were available—a higher proportion than that seen with influenza A virus. HCoV-229E was not associated with any cases of LRTI or URTI in otherwise healthy individuals and was found in over 70% of cases representing samples from immunocompromised patients (Fig. 3B).

Coronaviruses were not associated only with respiratory outcomes. Febrile convulsions were not reported for any patients testing singly positive for coronavirus, although one patient testing singly positive for HCoV-HKU1 had meningitis and one patient testing singly positive for HCoV-OC43 had seizures. Of 35 samples testing singly positive for HCoV-OC43, two were associated with vomiting, though one of these was taken from an immunocompromised patient.

Croup has previously been associated with HCoV-NL63 infection (19, 33, 37) but was not reported in association with any of the 41 samples with clinical data testing singly positive for HCoV-NL63; 3 samples were associated with cough and 1 was associated with wheezing.

For children under 2 years of age with LRTI, coronaviruses represented the only virus detected in 20 of 916 (2.18%) samples, compared with a rate of 5 of 336 (1.49%) samples collected from patients over the age of 45 with LRTI. The difference was not significant ($P = 0.5$).

For all four coronaviruses combined, the detection frequency in samples from patients with respiratory symptoms (URTI and LRTI) exceeded the proportion seen with samples taken from patients whose symptoms were categorized as “none” (i.e., no respiratory symptoms; Fig. 3C), providing epidemiologic evidence for the role of these three viruses in the etiology of respiratory disease.

No differences were found between the $C_T$ values of coronavirus-positive samples associated with differing respiratory outcomes (data not shown).

**Coinfections.** High rates of coinfection with other respiratory viruses were observed for all coronaviruses (Table 2). Similar rates of LRTI and URTI were observed in single coro-
FIG. 3. (A) Comparison of the clinical presentations of study subjects infected with coronaviruses to the clinical presentations of those infected with respiratory viruses included in diagnostic screening (adenovirus [AdV], influenza A virus, influenza B virus, PIV-1, PIV-2, PIV-3, and HRSV). Only single infections are included in the analysis. The y axis indicates proportions of subjects with symptoms or diagnoses of LRTI or URTI or symptoms not associated with respiratory infection (“None”). (B) Proportions of samples testing singly positive for each of the respiratory viruses from patients with immunosuppression. (C) Proportions of samples from patients with LRTI or URTI or with no respiratory symptoms (“None”) testing singly positive for each respiratory virus.
Coronavirus infections (HCoV-HKU1 and HCoV-OC43) compared to cases in which a coronavirus was detected as part of a mixed infection. LRTI and URTI were observed at a higher frequency in single compared to mixed infections with HCoV-NL63 (Table 3).

Of all samples testing positive for coronavirus and another respiratory virus, 63.5% tested positive for HRSV. Coronavirus infection seasonality most closely resembles that of influenza (Fig. 2), and so the coinfection frequency of HRSV with coronaviruses is compared to the coinfection frequency of HRSV with influenza virus. Of 1,300 HRSV-positive samples, 63.5% tested positive for HRSV. Coronaviruses NL63 and HCoV-HKU1 were specifically associated with lower respiratory tract disease in the study population. Three "cold" viruses. Here we demonstrate that HCoV-OC43, HCoV-NL63 detection peaked in winter and spring (January to April), which contrasts with the summer-to-autumn seasonality reported to occur in the tropics (4, 18), though the autumn-to-winter seasonality of HCoV-HKU1 and HCoV-OC43 infections and lack of a discernible seasonality of HCoV-229E were epidemiologically comparable features of both regions.

In 42% and 38% of infections with HCoV-OC43 and HCoV-HKU1, other respiratory viruses were codetected, most commonly HRSV. There were significantly more HRSV-positive samples that tested positive for coronavirus compared to the numbers of samples testing negative for all viruses included in routine diagnostics (one-tailed test; \( P = 1.34 \times 10^{-5} \)). The high rate of coinfection of coronaviruses with HRSV observed here was previously demonstrated (17). No differences in clinical outcome were observed for those coinfected with HRSV and coronavirus compared with those singly infected with either virus, and so HRSV presumably facilitates coronavirus infections (or vice versa) without exacerbating morbidity. HCoV-OC43 was significantly more frequently detected in hospitalized males than in females, an attribute that HCoV-OC43 shares with HRSV (8, 21, 24). No difference was observed between the sex distribution of HCoV-OC43 in singly infected subjects and the sex distribution in those coinfected with HRSV, indicating that the observed sex bias was coincidental and is not attributable to high coinfection rates.

The coronavirus load in singly positive samples was not different from the coronavirus load in samples in which co-pathogens were detected. This suggests that infection with another respiratory virus does not affect the ability of coronaviruses to establish infection and further demonstrates that, in mixed infections in respiratory patients, detection of coronaviruses should not be interpreted as representing an incidental infection that does not contribute to disease.

HCoV-229E differed clinically from other coronaviruses and/or high-dependency units, with presentations that included severe lower respiratory tract disease and pyrexia, and case reports have associated all coronaviruses with high morbidity and/or fatal outcomes (23, 25, 26, 34).

HCoV-OC43 was the most commonly detected coronavirus, followed by HCoV-NL63 and HCoV-HKU1, with similar moderate detection frequencies, and HCoV-229E, with a comparatively low detection frequency; this observation has been previously reported (18). However, in contrast to the results of a previous study (18), we found that HCoV-HKU1 was detected as frequently in patients with LRTI as in those with URTI, and there was a lack of association between HCoV-229E infection and respiratory symptoms in otherwise healthy individuals. HCoV-NL63 detection peaked in winter and spring (January to April), which contrasts with the summer-to-autumn seasonality reported to occur in the tropics (4, 18), though the autumn-to-winter seasonality of HCoV-HKU1 and HCoV-OC43 infections and lack of a discernible seasonality of HCoV-229E infections were epidemiologically comparable features of both regions.

### DISCUSSION

Human coronaviruses are increasingly recognized as respiratory pathogens associated with a broadening range of clinical outcomes, whereas they were once recognized as "common cold" viruses. Here we demonstrate that HCoV-OC43, HCoV-NL63, and HCoV-HKU1 were specifically associated with lower respiratory tract disease in the study population. Three of the four coronaviruses were more frequently detected as the sole pathogen in subjects with LRTI than in those with no respiratory symptoms and were detected as often as PIV-1 and PIV-2 (Fig. 3A). Samples representing monoinfections were frequently (26/184 [14.1%]) from subjects in intensive care and/or high-dependency units, with presentations that included severe lower respiratory tract disease and pyrexia, and case reports have associated all coronaviruses with high morbidity and/or fatal outcomes (23, 25, 26, 34).

### TABLE 3. Detection frequencies of coronavirus cases of LRTI and URTI

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>Detection frequency (%)</th>
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<tbody>
<tr>
<td></td>
<td>HCoV-229E (all)</td>
</tr>
<tr>
<td></td>
<td>Single</td>
</tr>
<tr>
<td>LRTI</td>
<td>0</td>
</tr>
<tr>
<td>URTI</td>
<td>0</td>
</tr>
</tbody>
</table>

### TABLE 4. Coronavirus \( C_T \) values for singly infected and coinfect ed samples

<table>
<thead>
<tr>
<th>CoV ( C_T ) value</th>
<th>No. of samples with indicated result</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCoV-229E</td>
</tr>
<tr>
<td></td>
<td>Single infection</td>
</tr>
<tr>
<td>&lt;30</td>
<td>15</td>
</tr>
<tr>
<td>30–35</td>
<td>15</td>
</tr>
<tr>
<td>&gt;35</td>
<td>0</td>
</tr>
</tbody>
</table>

\( C_T \) values were available for most coronavirus-positive samples (229/286; Table 4). Few differences in \( C_T \) value were observed between singly infected coronavirus-positive samples and coronavirus-positive samples also testing positive for another virus (Table 4).
other respiratory viruses in general; 70% of detections were in samples from immunocompromised patients. HCoV-229E has previously been associated with infections of immunocompromised individuals (9, 26). The further association of HCoV-NL63 with this group was previously suggested in a report of an outbreak in young adults. V. C. C. Cheng, P. C. Y. Woo, S. K. P. Lau, L. M. Rhodes, 1974. Clinical disease in children associated with newly described coronavirus subtypes. Pediatrics 197(2):70–76.

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Development of a novel pan-coronavirus multiplex PCR allows unique insights into the epidemiology and clinical implications of infections by the four respiratory coronaviruses. Fluctuating circulation frequencies of coronaviruses during respiratory seasons are reported here for the first time. All coronaviruses are associated with high coinfection rates, and detection of HCoV-229E infection should be particularly considered for an alternative diagnosis for immunocompromised patients with respiratory tract disease.


