

Optimized Laboratory Detection Strategy for COVID-19 Patients Reduces the Rate of Missed Diagnosis

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Abstract

Background: Novel coronavirus pneumonia (NCP) is an emerging, highly contagious community acquired pneumonia (CAP) caused by severe acute SARS-CoV-2. Nucleic acid test currently played a crucial role in diagnosis of suspected COVID-19 patients. However, a high false-negative rate of this “gold standard” test has been reported and posed a major setback in blocking the spread of the virus. We here aim to describe an optimized laboratory detection strategy to reduce the false negative rate.

Methods: Suspected NCP patients were asked to collect both coughed up specimen and pharyngeal swab. Samples from the same patient were mixed and tested at a single pool. SARS-CoV-2 was then detected by real-time RT-PCR using two different detection kits. Only if both results were negative was the test reported as negative. The patients will be excluded after two consecutive negative tests at 24 hour intervals. We also used multiplex PCR to detect 13 common respiratory tract pathogens (RTP).

Results: Using this strategy, we confirmed 85 SARS-CoV-2 infections from 181 suspected patients, and 94.12% of patients were positive in the first test. The 96 excluded patients were followed up, and no additional NCP was found. We also found that 31.25% patients in 96 non-NCP patients were infected with at least one RTP that may cause CAP.

Conclusion: Our studies suggest that dual reagents screening with pooled coughed up specimen and pharyngeal swab samples reduced the false negative rate of nucleic acid testing. During the epidemic of NCP in Anhui province, there was a certain proportion of infection and co-infection of other common pathogens of CAP. In comparison with SARS-CoV-2 detection alone, combining multiple pathogen detection reduces the rate of miss diagnosis.

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which was confirmed as the cause of the unexplained pneumonia has resulted in a rapid outbreak throughout China and global spread, and posed a significant threat to public health[1–6]. According to a recent study, the transmission capacity of SARS-CoV-2 was higher than the severe acute respiratory syndrome coronavirus (SARS-cov)[7]. In the meantime, no specific antiviral treatment has been confirmed to be effective, treatment is currently mainly symptomatic[8–10]. Therefore, early diagnosis, early isolation and early management are crucial to curb rapid spread of the virus and improve the prognosis of the patients[11].

Novel coronavirus pneumonia (NCP) is an emerging community acquired pneumonia (CAP). The clinical symptoms of NCP include fever, dry cough, muscle pain and so on[8]. Ground-glass opacity and bilateral patchy shadowing were the radiological features of the NCP patients[8, 12]. Whereas some pathogens, such as RSV, parainfluenza virus may share similar imaging features (Fig. 1). SARS-CoV-2 nucleic acid test is recommended as the standard for diagnosis of the disease. oropharyngeal sample is commonly used as a screening tool. However, according to the recent studies, the “gold standard” test exhibits high false-negative rates, resulting in delayed or missed diagnosis and posed a major setback in blocking the

spread of the virus[9, 13–15]. There is thus a pressing need to evaluate and optimize the current protocols, reagents and approaches.

Many reasons may lead to false negative results of nucleic acid testing for SARS-CoV-2, including insufficient viral load in the specimen, nonstandard method during sampling, or reagent quality[13, 15]. To reduce the false negative rate, suspected NCP patients were asked to collect both coughed up specimen and pharyngeal swab. Samples from the same patient were mixed and tested at a single pool. SARS-CoV-2 was detected by real-time RT-PCR using two different reagents.

The winter is the season of high incidence of CAP, SARS-CoV-2 nucleic acid detection can't clearly suggest which pathogen is responsible for the SARS-CoV-2 negative patients, nor can it determine whether the positive patients have co-infection. In order to improve the etiological diagnosis, 13 different respiratory tract pathogens (RTPs) along with SARS-CoV-2 were simultaneously detected in suspected NCP patients.

In this study, we used an optimized laboratory detection strategy for suspected NCP patients, which allowed rapid diagnosis of infection and co-infection with SARS-CoV-2 and other common pathogens of community-acquired pneumonia, while significantly reduced the rate of missed diagnosis.

Methods

Patients

We recruited 181 patients with suspected SARS-CoV-2 infection from Jan 21 to Feb 29, 2020 at the First Affiliated Hospital of University of Science and Technology(USTC) of China in Hefei, China. This hospital is designated to treat NCP patients in Anhui Province. All of the suspected patients enrolled in this study met the criteria of the latest “New Coronavirus Pneumonia Prevention and Control Program”[16]. Written consents were obtained from all of the patients and the study was approved by the Ethics Review Committee of the First Affiliated Hospital of USTC.

Microbial etiological diagnostic strategy for suspected NCP patients

1. Detection of novel coronavirus nucleic acid

On the admission day, pharyngeal swab samples were collected by specialized physicians according to WHO guidelines. After collection, the pharyngeal swabs were placed into a collection tube with transport swab buffers. Patients were also asked to cough as forcefully as possible, and the secretions and other matter that has been coughed up from the lungs and respiratory tract were collected. The patients were specifically advised that all coughed up specimens which may included sputum, mucus, and saliva, should be collected. Then transport swab buffers were taken from the collection tube and mixed with the

coughed up specimens from the same patient. A volume of 300 microliter from the pooled tube was used to extract total RNA and total RNA was extracted using an automatic nucleic acid extraction instrument (TANBead, China). SARS-CoV-2 was then detected by real-time RT-PCR using two different detection kits. One kit selected ORF1ab gene as the target sequence for detection (Beijing Genomics Institution, China), and the other one used ORF1ab, E gene and N gene as the target sequences (Bioperfectus technologies, China). In brief, the total RNA was added to the reaction reagent and dual fluorescence PCR (Applied Biosystems 7500 Real-Time PCR Systems, USA) was performed according to the manufacturer's instructions. Only if both results were negative was the test reported as negative. If the first SARS-CoV-2 nucleic acid test is negative, the respiratory specimens will be collected again after 24 hours for another test. According to the Chinese "New Coronavirus Pneumonia Prevention and Control Program"[16], only when both nucleic acid test results are negative can the patient be excluded.

2. Detection of common pathogens in community acquired pneumonia using the SureX 13 respiratory pathogen multiplex kit

The SureX 13 respiratory pathogen multiplex kit (Ningbo Health Gene Technologies Ltd Ningbo, China) was used to detect 13 common pathogens of CAP, including influenza A virus (FluA), pandemic influenza A virus-2009 (09H1), seasonal H3N2 virus (H3), influenza B virus (FluB), respiratory syncytial virus (RSV), adenoviruses (AdVs), rhinovirus (HRV), bocavirus (HBoV), metapneumovirus (HMPV), parainfluenza virus (PIV, including PIV-1, 2, 3 and 4), coronavirus (CoV, including OC43, 229E, NL63 and HKU1), Chlamydia [Ch, including Chlamydia trachomatis (Ct) and Cp] and Mycoplasma pneumoniae. In brief, the extracted nucleic acid was mixed with primer pairs targeting the 13 tested RTPs and RT-PCR enzyme mix, and the PCR amplification was performed in Biosystems 7500 Real-Time PCR Systems, followed by capillary electrophoresis. PCR products were then separated by size on the Applied Biosystems 3500Dx Genetic Analyzer. The signals of the 15 labeled PCR products were finally measured by fluorescence and analyzed with GeneMapper 4.1 software (Termo Fisher Scientific, Waltham, MA, USA).

Follow-up

Clinical data were obtained from patients' medical records. The patients were followed up until Mar 31, 2020.

Results

Dual reagents screening with pooled coughed up specimen and pharyngeal swab samples reduced the rate of false negative.

In total, 181 patients were enrolled in this study. Of the 181 suspected patients, 85 patients were confirmed of SARS-CoV-2 infection by using our optimized strategy through real-time RT-PCR assays. Of these 85 confirmed patients, 80 (94.12%) patients were positive in the first test. 96 patients were excluded

after two consecutive negative tests at 24 hour intervals (Fig. 2). The 96 excluded patients were observed in hospital and followed up after discharge, and no additional NCP was found.

Detection of pathogens other than SARS-CoV-2 in community acquired pneumonia

Suspected patients were detected for 13 respiratory tract pathogens other than SARS-CoV-2. Of the 85 NCP patients, one patient was also infected with adenovirus and the other with rhinovirus. Of the 96 non-NCP patients, common CAP pathogens were detected in 30 patients. Among them, one single pathogen infection was detected in 28 patients, and two pathogens were found in 2 patients. The most common pathogens were influenza A virus (18.75%), influenza B virus (18.75%), *Mycoplasma pneumoniae* (18.75%) and metapneumovirus (15.63%), followed by several other pathogens (Figs. 2 and 3).

Treatment and outcome of patients

NCP patients were treated based on the severity of the disease according to latest “New Coronavirus Pneumonia Prevention and Control Program”. Non-NCP patients with a definite microbial diagnosis were treated according to the international community acquired pneumonia guidelines. By Mar 8 of 2020, 84 (98.82%) NCP patients was discharged from hospital (including 29 patients with severe NCP) and 1 (1.17%) patient died from the disease. The average hospitalization time of NCP patients was 16.79 days. The dead case was a 55-year-old man and was transferred to our hospital after confirmed as SARS-CoV-2 infection. He was diagnosed with severe pneumonia, severe respiratory failure and cerebral apoplexy. One day after admission to our intensive care unit (ICU), the patient died from a sudden cardiac arrest. 91 (94.79%) non-NCP patients were discharged from hospital, 4 (4.17%) were transferred to other hospitals for further treatment, and 1 (1.04%) died.

Discussion

The SARS-CoV-2 is a positive-sense, single-stranded RNA virus and is the seventh member of enveloped RNA coronavirus[4, 5]. The SARS-CoV, which caused an epidemic in 2002–2003, also belongs to this genus. Infection with these two viruses often leads to lower respiratory tract disease with poor clinical outcomes associated with old age and underlying health[17], while SARS-CoV-2 seems to be more transmissible than SARS-CoV[7]. The virus is transmitted mainly through respiratory droplets or close contact. A recent study revealed that the R_0 of SARS-CoV-2 is 3.77, meaning that each infector could transmit the virus to another 3.77 people, which is higher than the R_0 of SARS (2.0–3.0)[18]. As of May. 6th of 2020, data from the World Health Organization (WHO) have shown that more than 3 595 662 patients have been confirmed to be infected with SARS-CoV-2 in 215 countries/regions[19]. Therefore, accurate identification and subsequent isolation of NCP patients plays a vital role in blocking the spread of the virus.

Currently, the most commonly used golden standard for NCP diagnosis is a positive result of the real-time RT-PCR assay. However, Ai et al. reported that 3 suspected patients were negative in real-time RT-PCR

assay and were later confirmed by metagenomics sequencing[9]. Wang et al. also reported that some patients were negative in the first three tests and turned to positive on the fourth test[20]. A recent report by Li et al. which included 610 patients with COVID-19 revealed that the detection of SARS-CoV-2 by qRT-PCR had a high false negative rate, which is easy to cause missed diagnosis[13]. Some scholars believe that there is an urgent need for improvement experimental reagents or methods to minimize the risk of false negative results and ongoing transmission based on a false sense of security[14, 21]. In this study, we confirmed 85 patients infected with SARS-CoV-2 from 181 suspected patients. 94.12% of patients were positive in the first RT-PCR test. Moreover, none of the patients who was negative in our first two tests were later diagnosed as NCP after discharge from hospital. The low false negative rate of our assay might be due to the fact that we collected and detected both cough up specimens and pharyngeal swab samples. The pharyngeal swab was the most common sample used to detect for SARS-CoV-2 in China[22]. However, Wang et al reported that the SARS-CoV-2 RNA was detected only in 32% of oropharyngeal swabs[22]. On the one hand, this may be because the sampling techniques affects the quality of the pharyngeal swab sample. On the other hand, viral loads in the oropharynx varied at different times of disease progression. During the early stage of infection, a single patient nasopharyngeal swab may harbor close to 1 million SARS-CoV-2 viral particles. As the disease progresses, NCP patients' lesions tends to be located in the lower respiratory tract[14]. In order to avoid missed diagnosis caused by low viral load in pharyngeal swab, all samples coughed up by patients, including sputum, mucus, saliva, etc. were collected simultaneously in our study. Some studies reported that sputum specimen has a higher viral load and should be collected if possible [23]. A recent study reported that SARS-CoV-2 can be detected in the self-collected saliva of 91.7% (11/12) of patients and live virus was detected in saliva, suggesting that saliva is a promising noninvasive specimen for diagnosis in patients with 2019-nCoV infection[24]. Samples from the same patient were mixed and tested at a single pool. Pooling diagnostic tests has been applied in other infectious diseases and has been proven to work for RT-qPCR when reagents are expected to be in short supply[25]. Sample pooling has recently been reported for the early comprehensive screening of SARS-CoV-2 [26]. Furthermore, we detected SARS-CoV-2 with two different kits simultaneously, and only if both results were negative was the test reported as negative. If the results of the two reagents are not consistent, we will use another viral detection testing platform for re-examination. These measures overcomeed the problem of false negative caused by the test performance characteristics or quality of the reagent itself.

In our study, 13 RTPs detections were performed in 85 NCP patients and 2 (6.06%) patients were found to be co-infected with adenovirus or rhinovirus. Both of these two patients didn't develop into severe conditions, but recovered and were discharged 20 and 24 days after being treated with Coolidge plus Ribavirin. Our findings were consistent with a recent study which reported that none of 99 patients had co-infection of other respiratory viruses[4]. However, Ai et al. reported a high co-infection rate by using mNGS assay which found 5 out of 20 NCP patients co-infected with other viruses[9].

Of the 96 non-NCP patients, 31.25% (30/96) patients were detected at least one respiratory tract pathogen by the SureX 13 respiratory pathogen multiplex kit. The most common pathogen was influenza (including influenza A and B), followed by *Mycoplasma pneumoniae*, and metapneumovirus. Our findings

were consistent with previous studies that reported the detection rate of pathogens in CAP adults as 30–40%[27]. Respiratory viruses were detected more frequently than bacteria. The most common pathogens in winter were human rhinovirus and influenza virus[27, 28]. In addition, about 70% of the 96 non-NCP patients in this study did not have any specific etiological diagnosis, which may be due to the specimens that we used for the detection being pharyngeal swabs but not alveolar lavage fluid. The positive rate of pharyngeal swab specimen in diagnosing CAP is usually low[28]. The SureX 13 respiratory pathogen multiplex kit used in this study did not include all common CAP pathogens such as Legionella and Chlamydia psittaci. It was reported that detection of alveolar lavage fluid with mNGS was able to detect more respiratory pathogens in CAP patients[29]. However, due to the difficulty in obtaining such samples and the high cost of the detection, mNGS was not used in this study.

Earlier studies on NCP patients in Wuhan reported high mortality as 11.1%-14.6%[1, 4]. However, recent studies based on large sample sizes with cases throughout China found a markedly lower case fatality rate (3.92%)[19]. In this study, the mortality of our 85 laboratory-confirmed NCP patients was only 1.18%. This finding is consistent with a most recent study, reporting the mortality of 1.4% of 1,099 cases[8].

Conclusion

In conclusion, in the present study, we found that pharyngeal swab samples can be combined with specimens coughed up by patients to provide higher diagnostic yields. Meanwhile, we found a certain proportion of infection and co-infection of other common pathogens of CAP during the NCP epidemic in Anhui province. This suggested that improvement of accuracy in microbiological laboratory detection is necessary to guide specific and precise treatment for the CAP patients. Please note that this is a single-center study with limited number of patients. The conclusions of this study need to be further verified by expanding the sample size.

Declarations

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Ethics approval and consent to participate

This study was approved by the Ethics Review Committee of the First Affiliated Hospital of USTC. Written consents were obtained from all of the patients.

Consent for publication

Not applicable.

Potential conflicts of interest

The authors declare no conflict of interest.

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Figures

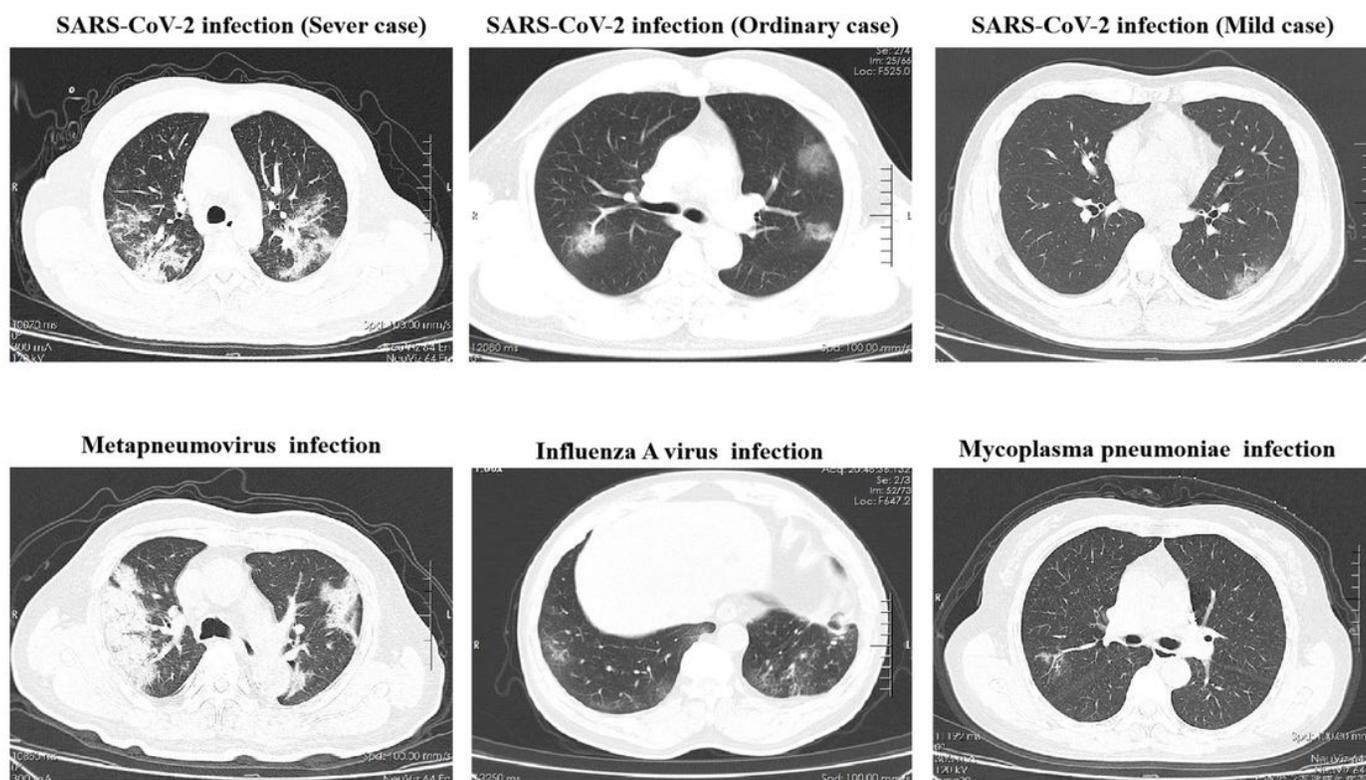


Figure 1

Chest computed tomographic Images of patients with COVID-19 and other pathogens.

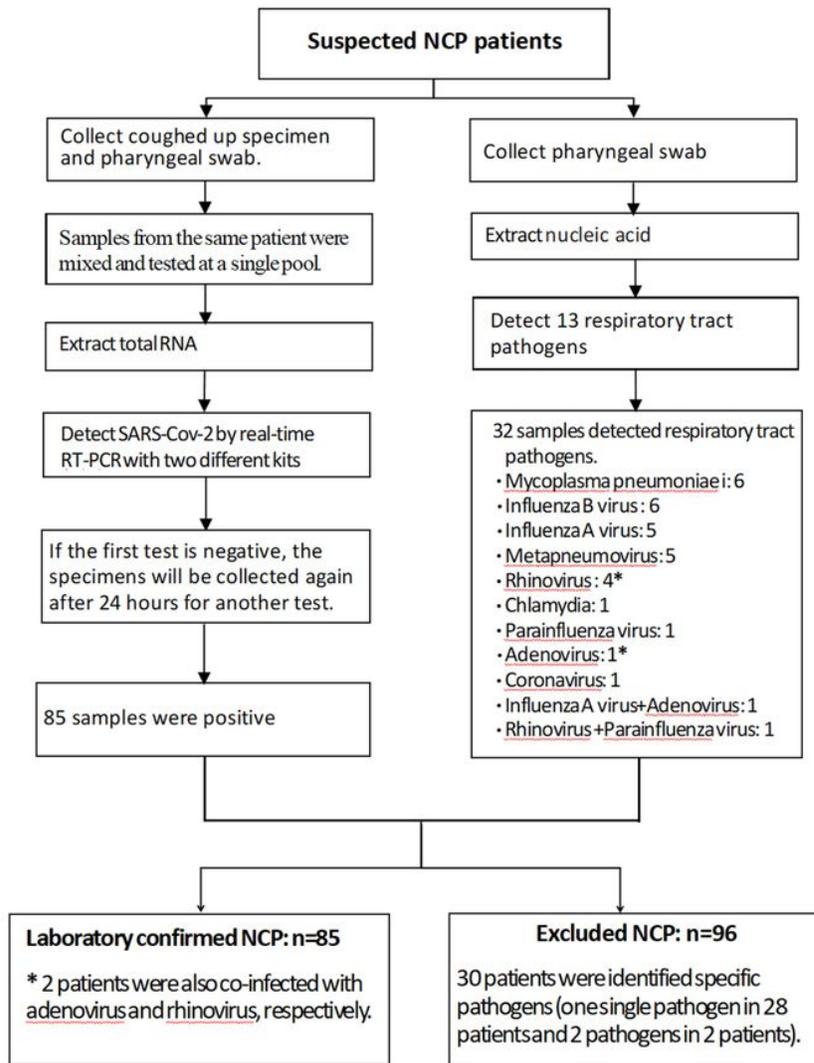


Figure 2

Flow chart for differential diagnosis of suspected NCP patients.

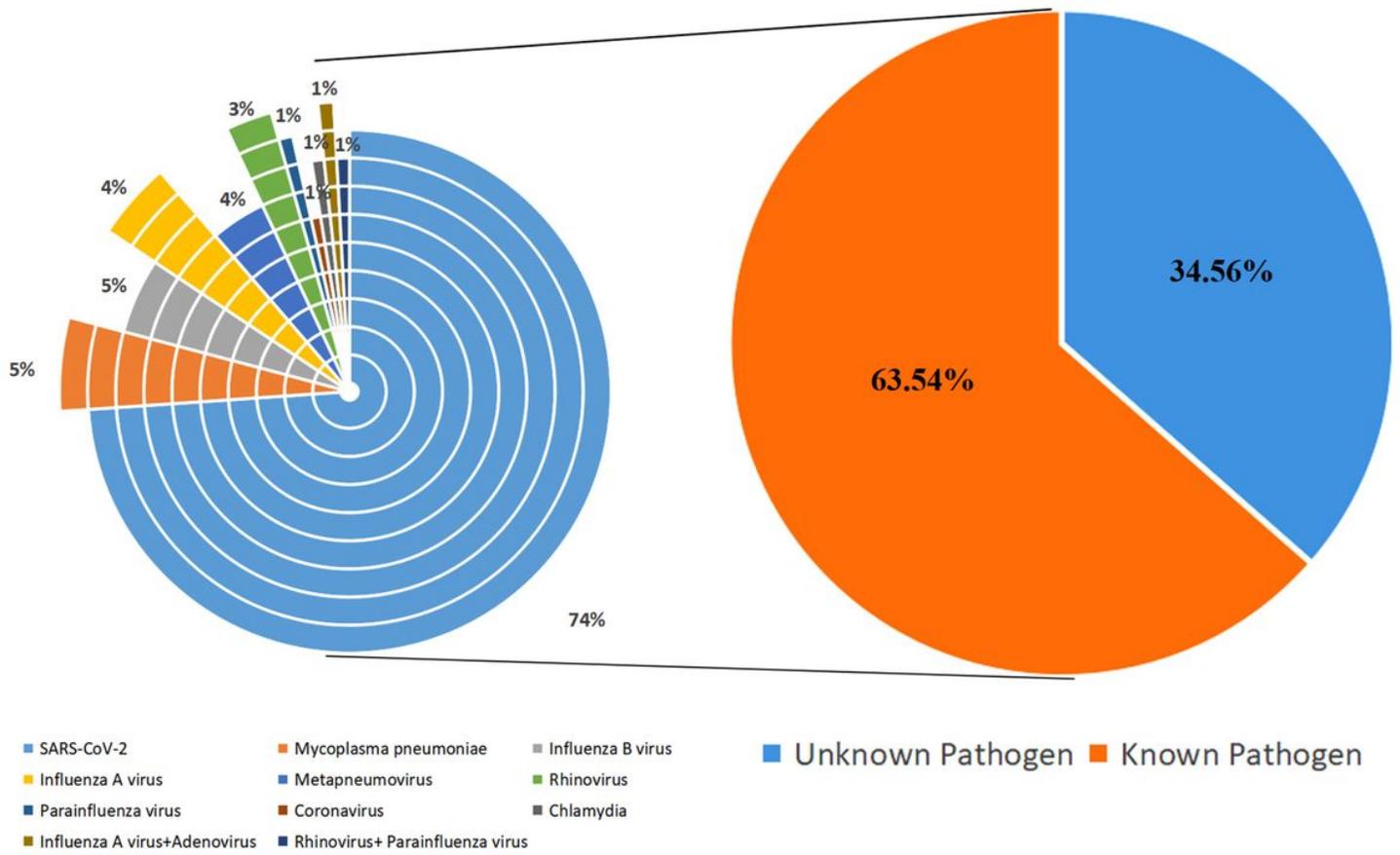


Figure 3

Distribution of pathogens in 181 hospitalized patients