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CASE REPORT

Molecular Testing of SARS-CoV-2 Infection from Blood Samples in Disseminated Intravascular Coagulation (DIC) and Elevated D-Dimer Levels

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SUMMARY

Background: **In 2020, the SARS-CoV-2 virus spread worldwide and infected more that 10 million people, causing more than 500,000 deaths worldwide. The infection has systemic effects on the respiratory and cardiovascular systems; thus, patients can present a variety of symptoms from asymptomatic to rapid deaths.**
In this paper, we present the first case of post-mortem SARS-CoV-2 molecular testing in Western part of Romania in a deceased with disseminated intravascular coagulation (DIC) and elevated D-dimer levels.
Methods: **During the autopsy which took place at the Institute of Forensic Medicine from Timisoara, Romania, blood sample was collected in a vacutainer with EDTA and sent to the Laboratory of Forensic Genetics from Victor Babes University of Medicine and Pharmacy, Timisoara, Romania. Viral RNA extraction was performed automated on the Maxwell 48 RSC Extraction System (Promega, USA) using the Maxwell RSC Viral Total Nucleic Acid Purification kit (Promega, USA). After RNA extraction, the samples were amplified on a 7500 real-time PCR (Applied Biosystems, USA) using the genesig® Real-Time PCR Assay (Primer Design, UK).**
Results: **The molecular testing showed a cycle threshold value of 23.4 (1.2 x 10^6 copies/mL), indicating increased viral loads, which correlated with the laboratory analysis results, especially with D-dimer levels.**
Conclusions: **In cases of coagulopathy of SARS-CoV-2, patients in hospitals should be monitored closely for thrombosis development. Thus D-dimer can be used as prognostic marker in monitoring the evolution of SARS-CoV-2 infected patients.**
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KEY WORDS

coagulopathy, severe acute respiratory syndrome, coronavirus 2 (SARS-CoV-2), disseminated intravascular coagulation (DIC), intensive care unit (ICU)

INTRODUCTION

In December 2019, a novel β-coronavirus was identified in Wuhan, China, which caused a cluster of pneumonia cases. The virus was named the 2019-novel coronavirus by the World Health Organization (WHO) on January

12, 2020. It soon emerged as a global health concern [2]. Over 1 million deaths were reported worldwide due to coronavirus SARS-CoV-2. Coronaviruses are a family of single-stranded RNA viruses that can infect both animals and humans, leading to respiratory, neurological, hepatic, cardiac, and gastrointestinal diseases [1]. The diagnosis of SARS-CoV-2 is based on clinical examination including chest X-ray examination, computer tomograph imaging (CT), and molecular laboratory test based on real-time reverse-transcription PCR (rRT-PCR).

The most common symptoms among infected patients are fever, dry cough, fatigue and dyspnea with or without nasal congestion [3,4]. Patients with mild symptoms do not present such signs, but those with severe symptoms present shortness of breath, weakened breath sounds, moist rales in lungs and a typical symptom of SARS-CoV-2 infection - the fever [5,6]. Chaolin et al., in their study on 41 patients, found the most common symptoms to be fever (98%), cough (76%), fatigue or myalgia (44%), and some atypical symptoms like sputum (28%), hemoptysis (5%), headache (85%), and diarrhea (3%). Lymphocytopenia was observed in 63% of the patients. The common complications observed in these cases were acute respiratory distress syndrome (29%), acute heart injury (12%), and secondary infections in 10% of patients. Of the patients studied, 32% needed treatment in the intensive care unit (ICU).

The chest X-ray examination revealed multiple small patchy shadows and interstitial changes present in the lung periphery in the early stages of pneumonia [8]. The chest CT scan revealed ground-glass opacity, infiltrating shadows, pulmonary consolidation, and pleural effusions. A study of CT scans in 21 patients diagnosed with SARS-CoV-2, coordinated by Chung et al., revealed that three patients (21%) had normal CT scans, 12 patients (57%) had ground-glass opacity on CT scans, and six patients (29%) showed ground-glass opacity and pulmonary consolidation [9].

Laboratory testing included virus isolation and viral nucleic acid detection. In case of the Sars-CoV-2 virus, numerous specimens, such as oropharyngeal swabs, nasal swabs, sputum, lung tissue, bronchoalveolar lavage fluid (BAL), blood samples, saliva, and feces, could be used for molecular detection [10]. Real-time reverse-transcription polymerase chain reaction (rRT-PCR) of nasopharyngeal swabs were used to confirm the infection [11]. RNA was isolated from different clinical specimens and determined by rRT-PCR. During the analysis, the cycle threshold values used as indicators had to be less than 36 to be interpreted as positive for SARS-CoV-2 RNA infection, while values ranging from 36 to 38 were considered suspicious cases and those greater than 38 were considered negative cases [12-14]. The values of the rRT-PCR cycle threshold were used as indicators of the SARS-CoV-2 RNA copy number in biological samples with lower threshold values. This coincided with an increase in viral copy numbers [25,26].

Swab samples need to be placed in universal or viral transport media. In Wang et al.'s study, oropharyngeal swabs were used more frequently in China during the SARS-CoV-2 pandemic as compared to nasopharyngeal swabs [15].

Serological tests can also provide the advantage of quick results and low-cost analysis. A recent study from China demonstrated that both immunoglobulin M (IgM) and immunoglobulin G (IgG) were found five days after the onset of the infection in 39 patients with SARS-CoV-2 [16]. These serological assays were used for antibody detection in the diagnosis of novel cases in SARS-CoV and MERS-CoV [17,18].

Disseminated intravascular coagulation (DIC) is one of the manifestations of SARS-CoV-2 infection, because it enables severe sepsis and acute respiratory distress, leading to a cascade of multifactorial events. In infected patients, SARS-CoV-2 associated coagulopathy is used to describe the changes in blood circulation and coagulation detected in the autopsy.

In this article, we present the first case of post-mortem SARS-CoV-2 molecular testing in Western part of Romania, from blood samples collected during an autopsy. The case had DIC and increased levels of D-dimer at the time of admission in the ICU.

MATERIALS AND METHODS

In February 2020, during an autopsy at the Institute of Legal Medicine in Timisoara, Romania, blood sample was collected in a vacutainer containing EDTA for the molecular testing of SARS-CoV-2. The blood sample was sent for SARS-CoV-2 molecular testing to the Laboratory of Forensic Genetics from Victor Babes University of Medicine and Pharmacy in Timisoara, Romania. The presence of patches in the external aspect of the body and the aspect of internal organs such as the lungs and the liver gave rise to suspicions of DIC with SARS-CoV-2. The patient had been admitted to the ICU with acute respiratory distress (ARDS) and had required orotracheal intubation and had died a few hours later. Antemortem, molecular SARS-CoV-2 testing was performed on this patient. The patient's ante-mortem laboratory results of tests taken at admission into the ICU are presented in Table 1.

RNA extraction

Manual pre-processing

Using a 1.5 mL Eppendorf tube (Eppendorf, Germany) [19], a lysis solution consisting of 200 µL of lysis buffer, 20 µL proteinase K solution [20] and 20 µL of internal extraction control (IEC) was prepared (Primer Design, United Kingdom) [21]. In another 1.5 mL sterile cryotube, 100 µL of blood sample was added to the lysis solution and the obtained solution was vortexed for 10 seconds. The mixture solution was then incubated for 10 minutes at 56°C in an Eppendorf Thermo Mixer C (Eppendorf, Germany) [19].

Table 1. Ante-mortem laboratory results on admission in ICU.

Biological parameters	Laboratory values at admission
C- reactive protein (CRP)	452 mg/L
Fibrinogen	15.2 g/L
D-dimer	15,992.7 ng/µL
Leucocytes	3,000/mm^3
Thrombocytes	82,000/mm^3
GPT	325 U/L
GOT	998 U/L
Creatine-kinase (CK)	44,734 U/L
Creatine-kinase muscle-brain (CK-MB)	976 U/L
Lactic acid dehydrogenase (LDH)	2,247 U/L
International normalization ratio (INR)	2.16
pH	6.7

Table 2. Amplification steps following recommendations for genesig® Real-Time PCR Assay (Primer Design, UK).

Steps	Time	Temperature	Cycles
1. Reverse transcription	10 minutes	55°C	1
2. Denaturation (Taq activation)	2 minutes	95°C	1
3. Denaturation	10 seconds	95°C	45
4. Annealing and extension	60 seconds	60°C	

Automated RNA extraction

The automated RNA extraction was performed using the Maxwell 48 RSC System (Promega, USA) [22] and the Maxwell RSC Viral Total Nucleic Acid Purification kit (Promega, USA) [20].

Amplification of the products

After the viral RNA extraction, the master mix solution was prepared using the Coronavirus COVID-19 kit (Primer Design, UK) [21]. The reagents were 10 µL of Oasig™ OneStep 2 x RT qPCR Master Mix and 2 µL of COVID-19 and IEC Primer & Probe. After the preparation, 12 µL of master mix solution was added to the PCR wells on a plate followed by 8 µL of sample, positive control template (PCT), and negative extraction control (NEC). The PCR plate was sealed with a MicroAmp™ Optical Adhesive film (Thermo Fisher, USA) [23], and was gently centrifuged to the PCR plate using the Axygen Axyspin Mini plate Spinner Centrifuge (Corning, USA) [24] to collect the contents at the bottom of the plate.

RESULTS

During the autopsy, macroscopic examinations found the lungs to be large and heavy because of the retained fluids. The lung surfaces presented signs of pleurisy. Fresh thrombi were also present in different organs such as the lungs, liver, and lower members. The pathologist concluded that the cause of death was DIC with septic shock and multi-organ failure due to SARS-CoV-2 infection.

At the microscopic examinations which were performed using hematoxylin and eosin (HE) staining, the lungs revealed hematic thrombi adherent to the vascular wall. The interalveolar septal walls were thick due to capillary stasis, as presented in Figure 1.

The amplification plot of SARS-CoV-2, with a cycle threshold value of 23.4 (1.2 x 10^6 copies/mL), is presented in Figure 2, indicating the increase in viral loads. In this case, SARS-CoV-2 viral loads were correlated with laboratory values.

DISCUSSION

Currently the 'gold standard' in detection of SARS-CoV-2 infection is rRT-PCR. Even if the presence of nucleic acids cannot be quantified, it is well-known that lower cycle threshold value correlates with an increased virus load in the biological specimen during the amplification process [26].

Since the beginning of the outbreak, many studies have demonstrated that abnormal coagulation parameters are associated with SARS-CoV-2 infection. Reports from different Wuhan hospitals have shown that of the 99 patients hospitalized, 6% presented an increased activated partial thromboplastin time (aPTT) and 5% had increased prothrombin (PT); 36% presented elevated D-dimer levels, interleukin-6 (IL-6), erythrocyte sedimentation rate, and C-reactive protein [27].

Another report from two hospitals from Wuhan, that included 191 patients, reported a mortality rate of 28% (54 patients) [28]. From presented factors associated with mortality, the report included elevated D-dimer > 1 µg/mL on admission, increased PT levels and increased IL-6. In these cases, there were pre-existing comorbidities such as age, diabetes, hypertension, and coronary artery disease. An increased D-dimer level was associated with increased mortality rate in all the cases [29]. From the Institute of Legal Medicine in Hamburg, Germany, Edler et al. presented a study of the first 80 autopsies of SARS-CoV-2 in the city, where macroscopic examinations revealed a large spectrum of changes such as bilateral pneumonia, bronchitis, and emphysema in the lungs [30]. The study also suggested an adverse im-

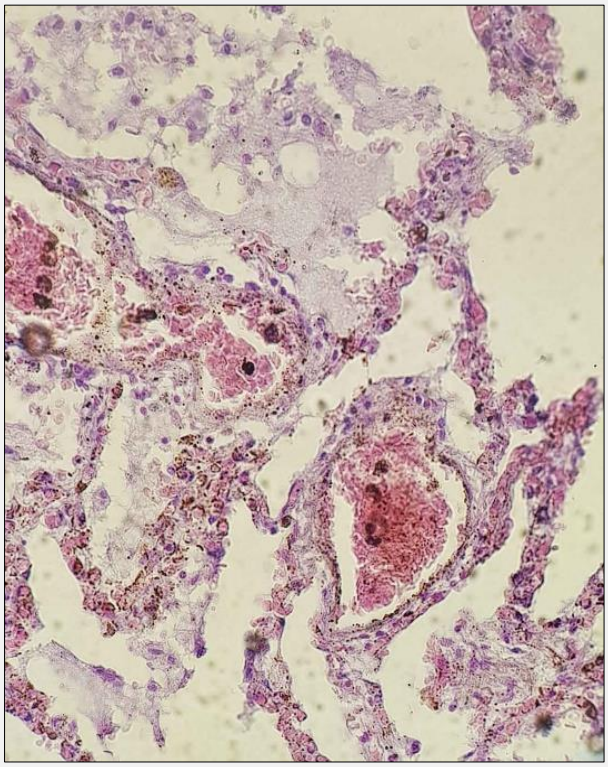

Figure 1. Microscopic lung section with H&E staining (40 x magnification).

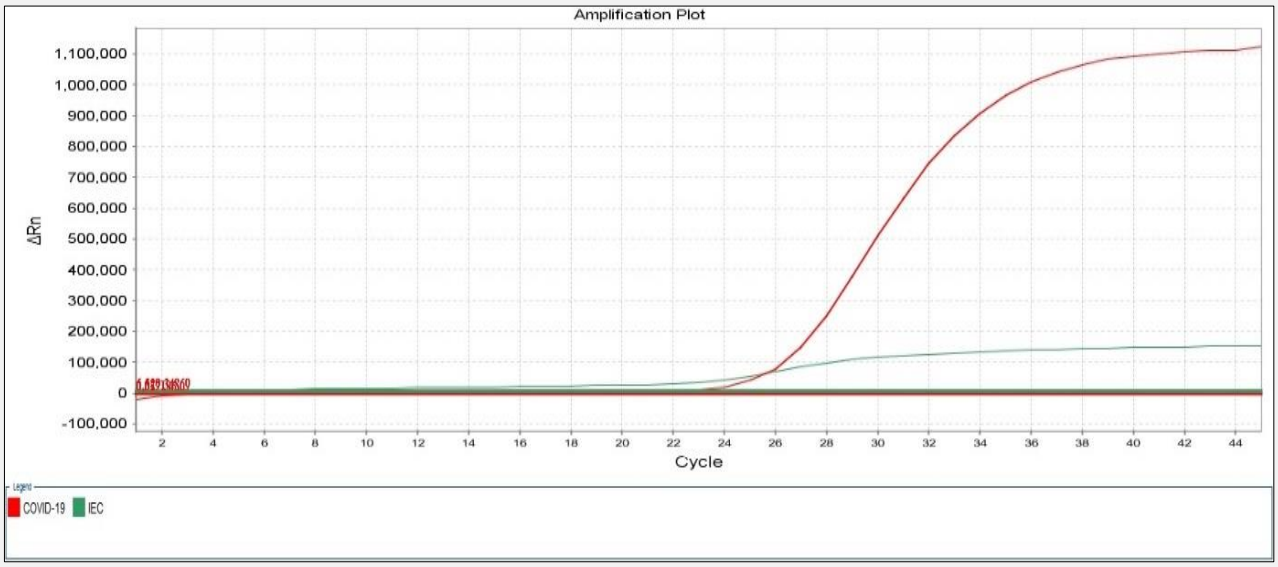

Figure 2. Amplification plot of SARS-CoV-2 on 7500 real-time PCR (Applied Biosystems, USA).

pact on the internal organs: congestion of the liver and spleen, signs of arteriosclerosis in kidneys, and the presence of fresh thrombi in the lower legs and other organs. Edler et al. reported that SARS-CoV-2 pneumonia was detected in more than 83% of the cases. In most cases, the virus affected the lungs as it entered the respiratory epithelium through the angiotensin-converting enzyme 2 (ACE2) receptor [31].

Other research groups reported that pulmonary embolism and coagulopathy were very frequently seen in SARS-CoV-2 patients [32].

Many reports explained the importance of detecting D-dimer levels in patients infected with SARS-CoV-2 on their admission in hospitals, as it could be used as a prognostic marker to monitor the progression and outcomes of the disease.

Our autopsy findings correspond to other post-mortem reports in cases of SARS-CoV-2 infection with coagulopathy with multiple organ failure.

To our knowledge, this is the first post-mortem case of SARS-CoV-2 infection with DIC, multi-organ failure and elevated D-dimer levels, detected in Western part of Romania by molecular testing.

CONCLUSION

Our data suggest that D-dimer could be used as prognostic marker after the patient's admission to the ICU because its rapid increase during SARS-CoV-2 infection with coagulopathy is associated with a significant risk of mortality over the following few hours due to multiple organ failure.

Declaration of Interest:

The authors report no conflict of interest.

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