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Review

Positive direct antiglobulin test in COVID-19 patients: Decision-making process

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ABSTRACT

In this unprecedented crisis of severe acute respiratory syndrome coronavirus 2 and its associated coronavirus disease 2019 (COVID-19), polymerase chain reaction and then serological testing platforms have been massively developed to face the important screening demand. Polymerase chain reaction and serological testing platforms are not the only actors impacted by the crisis, transfusion services are facing important difficulties. A positive direct antiglobulin test is frequently observed for patients encountering COVID-19. Patients with severe symptoms may develop anaemia and become good candidates for blood transfusions. The interpretation of a positive direct antiglobulin test for patients recently transfused and suffering from COVID-19 is complex. The differentiation between COVID-19 induced antibodies and possible associated transfusion alloantibodies is therefore crucial. In this context, the elution technique incorporated in an appropriate decision-making process plays its full role. This intricate topic is presented through a case report followed by literature review and finally decision-making process for COVID-19 patients necessitating red blood cells administration.

Abbreviations

Ab	Antibody
Ag	Antigen
AHG	Anti-Human Globulin
AIHA	Autoimmune Haemolytic Anaemia
CAS	Cold Agglutinin Syndrome
COVID-19	Coronavirus Disease 2019
CRP	C-Reactive Protein
DAT	Direct Antiglobulin Test
ECMO	Extracorporeal Membrane Oxygenation
IAHA	Immuno-Allergic Haemolytic Anaemia
ICU	Intensive Care Unit
IAS	Irregular Antibody Screening
IAT	Indirect Antiglobulin Test
Nl	normal
PCR	Polymerase Chain Reaction
RBCs	Red Blood Cells
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SOP	Standard Operating Procedure

1. Introduction: case report

1.1. Presentation

A 43-year-old man was hospitalised for hepatorenal syndrome. The patient was confirmed positive to SARS-CoV-2 at the admission by PCR testing. In the first weeks, the patient received oxygen supplementation (2L per day) and presented only minor COVID-19-related symptoms: dyspnoea on exertion, lack of appetite and asthenia. During his stay, the patient faced many cirrhosis complications such as spontaneous bacterial peritonitis, urinary tract infection, terlipressin-induced ischemic skin necrosis

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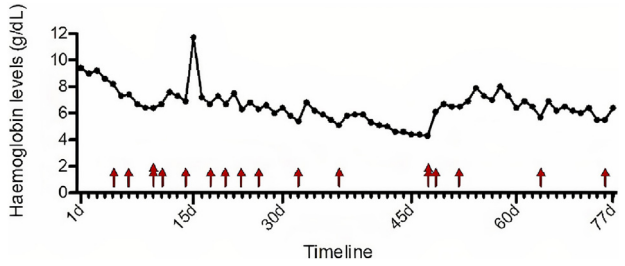

Fig. 1. Time follow-up of haemoglobin levels and administration of red blood cells.

Table 1
Haemolysis parameters.

Parameter	Observation	Argument for Haemolysis
Reticulocyte	From 2 to 4 *10^3/μL (Nl: 30–100 *10^3/μL)	Should be elevated but acute renal impairment may diminish EPO levels
Haptoglobin	1,91 g/L and 2,03 g/L (Nl: 0,30–2,00 g/L)	Should be collapsed but hepatic impairment decreases haptoglobin synthetizing function while inflammatory syndrome has upregulated effect on haptoglobin level
LDH	Elevated but constantly decreasing	Should be increased but levels lessen correlated to hepatic function improvement
Bilirubin	Elevated but constantly decreasing	Should be increased but levels lessen correlated to hepatic function improvement
Haemoglobinuria and bilirubinuria	Not significant	May be observed in severe haemolysis
Blood smear	No significative number of schistocytes	Generally positive in AIHA
DAT	Positive	Positive in AIHA

EPO: Erythropoietin/DAT: Direct Antiglobulin Test/AIHA: Auto-Immune Haemolytic Anaemia.

with associated haemorrhage, oesophagitis, polyneuropathy, hyponatremia, coagulation disorders.

The patient presented aregenerative macrocytic anaemia requiring regular blood transfusions (see Fig. 1). The anaemia's origin was multifactorial: chronic inflammatory syndrome (CRP level fluctuating between 22,4 mg/L and 124,4 mg/L), hypersplenism secondary to portal hypertension, previous alcohol consumption, malnutrition, vitamin B12 and folate deficiency, impaired coagulation, acute renal impairment, liver cirrhosis and bleeding upon testicular wound. There was no argument for a manifest haemolysis and it was therefore not retained as a possible cause of anaemia (see Table 1). The positive DAT was not associated with the presence of warm autoantibodies by the clinicians/laboratory (see Table 2 for

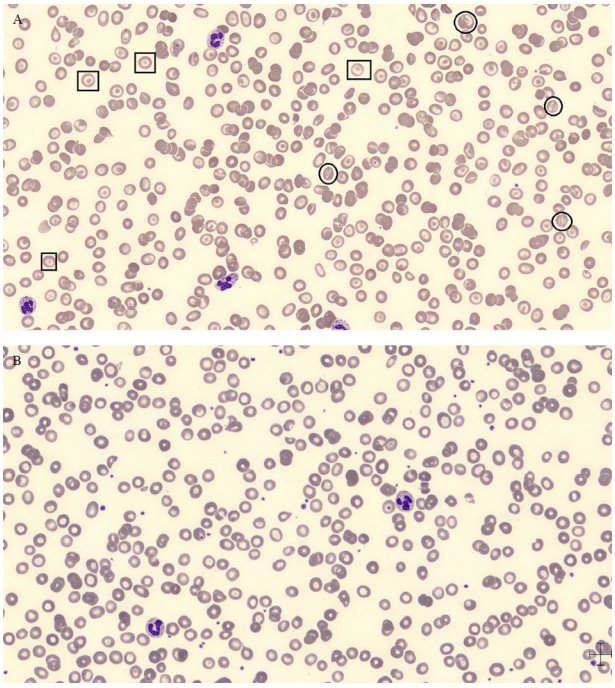

Fig. 2. Blood smear features during and after COVID-19 episode. A. Blood smear features during COVID-19 episode. Squares = Target cells; Circles = Knizocytes. B. Normalisation of blood smear features after resolution of COVID-19 episode.

the timeline). The blood smears did not show significative number of schistocytes but showed high number of target cells and significant number of knizocytes along with the formation of few rouleaux and a striking poikilocytosis (see Fig. 2). Knizocytes, rouleaux formation and poikilocytosis have been recently associated with COVID-19 [1]. Target cells are frequently observed in liver diseases. These red blood cell morphological features were not observed anymore after COVID-19 episode (see Fig. 2). LDH and indirect bilirubin levels were elevated but constantly decreasing and were most likely due to severe hepatic impairment. Reticulocytes were very low (from 2 to 4 *10^3/μL with Nl 30–100 *10^3/μL)) but should be assessed carefully considering patient's acute renal impairment. After injection of darbepoetin alfa 100 μg/0,5 ml, reticulocyte count increased to 191*10^3/μL and then lessened back to normal values. Haptoglobin levels were measured twice with normal to slightly increased values (1,91 g/L and 2,03 g/L with Nl 0,30–2,00 g/L). However, the haptoglobin measurements should be interpreted with caution because of hepatic impairment and inflammatory status that respectively decrease and upregulate haptoglobin production. Haemoglobinuria and bilirubinuria trends varied from positive to negative but should again be analysed carefully considering the acute renal impairment with acute tubular necrosis. Bone marrow aspirate examination did not reveal erythroid regeneration in response to anaemia and showed important inflammation fea-

Table 2
Testing performed by the hospital blood transfusion service.

Days since hospitalisation	Polyvalent DAT	IgG DAT	C3d DAT	IAS	Plasma antibody screen	Elution antibody screen
25 days	+	+	+	–	NA	Not specific in IAT
31 days	+	+	+	+	Not specific in IAT	Anti-Jka + non specific reaction
36 days	+	+	+	+	Anti-K and anti-Jka in IAT	NP
44 days	NP	NP	NP	+	Anti-K and anti-Jka in IAT	NA
47 days	+	+	+	+	Anti-K and anti-Jka in IAT and ficine	NP
55 days	+	+	–	+	Anti-K and anti-Jka in IAT	Anti-K and anti-Jka in IAT
58 days	+	+	–	+	Anti-K and anti-Jka with dosage effect in IAT and ficine	Anti-K and anti-Jka in IAT
65 days	NP	+	–	+	Anti-K in IAT	NP

DAT: Direct Antiglobulin Test/IAS: Irregular Antibody Screening/IAT: Indirect Antiglobulin Test/NA: Not Applicable/NP: Not Performed.

tures. Increase of proerythroblasts in the bone marrow is normally observed in response to anaemia as they are highly sensitive to erythropoietin secreted by the kidney under hypoxic conditions. However, our patient suffered from acute renal impairment leading to diminished EPO secretion.

1.2. Testing performed at the hospital blood transfusion service

ABO group control and irregular antibody screening (IAS) were performed before any transfusion of red blood cells (RBCs). IAS had already been tested negative 15 times before first evidence of antibody. A few days before patient's first positive IAS, DAT was performed to investigate the origin of chronic anaemia as the cause was still not obvious for clinicians. The DAT was mixed positive with IgG and C3d specificity. At that time either a positive DAT in a context of AIHA or a post-transfusion alloantibody was suspected but there was no significant manifestation of haemolysis. An acid elution process was performed but the detected immunoglobulins showed neither specificity nor panagglutination. Six days later, IAS and DAT were tested again. This was the first positive IAS for this patient and an antibody screen was performed both with plasma and eluate. The indirect antiglobulin test (IAT) was non-specific with the plasma but an anti-Jka alloantibody was discovered in the eluate. The elution process allowed the hospital blood transfusion service to consider the presence of a transfusion alloantibody and therefore to select Jka negative cross-matched RBCs for patient's safety. The IAT with the plasma revealed four days later the anti-Jka alloantibody but also the appearance of an anti-K alloantibody (see Table 2).

This case report triggers the importance of performing an elution process for poly-transfused patients when an IgG-type DAT is evidenced. The appearance of an alloantibody in the plasma may be delayed if all antibodies are fixed upon their respective antigen at the surface of the RBC membrane. The elution process is a high-burden working process and an appropriate decision-making algorithm is therefore important to limit the situations where it is effectively applicable. It is even more important in this current context in which the patients infected by SARS-COV-2 often present a positive DAT and are prone to multiple transfusions.

2. Discussion

2.1. DAT basics

About seventy-five years ago, the Direct Antiglobulin Test (DAT) was described by R. Coombs who had the innovative idea to use a second antibody, a rabbit AHG, to amplify the agglutination between RBCs sensitized by antibodies [2]. This laboratory testing is now widely used and offers valuable information in many differential diagnosis. The DAT has been slightly redesigned over time. It is still performed by traditional tube agglutination which is the reference method but new more sensitive testing methods now exist such as gel microcolumn method [3–5], solid-phase method or more recently flow cytometry [6–8]. Nowadays, the DAT consists of testing patient's RBCs to investigate the presence of immunoglobulins or complement proteins at the membrane's surface. The test is performed firstly using polyspecific reagents (recognizing both immunoglobulin and complement fragments) and subsequently with monospecific reagents if the polyspecific reagent shows RBCs agglutination [9,10]. Main situations resulting in positive DAT are Autoimmune Haemolytic Anaemia (AIHA), Immunoallergic Haemolytic Anaemia (IAHA), Alloimmune-mediated Haemolytic Transfusion Reaction, and Haemolytic disease of the foetus/newborn [9–11]. Recently, correlation between COVID-19 and positive DAT has been established.

2.2. Positive DAT in COVID-19 patients

Sensitisation of RBCs by immunoglobulins or complement proteins has been reported several times in different infectious contexts, such as viral and bacterial infections. Infection by Mycoplasma pneumoniae has been frequently associated with autoimmune haemolytic anaemia induced by cold agglutinins that fix complement with anti-I and anti-i specificity [12–15]. This phenomenon, also called Cold Agglutinin Syndrome (CAS), is a secondary AIHA [16]. Infectious mononucleosis by Epstein-Barr virus has also been frequently associated with CAS [17,18,19,20,21]. Similarly, several authors have reported the presence of positive DAT consequently to other infectious agents such as Cytomegalovirus [22–25], Plasmodium [26–28], B19 parvovirus [29], human immunodeficiency virus-1 [30–32], Brucella [33–36], adenovirus [37], Influenza A [38,39], Varicella virus [40–42], Rubella virus [43–46], Legionella pneumophila [47,48] and Chlamydia pneumoniae [49,50].

Positive DAT has recently been observed among SARS-CoV-2 infected patients. Case reports associating SARS-CoV-2 infection with development of secondary AIHA are continuously increasing in the literature. Some authors report the appearance of secondary AIHA with a positive DAT showing complement specificity (CAS) [51–53]. Other papers report a mixed-type AIHA [54,55,56,57,58]. Finally, one case report describes secondary pure-IgG DAT in post-COVID-19 AIHA [59]. The presence of other pathogens responsible of respiratory infections had been excluded in most case reports, supporting the relationship between the SARS-CoV-2 infection and secondary AIHA [51,53–55,58,59]. This has been described both in adult and paediatric populations. A case series of seven patients showed positive DAT in all cases, 2 for IgG, 2 for C3d and 3 were mixed type. The authors concluded that anti-erythrocyte antibodies were warm antibodies in 4 cases and cold agglutinins in 3 cases [60]. Platton et al. collected blood samples of 20 patients hospitalised in intensive care units confirmed for SARS-CoV-2 infection by PCR and 20 patients hospitalised in intensive care units negative for SARS-CoV-2 infection. They observed a positive DAT in 80% of infected patients against 35% in non-infected patients. In the infected-patient group, the DAT was always IgG-positive except in one, which was positive for C3d. Eluates didn't show any specificity when tested by IAT [61]. One larger scale study has been published by Berzuini et al. studying 113 patients infected by SARS-CoV-2. They found 52 of 113 (46%) patients who presented a positive DAT using a microcolumn screening assay, with 88% of IgG specificity. Four patients were tested positive for both IgG and C3d (8%) while only 2 patients were positive for C3d. Positive DAT was confirmed either by a second agglutination technique or by flow cytometry. As demonstrated by Platton et al., the eluates of DAT-positive patients did not react against panel of reagent RBCs [62]. Results from our hospital internal study among 225 SARS-CoV-2 confirmed patients (99 from ICU and 126 in classic hospitalisation wards (CHW)) showed a positive rate for DAT of 44% [63]. The positivity rate was significantly higher in the ICU patient group (56% positivity) against 35% in other units. A total of 58,8% of positive DAT showed IgG-specificity, 32,4% a mixed IgG-C3d specificity while only 8,8% were C3d specific. Same conclusion was drawn for eluates, which were not specific against panel of reagent RBCs.

Results of all these case reports and studies demonstrate a high percentage of positive DAT among patients with COVID-19. The case reports with demonstrated clinical and biological haemolysis tend to demonstrate rather C3d or mixed-type DAT. However, the number of patients studied is very limited. In larger scale studies, most COVID-19 patients show IgG-specific DAT and in a lesser

extent mixed IgG-C3d specificity. Clinical or biological manifestations of AIHA are not always observed in these substantial studies.

Warm and cold AIHA associated with SARS-CoV-2 infection occur after the beginning of the infection and seem to correlate with the cytokine storm [59]. Underlying mechanisms behind positive DAT among COVID-19 patients are currently not well understood. Several hypotheses have been proposed such as molecular mimicry [64,65], hyperinflammation induced by cytokine storm or alteration of the RBCs membrane with exposition of cryptic antigens [66,67]. Bastard et al. recently discovered auto-Abs against type I interferon among patients with life-threatening COVID-19 pneumonia [68].

2.3. COVID-19, haemoglobin drop, transfusion need, alloimmunisation and positive DAT

Lower haemoglobin levels have been demonstrated among patients infected by SARS-CoV-2. Algassim et al. found significantly lower haemoglobin levels in SARS-CoV-2 infected patients admitted in ICU compared to those in CHW. Drop in haemoglobin levels is associated with poorer prognosis. One hundred sixty-three patients out of 250 (65%) were anaemic in ICU against 111 out of 257 (43%) in CHW [69]. Lower haemoglobin levels for severe COVID-19 have been highlighted in many other studies [70–73]. A meta-analysis about anaemia in COVID-19 collected data from 40,450 patients. The mean haemoglobin level was 12,97 g/dL but significantly lower levels were observed in severe COVID-19 cases [74]. Berzuini et al. reported that 44 of 113 COVID-19 patients received at least one blood transfusion with significantly higher percentage among patients presenting a positive DAT [62]. In other studies, the percentage of COVID-19 patients needing blood transfusion varies between 6,2 and 11,1% [75–78]. Blood transfusion needs for COVID-19 patients are related to extracorporeal membrane oxygenation (ECMO) and coagulation dysfunction [77].

Multiple transfusions and positive DAT among COVID-19 patients complicate immune-hematologic investigations for hospital blood transfusion services. Some situations justify the need to exclude the presence of underlying alloantibodies when DAT is positive. For example, further investigation is required when the patient presents post-transfusion haemolysis signs or in case of insufficient post-transfusion increment of haemoglobin levels. The Kidd (Jk) blood group may generate difficult situations as illustrated by our case report [79]. The elution process is appropriate in the sense that it eluates the potential alloantibodies coated on RBCs while COVID-19 associated antibodies don't give any RBC antigen specificity on commercial panels after elution [61,62]. COVID-19 autoantibodies are probably attached with low affinity at the RBC membrane and they may be removed during washing step of the elution process. Berzuini et al. also observed that eluates from COVID-19 patients did not react against commercial panel RBCs while they reacted against RBCs prepared from DAT-negative COVID-19 patients. The hyperinflammation state during the disease probably modifies the RBC membrane with exposure of cryptic antigens allowing the binding of complement proteins or immunoglobulins [62]. A decision-making process should be elaborated in hospital blood transfusion services for COVID-19 patients presenting a positive DAT and needing blood transfusion.

2.4. Elaboration of a decision-making process for DAT-positive COVID-19 patients needing RBCs administration

The algorithm should include DAT-positive COVID-19 patients that will need immediately or in the near future administration of red blood cells because of post-transfusion haemolysis risks. The transfusion timeline may be adequate as a first step in the algorithm. Post-transfusion alloantibodies usually appear 2 to 3 days after the transfusion and elution process is thus not necessary if RBCs have been transfused for less than 3 days. The potential alloantibodies are first entirely fixed on RBCs. They are not circulant yet in the plasma/serum and elution process may therefore be justified. We assume that any transfusion alloantibody will appear in the plasma or serum of the patient after 30 days, which renders the elution process worthless after that timeframe. Serum investigation must always be conducted in parallel. IAT should be performed after a positive IAS.

In addition to the timeline, the high-burden load of elution process should be taken into consideration when designing the decision-making process. This why clinical and biological haemolysis parameters along with post-transfusion haemoglobin level monitoring are further steps to be considered into the decision-making process before conducting elution techniques. These criteria should be discussed with clinicians and appropriate follow-up of patients should be in place. When the algorithm triggers the start of the elution process, the result should be documented for future consideration if other transfusions are required. If the elution process results in the identification of a transfusion antibody, hospital blood transfusion services should take appropriate measures such as respect of patient RhK phenotype and serologic cross-match between donor red blood cells and patient eluate/plasma.

There are similarities between the plasma of AIHA patients and SARS-CoV 2 patients. Both can present a positive DAT and may be polytransfused. Furthermore, it has been proven that patients with AIHA are at risk of developing transfusion alloantibody even when antigen-matched units are selected [80]. Autoantibodies and alloantibodies coexist in about 1/3 of patients with AIHA [81]. Including the AIHA on this decision-making process would be a potential improvement. However, AIHA management is not exactly similar. The autoantibodies present in AIHA have often panagglutinin specificity after elution process while COVID-19 autoantibodies are removed and don't give any agglutination when eluate is tested. Auto-adsorption is often required to get rid of the autoantibodies in AIHA in order to determine the presence and the specificity of potential alloantibodies [82,83].

3. Conclusion

Tailored decision-making process can be implemented in hospital blood transfusion services to help with the management of polytransfused COVID-19 patients presenting a positive DAT. Adequate management process should be in place to avoid severe haemolytic transfusion reactions. This paper aims to help blood transfusion services in their investigations and therefore to improve the quality of patients treatment.

Authorship contributions

J.C. was the first author and the designer of decision-making processes. A.B., P.S., M.v.D. and V.D. contributed to the design of the decision-making process and reviewed the manuscript structure and contents. L.C. and B.D. contributed to the review of the clinical aspects of the paper.

Disclosure of interest

The authors declare that they have no competing interest.

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