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Research article

Impact of COVID-19 pandemic on transplant laboratories: How to mitigate?

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ABSTRACT

A positive flow cytometry crossmatch (FCXM) due to donor specific antibodies (DSA) constitutes a risk for kidney transplantation; such a finding may indicates an unacceptable donor for this patient. However, positive FCXM in the absence of DSA is considered discordant and need further investigations. During COVID-19 pandemic, we observed 22% discordant results out of 445 FCXM performed during eight months period in our laboratory and another 7% were invalid due to high background negative control (NC). No study has addressed the impact of COVID-19 pandemic on FCXM and the overall pre-kidney transplant workups or described a solution to deal with these non-specific reactivities. Herein, we analyzed all FCXM results in SARS-CoV-2 seropositive patients and addressed how this pandemic affected significantly the pre-kidney transplant workups, highlighting both technical and financial implications. We also shared our modified FCXM procedures using dithiotheritol (DTT) sera treatment or blocking donor cells with negative control human serum (NCS) which we found to be successful to abrogate 98% of all discordant FCXM results and to validate all invalid results due to high background NC.

In conclusion, COVID-19 pandemic has affected our HLA laboratory significantly by creating many false positive or invalid crossmatch results. Transplant laboratories must consider this before test interpretations and immune risk assessments. We recommend the use of DTT serum treatment to remove nonspecific bindings in the sera of kidney transplant candidates and the use of NCS-blocked donor cells to correct high background when performing FCXM in transplant candidates or donors with recent history of SARS-CoV-2 immunization respectively.

1. Introduction

The global SARS-CoV-2 pandemic has affected significantly all aspects of our life including global economy, social life, health care systems and solid organ transplant laboratories were no exception. Data on COVID-19 impact on HLA sensitization is scarce and sometime controversial [1–3]. Moreover, the limited available data on the influence of SARS-CoV-2 on laboratory tests is confined to

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Abbreviations

BMP	Blocking modified procedures
cPRA	Calculated PRA
DSA	Donor specific antibodies
DTT	Dithiothreitol
DMP	DTT -modified procedures
EDTA	Ethylenediamine tetraacetic acid
FCXM	Flow cytometry crossmatch
LSM	LABScreen Mixed
MCS	Median channel shift
MCF	Mean channel fluorescence
NGS	Next generation sequencing
NCS	Negative control serum
PCS	Positive control serum
SAB	Single antigen bead

two case reports of emergence of positive crossmatches in previously non-sensitized transplant candidates [4,5] while the longest series on HLA-antibody productions post COVID-19 infection include only 18 wait-listed transplant candidates [6]. Therefore, comprehensive studies focusing on the influence of SARS-CoV-2 on laboratory assays and the overall impact on pre-transplant work-ups are still lacking.

Due to the inherent capacity of T-cell receptors to cross-react with multiple antigens, polyclonal T cell activation following viral infection is not uncommon; the latter comprises a broad range of T-cells expressing unrelated epitopes including HLA-specificities commonly referred to as heterologous immunity [7]. Cytomegalo-virus (CMV) and herpesvirus in particular are well known for inducing heterologous immunity [8,9]. Vaccine related allo-immunization had also reported through multiple mechanisms including heterologous immunity on the level of B-cells similar to T-cells, adjuvant impact on innate immunity, and bystander activation of quiescent existing memory B- cells [7]. Many authors believe that activation of preexisting memory B- cells is the most important mechanism leading to HLA-antibody production following viral exposure [10]. Much of our knowledge on vaccine-induced anti-HLA Antibodies came from experience with influenza vaccines with some studies reported an incidence between 11.9%and 17.3% of de novo anti-HLA antibody following H1N1 vaccination [11–15].

The knowledge on the immune response against COVID-19 infection still evolving, but it has been shown that the virus can induce a unique immune dysregulation and many recent studies on SARS-CoV-2 demonstrated that COVID-19 infection could induce heterologous immunity to unrelated pathogenic bacteria [16]. Others and we have recently shown that COVID-19 infected patients including wait-listed transplant candidates mounted a strong durable humoral immune response that persisted for a few months [17–19]. Moreover, many of COVID-19 infected patients displayed some sort of immune dysregulation characterized by release of pro-inflammatory cytokines, broad activation of B-cells and stimulation of innate immunity leading to activation of complement cascade and other inflammatory markers [20,21]. In view of ongoing COVID-19 vaccination program in many countries, there remain to be another concern of vaccination induced immunization or enhancement of immune response following natural COVID-19 infection. Interestingly the single-stranded mRNAs in SARS-CoV-2 vaccines was found recently to be potent stimulators for Toll-like receptors on B cells, with a potential for robust bystander activation of preexisting memory B cells [22]. Accordingly true allo immunization or reactivation of anamnestic response following SARS-CoV-2 exposures need to be considered during interpretation of HLA related tests including Flow cytometry crossmatch (FCXM).

Although FCXM is considered the gold standard test to assess donor –recipient compatibility, the test has some limitations such as occurrence of false positive results due to non-specific bindings of non-HLA antibodies including therapeutic monoclonal antibodies or immune complexes present in patients' sera to constitutively expressed Fc or immunoglobulin receptors on B cells [23,24]. Blocking these receptors to remove the non-specific bindings routinely performed by many laboratories through saturating the receptors with certain reagents such as specific anti-Fc receptor antibodies, excess (unpurified) IgG in the form of human serum or pronase treatment prior to staining the cells with labeled antibodies [25–29].

We recently found that SARS-CoV-2 exposure could induce new HLA-antibody formation [30] and frequently lead to occurrence of discordant FCXM results. We also observed high NC background invalidating many FCXM results. Because, we seek the highest accuracy before issuing histocompatibility reports in our laboratory, all discordant FCXM results with the status of donor-specific antibodies (DSA) thoroughly investigated during COVID-19 pandemic. We also tried to develop a way that can abrogate these non-specific reactions. In this study, we aim to highlight both the technical & financial implications of SARS-CoV-2 exposures on a reference laboratory and to share our modified crossmatch procedures that eliminated almost all non-specific reactions and abrogated non-valid crossmatches related to SARS-CoV-2 exposures in both patients and donors respectively.

To the best of our knowledge there is no study addressed the impact of this pandemic on crossmatch and the overall pre-transplant work-ups neither there is a report on finding a technical solution to be used routinely during performing FCXM procedures to remove such impact, a problem likely to continue in view of the ongoing global pandemic.

2. Methods

The pre-kidney transplant work-ups in our laboratory include donors and recipients HLA typing by next-generation sequencing (NGS) (our laboratory is ASHI accredited for NGS-typing) and antibody screening and identification for patients using Luminex® bead-based multiplexing technology. Additional testings include physical FCXM within four weeks before the due date of transplantation for living donor transplantation and quarterly monitoring of HLA antibody by single antigen bead (SAB) for waitlisted patients. Correlation of physical FCXM with virtual crossmatch (VXM) results always practiced before reporting immunological assessment results.

In our study, we collected the data of 445 consecutive allo-FCXM belonging to SARS-CoV-2 antibodies positive-living kidney transplant candidates performed during the period of 20/3/2020-20/11/2021. Among those, we found 107 cases of discordant FCXM results, which refers to disagreement between VMX findings and FCXM results, that's to say patients with no or low titer DSA (excluding public epitope sensitization) had positive FCXM results. Additionally, we encountered 32 invalid FCXM results, which refers to FCXM assays that yields a high negative control reading that exceeds our laboratory pre-established ranges making test results uninterpretable. Fig. 1 (A) shows an example of invalid FCXM that was corrected with our modified protocol (B). Fig. 2 shows a flow diagram that summarizes study population.

All experiments and consenting processes were carried out in compliance with the established ethical regulations and practices. The study was approved by the institutional review board at King Fahad Specialist Hospital-Dammam (Reference number: LAB0325).

Physical allo-FCXM was performed as previously describe [31]. Briefly, peripheral blood donor cells, were isolated using EasySep (StemCell Technologies), pronase treated (0.5 mg/ml; Sigma-Aldrich) for 15 min at 37 °C, followed by 3 min DNAse treatment (2.75 mg/ml; Sigma-Aldrich). The washed cells (2.5×10^5) were then incubated with 25 μl patients' sera for 20 min at room temperature, washed and incubated with a cocktail of anti-CD3-PerCP5, anti-CD19-PE(BD Biosciences), and goat F(ab')2 fragment specific anti-human IgG (Fcγ)-FITC (Jackson IR Laboratories) at 4 °C for 25 min. Analysis was performed using BD FACS DIVA software (BD), and 1024 channel log scale, on FACSCantoII. A Median channel shift (MCS) of ≥66 and ≥ 96 considered positive for T cells B cells respectively. Ready-made NCS from Invitrogen (ThermoFisher, USA) and positive control serum (PCS) (prepared by polling 10 sera of highly sensitized patients with cPRA >90% broadly reactive for both class I and II HLA-antibodies) were used to establish baseline median channel fluorescence (MCF) and as quality controls for the anti-human IgG (Fc)-FITC secondary antibody respectively. Auto-FCXM is performed with same procedure for allo-FCXM described above utilizing serum and cells from the same patient, to exclude false positive allo-FCXM due to autoantibodies.

The HLA class I and class II antibodies were measured using LABScreen Mixed (LSM) Class I and Class II assays (One Lambda Inc., Canoga Park, CA) according to manufacturer instructions and positive reactivities were further tested using SAB. In our laboratory, sera for HLA antibody detection routinely treated with dithiotheritol (DTT) plus 1:4 dilution for highly sensitized patients to overcome prozone problems [32]. A mean fluorescence intensity (MFI) of 1000 in SAB assay scored positive but DSA between 500 and 1000 MFI reported as "weak reactivity" DSA.

VXM was performed by comparing donor HLA antigens against the potential recipient's antibody specificities (HLA-A, -B, –C, -DR, -DQA, -DQB, DPA, and -DPB) as detected by SAB assay and using a cutoff 3000 MFI for A, B, DR and 5000 for Cw, DQ, DP specificities to consider positive VXM as described previously [33]. Briefly, patients who have one or more DSA that are greater than the aforementioned cutoffs will be considered positive whereas negative VXM means absence of DSA or presence of DSA below those cutoffs. Other factors such as DSA titer underestimation (due to shared epitope) or antibody genuineness are also considered when analyzing VXM data.

Discordance between FCXM and VXM qualifies the corresponding serum to be enrolled in two modified FCXM procedures aiming to abrogate the nonspecific reactions. Antibody against SARS-CoV-2 were detected using DiaSorin SARS-CoV-2 S1/S2 IgG/IgM kits on

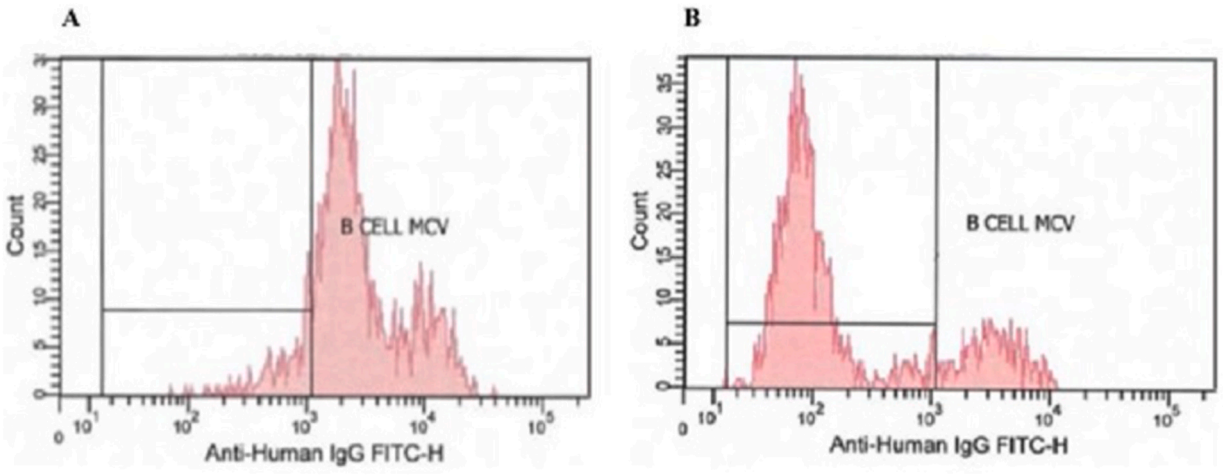

Fig. 1. Flow cytometry histograms of negative control serum (NCS) reacting with one donor-cells (A) before (invalid) and (B) after blocking donor cells (valid) as described in blocking modified procedure.

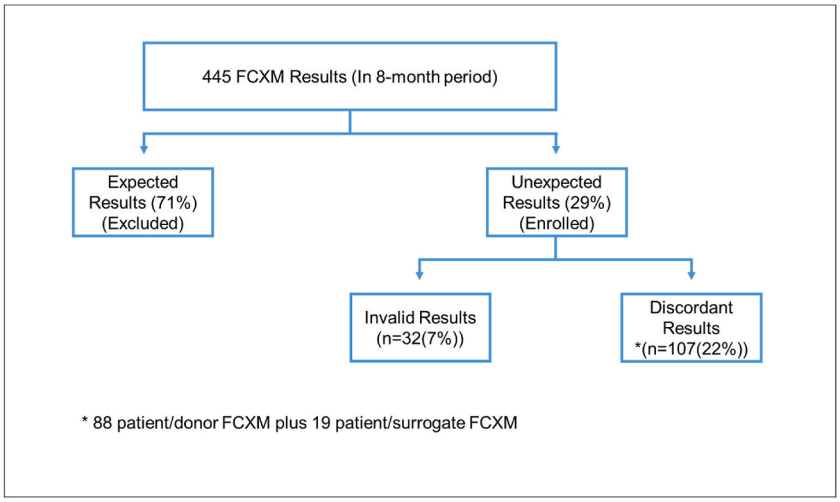

Fig. 2. Study flow diagram.

LIAISON XLR platform. Table 1 illustrates the total number of FCXM included in the study and the number and characteristic of sera that gave discordant (false positive) or invalid FCXM results (high background).

Modified FCXM procedures:

All sera from 88 SARS-CoV-2 seropositive recipients that gave confirmed discordant FCXM results were investigated by repeating FCXM with two different modified procedures as follows:

1 DTT modified procedure (DMP): sera were treated using DTT at final concentration of 5 μM prepared from 1 M stock (Sigma-Aldrich, St. Louis, MO) by diluting 50 μl in 950 μl PBS. This working solution was stored at 2–8 °C to be used within two weeks. Treatment involved addition of 20 μl of DTT working solution to 180 μl of patient sera, incubating at 37 °C for 30 min and spinning at 13,000 RPM for 10 min before being used in FCXM.
2 Blocking modified procedure (BMP): involved blocking donor cells with blocking solution prepared by adding 500 μl of NCS to 4500 μl of PBS. After pronase treatment, donor cells were washed twice with 2% FBS, then we added 2 ml of the blocking solution to the dry pellets and incubated them at 37 °C for 15 min. Cells then were washed and the concentration was adjusted using 2% FBS before being used for FCXM.

Because treatment with BMP was not so effective in removing the nonspecific reactions for the discordant FCXM sera, we discontinued using this protocol after performing 40 FCXM and continued with DMP only for the remaining discordant cases. BMP,

Table 1
Number of FCXM included in the Study and the Number and Characteristics of Sera that gave Discordant (false positive) or Invalid (high NC[a]) FCXM Results.

	Number	Percentage
Total Number of FCXM investigated	**445**	
Discordant FCXM results	107	22%
Invalid FCXM results	32	7%
All Problematic FCXM	139	
Patients with Discordant FCXM Results	**88**[b]	**29%**
Patient Characteristics		
Pediatric patients	26	30%
Adult patients	62	70%
Male/Female	42/46	48%/52%
Sensitized Patient with Weak[c] DSA	19	22%
Patient with Negative DSA	69	87%
Characteristic of the Discordant FCXM		
B + T-	57	64%
B + T+	24	27%
T + B-	7	9%
Total	**88**	**100%**

[a] Negative control.
[b] 19 sensitized patients out of 88 giving discordant FCXM with weak DSA underwent surrogate cells FCXM in addition to the initial FXCM with their corresponding donors.
[c] Weak DSA = MFI less than 2000.

however, was effective in reducing high background for the invalid FCXM (cases with MCF of NCS exceeds the pre-established range). Thus, we continued to use BMP to block donor cells in the 32 invalid FCXM cases.

The two modified FCXM procedures were performed simultaneously for sera in patches within 2–3 days of receiving the corresponding donor cells. The results were tabulated and the MCS after the two modified procedures compared with initial MCS of FCXM using neat sera. To exclude unwanted effect of DMP on true positive or true negative FCXM results, we crossmatched 10 known positive sera and diluted PCS with surrogate cells selected from potential donors after treating the sera with same DMP. Additional 30 known negative DTT treated sera were used as negative control. Part of the investigation included performing FCXM with third-party surrogate cells for 19 sera that gave discordant FCXM in the context of weak DSA to better characterize the allo-specificities of the positive FCXM and exclude technical error inducing false positive results.

3. Results

We investigated 139 problematic FCXM cases out of 445 collected cases. These problematic FCXM include 107 (22%) discordant results belonging to 88 patients (VXM negative but positive physical FCXM in the context of negative or borderline positive auto-FCXM) and 32 (7%) invalid FCXM due to high NC.

DTT-treatment converted 86 (98%) out of 88 false positive FCXM results to either negative or lowered the MCS to a level consistent with MCS of the auto-FCXM performed on the same sera. The remaining two false positive that did not respond to DTT treatment were converted to negative with additional donor cells blocking as in procedure 2. Only 19 (48%) discordant results out of 40 discordant XM corrected after using BMP alone. Table 2 summarized all results after using the two modified XM procedures. The true positive FCXM results in the 10 known positive samples and in PCS remained positive up to 640 dilution of PCS when FCXM performed with DMP indicating no impact on true positivity. Fig. 3 demonstrates that DMP had no effect on true positive FCXM for both T-cell (A) and B-cell FCXM (B). All 32 invalid FCXM results due to high NC sera corrected after blocking donor cells with NCS (BMP). Fig. 1 demonstrated pattern of FCXM of NCS before (A) and after (B) BMP for a patient evaluated for transplantation from a female potential donor who received the second dose of COVID-19 vaccine four weeks before FCXM.

Fig. 4 (A and B) summarizes a scenario of discordant FCXM with one non-sensitized transplant candidate (patient 1); whereas Fig. 5 (A and B) shows the scenario of discordant FCXM with a sensitized transplant candidate (patient 2). Both patients showed positive FCXM in the context of weak reactivity DSA.

Utilizing our modified protocols to refine their results, our center was able to successfully transplant 42 kidney patients who initially had either false strong positive FCXM results or uninterpretable (invalid) FCXM results without a need for additional immunosuppression, with good kidney function during one year post transplant follow-up.

4. Discussion

FCXM is the most sensitive method to detect DSA by measuring the reactivity of recipient serum against donor lymphocytes in pre-kidney transplantation work-ups. However, inconsistencies between DSA performed by SAB and FCXM are not uncommon in laboratory practice, which are generally attributed to technical limitations of both assays including either missing antibodies in SAB or non-specific bindings in FCXM. False interpretation of positive FCXM in the absence of DSA or missing true sensitization could lead to inappropriate denial of transplantation or put patients at risk of rejections respectively, therefore laboratories using FCXM should always be aware of assay limitations to avoid incorrect report.

False negative SAB results are commonly attributed to interference of HLA antibody detection leading to false low/negative results of high titer HLA antibodies, a phenomenon known as the prozone effect. The prozone effect is mainly mediated by serum-derived complement complexes masking the site of the antigen-antibody interaction on Luminex beads, thus hindering the access of the secondary antibody [34,35]. Others have speculated that IgM, antibodies might play a role in the prozone effect, presumably by competing with IgG antibodies for binding to HLA molecules [32,36]. Multiple strategies have been shown to reduce the prozone effects including use of calcium chelators, such as ethylenediaminetetraacetic acid (EDTA), heat inactivation, the use of reducing agents (DTT), or serum dilutions [37,38]. Although most laboratories pretreat sera before SAB assay to remove interfering factors present in patient sera that might cause false negative results, it is not known if laboratories treat equally the same sera used for FCXM despite being theoretically subjected to the same interfering factors. This is possibly because false negative FCXM is generally not a concern due to the high sensitivity of the method and assay improvement in general through adjusting cells to serum ratio, use of

Table 2
Total number of FCXM tests performed with the two modified FCXM procedures and their effectiveness in correcting discordant or high background results.

	Total Number	Corrected (Number)	Corrected (Percentage)
Discordant FCXM run with DMP	88	86[a]	97.7 %
Discordant FCXM run with BMP	40	19	48%
Discordant FCXM run with DMP + BMP	2	2	100%
Invalid[b] FCXM run with BMP	32	32	100%

[a] Three out of seven (T + B-) FCXM were corrected after repeating the modified procedures without adding pronase.
[b] NC signal exceeded the pre-established range.

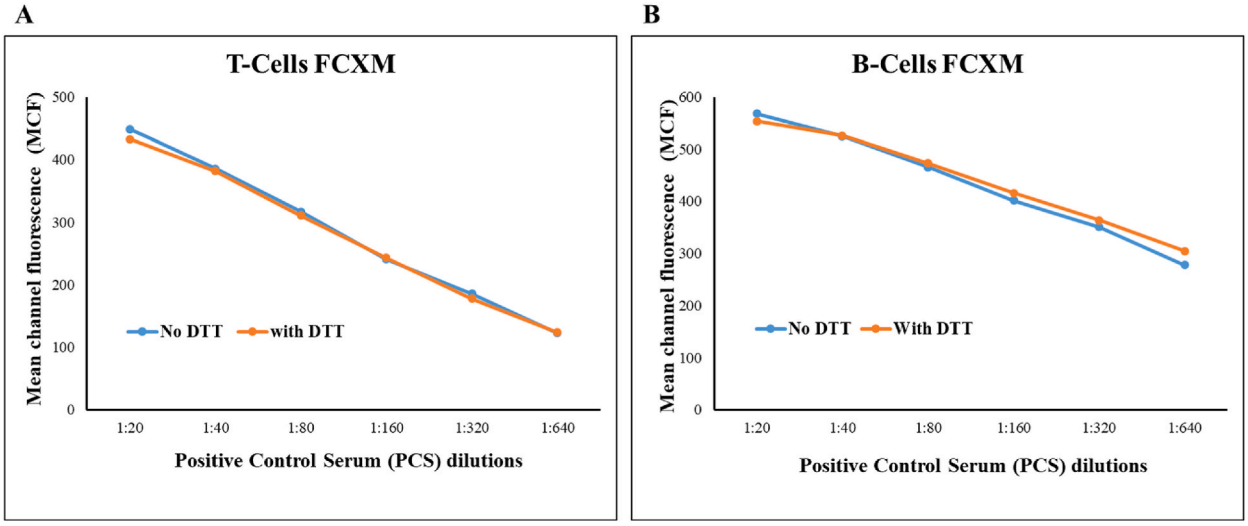

Fig. 3. The effect of DTT-serum treatment (DMP) on PCS at serial dilutions when crossmatched with surrogate cells. The effect is expressed as MCF of (A) T and (B) B-cells FCXM for each dilution from the DMP-FCXM as compared to MCF of FCXM performed with no DTT treatment.

A

Patient 1:
25 y single female, ESRD of unknown cause, history of recent COVID-19 infection,

HLA Typing:

Patient: A3, A68; B50, B51; Cw6, Cw15; DR4, DR13 DR52, DR53. DQ4, DQ6; DP4

Donor: A2,A26; B7, B51; Cw6,Cw14; DR7, DR53; DQ2; DP2,DP3

LSM: Negative for class I & II. **SAB:** showed (weak reactivity DSA A2)

Serology findings: SARS-Co-v2 Ig G >400, Positive IgM.

	T -FCXM (MCS 66)	B-FCXM (MCS 96)
Allo-FCXM with donor	129	182
Auto- FCXM	22	77
Allo FCXM with 1st surrogate cells (A2 positive)	160	246
Allo FCXM with 2nd surrogate cells (A2 positive)	105	135
Allo FCXM with 3rd surrogate cells (A2 negative)	32	103
Allo FCXM with 2nd surrogate cells with DDT	33	53
Allo FCXM with 2nd surrogate cells with blocking	33	44
Allo FCXM with original Donor with Blocking	74	95
Allo FCXM with original Donor with DDT	34	48

B

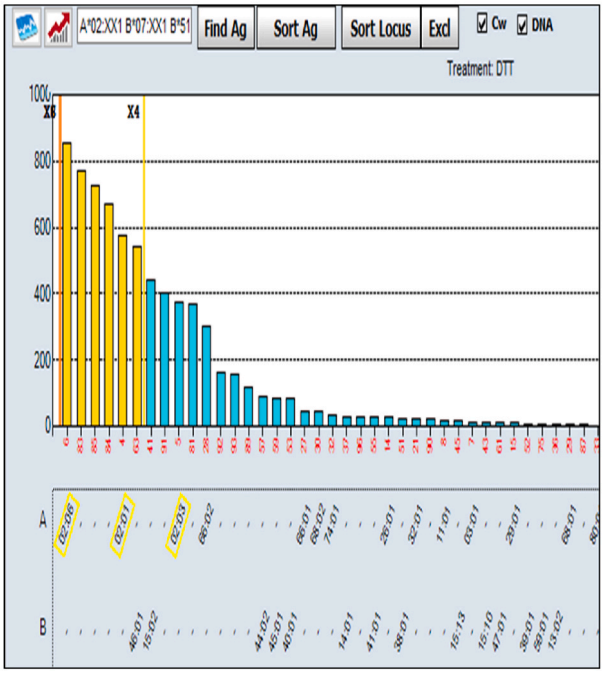

Fig. 4. Illustrates the results of Pretransplant work-ups in a patient/donor pair (patient 1). (A) Shows patient demography, HLA-typing and summary of T/B- FCXM results expressed in MCS when performed initially with the donor and after investigation. The patient had high IgG against SARS-COV-2 but negative HLA-antibody screening (LSM). (B) Demonstrates HLA-class I –antibodies by SAB, there were only week reactivity to two A2-carrying beads that cannot explain the initial positive FCXM. The sera demonstrated positive crossmatch with three surrogate cells irrespective of A2 positivity. (Positive results are in bold). Treating the sera with DTT or blocking lead to variable reduction in MCS of FCXM.
FCXM: flowcytometry crossmatch
SAB: single antigen bead
MCS: mean channel shift
LSM: lab screen mix.

A

Patient 2:
40 Y multiparous female , ESRD secondary to SLE , received two doses of COVID-19 vaccine. Before surgery, she contracted natural COVID-19 infection

HLA-Typing:
Patient: A24, A26; B7, B8; Cw7; DR17, DR52, DR15, DR51; DQ2, DQ6 DP4, DP13.
Donor: A1, A3; B8,B37; C7,Cw15; DR10,DR5,DR51; DQ2,DQ5

SAB: Positive for (A2,A68,A69, B57,B58, DR7), Weak Reactivity DR10
Serology: ANA 320

Allo-FCXM with donor	T –XM (MCS 66)	B-XM (MCS 96)
Post Vaccination Allo FCXM	256	229
Auto- FCXM	163	184
After COVID-19 Allo-FCXM	345	317
Allo FCXM + BMP	216	142
Allo FCXM + DMP	318	247
Allo FCXM with original Donor with BMP + DMP	116	143
Allo FCXM without Pronase	116	Invalid

B

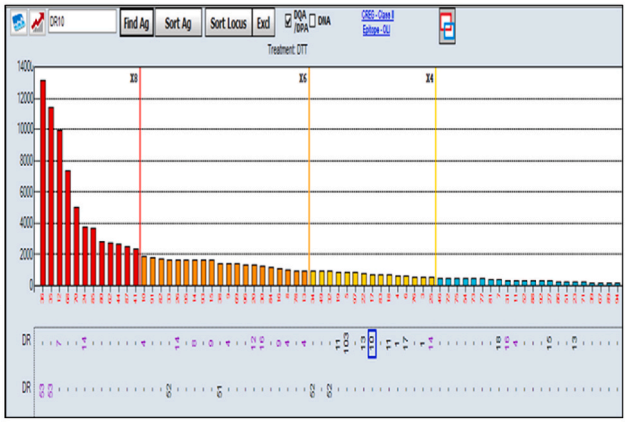

Fig. 5. Illustrates the results of pre-transplant workups in one sensitized patient (Patient 2) who had multiple HLA-antibodies and referred after receiving the second dose of SARS-COV-2 vaccine. (A) Shows patient demography, HLA-typing and T/B FCXM results expressed in MCS when performed initially with the donor and after investigation. (B) Shows results of SAB for HLA –class II antibody demonstrating weak reactivity DSA (DR10) highlighted in blue box, which cannot explain the initial positive FCXM. No class I DSA was detected. The sera initially demonstrated positive allo and auto crossmatch (SLE-patient). One week before the due day for transplantation she acquired natural COVID-19 infection, the allo crossmatch was repeated and demonstrated increased positivity (Positive results are in bold). The crossmatch was repeated using both DMP and BMP. Only combination of both protocols was successful to lower MCS near to auto crossmatch MCS.
FCXM: flowcytometry crossmatch
SAB: single antigen bead
MCS: mean channel shift
DMP: DTT-modified protocol
BMP: Blocking modified protocol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

different secondary antibodies (affinity purified goat F(ab')2 fragment anti-human-IgG) and other improvement strategies [28]. On the other hand, despite the marked reduction in the incidence of false positive FCXM due to the routine use of 0.5–1 mg/Ml pronase (a proteolytic enzyme that remove Fc receptors from the cell surface), false positive B- FCXM still occurring. This could be due to incomplete digestion of FC receptors as the action of pronase is dose dependent or possibly due to other mechanisms [39–41]. Moreover, it has been shown that pronase treatment is prone to give false-positive T-FCXM probably due to the participation of non-HLA antibodies, autoantibodies, or unmasking cryptic epitopes on T-cells [42,43] or even to give false negative FCXM results through reducing HLA expression on lymphoid cells [44].

Because we routinely DTT treat all sera for HLA-antibody test plus dilution for highly sensitized patients, the impact of prozone (missing HLA – antibodies) is very unlikely in our study. Therefore, we assumed that the discordance is mainly due to false positive FCXM and not due to missing antibodies particularly in the absence of sensitization history in many transplant candidates in our study.

The occurrence of false positive FCXM in our laboratory never exceed 5% and usually attributed to rituximab treatment, however in this study 22% of performed FCXM during the COVID-19 pandemic were false positive with another 7% of FCXM were invalid preventing us from releasing confidentently the immunology reports. This unusual situation stimulated us to investigate the problem. The investigation and modification we made to our FCXM procedures to get rid of the false positivities imposed significant financial and technical burden in our laboratory. All these discordant crossmatches initially repeated with a second serum sample or were crossmatched with surrogate cells when weak DSA exist to exclude technical error before enrolled into the tow-modified protocols to abrogate the nonspecific reactions. Our investigation for 139 problematic FCXM constituting 29% of all FCXM performed within eight -months period resulted in actual performance of 360 different procedural FCXM for an initial 120 non or minimally sensitized patients which reflected in three times increase in technical works and cost increment from about $70,000 to near $ 210,000 (Fig. 6). The turnaround time also changed from a conventional 3 days per/single FCXM test to 5–9 working days, which delayed transplantations of many patients.

Our hospital regulations mandate two-dose COVID-19 vaccinations for any individual attending medical care (patients and their corresponding donors), therefore, all patients and their donors in this study were immune by vaccination with or without infection.

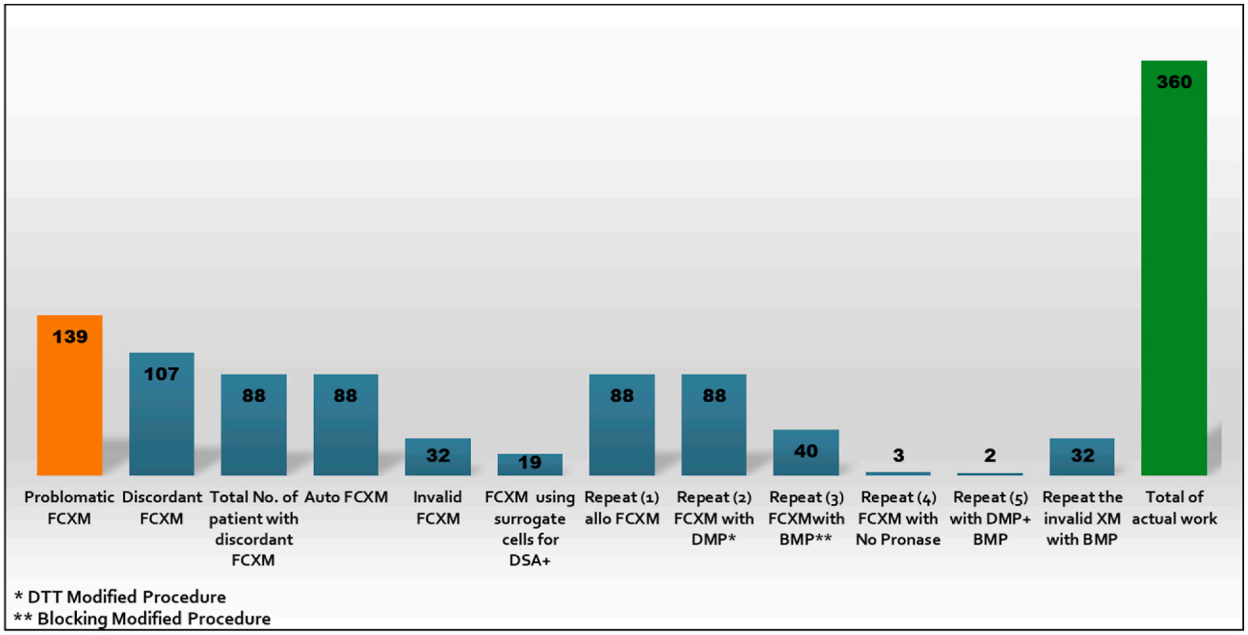

Fig. 6. Bar chart indicating the actual number of FCXM performed during the investigation of 88 discordant and 32 invalid FCXM. Each bar represent one category of FCXM based on procedural modifications to remove false reaction.

Based on this, we assumed that the immune responses to the virus with production of antibodies, complement and other inflammatory products were responsible for the non-specific reactivities, and could be removed by one of the methods utilized to remove interferences in SAB. The use of DTT to abolish false positive FCXM was used long time ago in complement-dependent cytotoxicity crossmatch assay to remove non-harmful IgM- HLA antibodies [45]. As a reducing agent DTT disrupts the disulfide bonds between amino acid residues necessary for structural conformation of some proteins and the bonds holding pentameric IgM molecules, thus prevents the nonspecific bindings to lymphocytes caused by irrelevant antibodies or immuncomplexes. We found 98 % of the false positive results abrogated by DMP, therefore confirmed our assumption of serum related factors creating non-specific reactions due to recent SARS-CoV-2 exposure and possibly enhanced by vaccinations in some donor/recipient pairs. Besides being simple and cheap, by using DTT, we have the advantage of using the same serum used for antibody test.

Failure to abrogate false positive FCXM in two cases that responded lately to the combined procedures may be due to simultaneous (donors/recipients) viral-exposures and therefore the addition of BMP resolved the problem. Although DTT treatment have, the potential to disrupt the three-dimensional structure of HLA and IgG [45,46], this is unlikely occurred in our modified procedure in view of persistence positive results in the PCS even at high dilution.

Tissues and cells blocking strategies in immunohistochemistry aimed to prevent nonspecific bindings by blocking potential sites in the tissues or cells are very well known to allow only specific antibody bindings and prevent nonspecific bindings of other factors that can attach by simple adsorption, charge-based, hydrophobic, or other types of interactions. In principle, any protein that does not bind specifically to the target antigens or the secondary antibodies in the assay can be used for blocking. Because serum is rich in albumin and carries antibodies that readily bind to nonspecific protein-binding sites thus prevent the nonspecific bindings of the secondary antibodies, normal human serum (NHS) at 1–5% (w/v) is a common blocking reagent used in immunohistochemistry techniques [47, 48].

The high NC signals in FCXM we faced in thirty-two FCXM likely related to donor issues leading to high background. As previously mentioned all donors were immune and therefor, had the same impact in their samples used in the assay. The use of NCS used in our blocking protocol abolished all NC reactivity in 100% of the treated samples allowing us to interpret confidently initially invalid FCXM results. Our validated modified procedures helped 42 patients to be transplanted with no additional therapeutic intervention.

Lastly true sensitization or activation of existing memory B-cells leading to development of HLA-antibodies and DSA or even occurrence of rejections remain a possibility post COVID-19 infection [49,50]. It is the job of a transplant laboratory to discriminate between true immunizations versus non -specific reactions before guiding transplant clinicians through using multiple laboratory tests and interpreting physical FCXM in conjunction with VXM in patients with recent history of COVID-19 infection and/or vaccination. This study only highlighted the importance of being vigilant when unexpected FCXM in COVID-19 infected/vaccinated patients and donors encountered to avoid unjustifiable denial of transplantation however, risk of reactivation of memory response post COVID-19 infection and/or vaccination cannot be inferred from this study and need to be followed in large controlled prospective studies. Finally, one future direction for this work is also to validate the modification described here with functional FCXM assays that are able to examine DSA lytic ability and its ability to fix complement [51–53].

In conclusion, although there was no obvious clinical impact for COVID-19 immunization on transplant outcomes however, it is

certainly that COVID-19 pandemic has affected our transplant laboratory and disturbed the flow of our pre-transplant work-ups significantly. Giving the ongoing nature of the pandemic and implementation of SARS-CoV-2 vaccination programs, there continues to be a chance of having these interferences with FCXM and possibly other laboratory assays. We recommend treating sera with DTT up front to minimize the repeat and to block donor cells using our BMP for high NC FCXM if there is history of recent SARS-CoV-2 vaccination or infection in patients or donors. Implementations of these modified FCXM-protocols dramatically mitigated the huge COVID-19 impact in our hand.

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Data availability statement

Data of this study is available and can be request by contacting the corresponding author.

CRediT authorship contribution statement

Rabab Ali Al Attas: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Conceptualization. **Raha Al Dhafir:** Visualization, Validation, Methodology, Formal analysis, Data curation. **Amani Mohammed:** Methodology, Investigation, Formal analysis, Data curation. **Dalal Abduladheem:** Methodology, Investigation, Formal analysis. **Mohammad Awaji:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Data curation, Conceptualization. **Kenana Alajlan:** Methodology, Investigation. **Ahmed Otaibi:** Resources, Project administration, Methodology, Investigation. **Rafa Bamardouf:** Methodology, Investigation, Data curation. **Abdulnaser Abadi:** Writing – original draft, Project administration, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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