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Proficiency testing in histocompatibility and immunogenetics: current status and future perspectives

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Proficiency testing in histocompatibility and immunogenetics: current status and future perspectives

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Editorial: Proficiency testing in histocompatibility and immunogenetics: current status and future perspectives

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Editorial on the Research Topic
Proficiency testing in histocompatibility and immunogenetics: current status and future perspectives

Proficiency testing (PT) has been part of Histocompatibility and Immunogenetics (H&I) since the early days. Starting from spontaneous interlaboratory comparisons, it gradually moved to more elaborate quality control exercises. Immunogenetics PT finally evolved into a formalized system comprising a range of testing schemes, the rules of which have been fine-tuned and approved with the assistance of professional societies. This collection of papers presents contributions covering major areas of current H&I practice in the PT context. The authors are active immunogeneticists, mostly working as clinical laboratory scientists, but in some cases also those working in PT program development. Contributions come both from Europe and North America, and while the H&I is undoubtedly of international character, and experience and lessons learned from PT are shared, we will briefly summarise particular contributions by geographical region in the following text.

Zoet et al. describe principles of the external proficiency testing (EPT) organized by Eurotransplant; one of the oldest EPT programs (established in 1978) consisting of schemes for HLA typing including serology, for CDC crossmatching, and for HLA-specific antibody detection and identification. Voorter et al. reports on an EPT scheme run from Maastricht, the Netherlands, which is aimed namely at the high-resolution typing of HLA class I (HLA-A,-B,-C) and class II (HLA-DRB1, -DRB3,4,5, -DQA1, -DQB1, -DPA1 and -DPB1) alleles, including allelic resolution typing for HLA class I–a feature being unique. In their perspective article, d´Ath et al. highlight some of the technological and clinical milestones in HLA typing to show the history and continual evolution of EPT schemes provided by the UK NEQAS for H&I. These include not only continually evolving DNA based typing methods, but also expansion into crossover discipline application areas, as exemplified by PT on pharmacogenetic testing. The authors also emphasize the need to move an EPT service from solely covering the technical elements of the laboratory testing, to include appraisal of result interpretation and clinical advice, which is indeed the opinion shared by most stakeholders in the PT field.

While Eurotransplant, UK NEQAS and the Maastricht schemes cover multiple countries/regions, there have been national EPT schemes in Europe. Hereby, Martin et al. characterizes Spanish EPT program GECLID which, besides covering HLA typing and crossmatching, runs schemes for human platelet antigen (HPA), killer inhibitory receptor (KIR) typing, and chimerism testing. The report by Vrana et al. summarizes the 10-year experience of Czech organizers of the EPT scheme "Detection of HLA Alleles Associated with Diseases" focused on standardization, harmonization, and improvement of the overall quality of the HLA investigations for the coeliac disease diagnosis. In this regard, the relevance of population genetics for EPT is brought by Mrazek, who emphasizes the need to reflect population-based differences in disease-associated HLA alleles, distribution and linkage disequilibrium of HLA alleles in particular populations, and interpretation of the presence of less common HLA variants/haplotypes. Bogunia-Kubik et al. present a perspective on the development and organizational principles of the Polish national system of supervision and control of histocompatibility laboratories, discuss problems which may occur and suggest prospects for the future. Oguz puts proficiency testing in H&I testing again in the wider context of Quality assurance and emphasizes the importance of EPT for accreditation, e.g. by the European Federation for Immunogenetics (EFI), and reviews existing and potential EPT programs.

The opinion piece by Doxiadis and Lehmann, closing the "European" part, is a natural bridge between the contributions from the two continents as it is generally applicable. The authors (among others) endorse the digital (electronic) handling of PT data. They call for greater flexibility of PT programs in order to reflect the changing nature of the field, including s.c."experimental" PT in cases of new methodologies They also suggest exploiting the potential of experience from PT to contribute to formulation of organisational policies, e.g. in transplant setting.

H&I EPT in North America is largely provided by the American Society of Histocompatibility & Immunogenetics (ASHI), the College of American Pathologists (CAP) and the UCLA exchange programs, with international laboratories around the globe often subscribing to these North American programs as well. These programs, and their subscribers (or participants) acknowledge that swift advances in testing, data and analytic diversity necessitate EPT diversification in the H&I space; an important consideration given that Centers for Medicare & Medicaid Services (CMS) and the Clinical Laboratory Improvement Amendments (CLIA) in the United States require high complexity laboratories to subscribe to EPT, or alternative EPT, for all methods of testing utilized.

The ASHI EPT program, as detailed by Hod-Dvorai et al., highlights EPT survey data as an essential educational tool in testing and clinical consultation, with its virtual crossmatch challenge offering global participants blinded simulation cases to form HLA-based immunologic transplant risk assessments with paired anti-HLA antibody and lymphocyte crossmatch results from ASHI EPT antibody identification and lymphocyte crossmatch surveys. The 75-year history and evolution of the CAP histocompatibility PT program is reviewed by Sullivan et al., with current and future perspectives on antibody, molecular, engraftment, parentage/relationship, disease association and drug hypersensitivity testing and analytic trajectories discussed. Zhang et al. chronicle expansion of the UCLA Cell Exchange program, founded in 1974 with a focus on international collaboration, which has transformed into a provider of HLA molecular typing, antibody, cytotoxicity, flow cytometric crossmatch and KIR gene typing challenge to over 30 countries around the globe. Authors highlight gaps in EPT provision of non-HLA antibody, eplet analytics and Swine Leukocyte Antigen (SLE) challenges. Surge in next-generation sequencing (NGS) and third-generation sequencing access and application parallel more frequent identification of novel alleles, presenting opportunities and challenges for EPT. Tran et al. highlight the frequency of NGS-based novel allele detection in British Columbia, Canada, while also providing protocols for standardization of typing confirmation, assessment of novel polymorphism impacts to HLA proteins and submission to the Immuno Polymorphism Database-Immunogenetics/HLA (IPD-IMGT/HLA). Kakodkar et al. underscore the utility of NGS sensitivity in chimerism assessment of allogenic hematopoietic stem cell transplantation (allo-HSCT) recipients and call for EPT programs to develop challenges designed to support accuracy in key cell subset chimerism testing.

All the fourteen articles collected in this unique Research Topic are a testament to EPT adaptation in the technology and data innovation wellspring of H&I, but appropriately present EPT programs with a call to action for provision of new and enhanced PT challenges. The Editors believe that the collection and/or individual reports will be useful for the truly international and global H&I community. As the field has been constantly evolving, the Immunogenetics section of the Frontiers in Genetics would be happy to continue considering contributions on the diverse topic of PT in H&I. Submissions from authors representing other/additional continents would be valuable and most welcome.

Author contributions

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The history and evolution of HLA typing external proficiency testing schemes in UK NEQAS for H&I

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The UK National External Quality Assessment Service (NEQAS) provide an external proficiency testing (EPT) service for clinical laboratories. UK NEQAS for Histocompatibility and Immunogenetics (H&I) has been providing EPT schemes for over 45 years and has grown during this time to provide 19 EPT schemes. Accurate human leucocyte antigen (HLA) typing is critical to support safe clinical services, including transplantation, therefore high quality, relevant EPT schemes are required as part of a laboratory's quality assurance. This article reviews the development of the HLA typing EPT schemes, from the first HLA phenotyping scheme in 1975, via the first HLA genotyping scheme in 1992, through to the introduction in 2017 of HLA third field assessment results from next-generation sequencing technology. In addition, the introduction of EPT schemes to cover HLA associated diseases and pharmacogenetic reactions, including HLA-B27, HLA*B*57:01 and HLA-DQ for coeliac disease are discussed. The accuracy of laboratory EPT results for HLA phenotyping are >96% (2018–2022), HLA genotyping >99% (2020–2022), HLA-B27 testing >99% (2018–2022) and B*57:01 testing >99% (2017–2022). However, for HLA genotyping for coeliac disease 22%–46% of laboratories made errors in 2020–2022. On investigation, the high rate of unsatisfactory performance was attributed to laboratories lacking specific knowledge to interpret HLA genotyping results and accurately report HLA types for coeliac disease. A misleading commercial kit insert was also identified. The assessment of scheme results has uncovered several issues which have been addressed with the intention of educating participants and improving clinical services. The UK NEQAS for H&I EPT schemes have evolved over the past four decades to reflect changes in HLA typing technology, laboratory clinical practice and to cover post-analytical interpretative elements of HLA typing.

KEYWORDS

external proficiency testing (EPT), external quality assurance (EQA), HLA, genotyping, phenotyping

Introduction

The HLA genes are the most polymorphic genes in the human genome. The remarkable allelic diversity of the Class I and II loci has been revealed by molecular genetic analyses, made possible by the development of recombinant DNA technology, chain-termination Sanger sequencing, Polymerase Chain Reaction (PCR) amplification, and more recently next-generation sequencing (NGS). The current understanding of the genetic organisation and polymorphism of this region is built on the pioneering work of the Immunogeneticists who used serological and cellular typing to begin to define the HLA loci and the allelic variants.

Today, HLA typing is typically performed by specialist H&I laboratories providing support for services, comprising solid organ and haematopoietic stem cell transplantation (HSCT) including volunteer stem cell donor registries, platelet refractoriness, HLA disease and pharmacogenetics associations. Accurate laboratory results are therefore critical to guide patient management, through transplant compatibility assessment, disease diagnosis, and directing treatment.

External Proficiency Testing (EPT) or external quality assessment is a critical component of a quality management system and is required by many regulators, including International Organization for Standardization (ISO) 15189 accreditation (Schineider et al., 2017). EPT monitors laboratory performance using "blind" samples intended to simulate clinical equivalents. Laboratory test results are evaluated by the EPT provider and reports issued to participating laboratories detailing their performance. Continued participation in EPT and corrective action in the event of any performance issues supports improved laboratory performance over time. The EPT process helps to ensure that laboratory testing is comparable, safe, and clinically effective no matter where testing is performed.

UK NEQAS for H&I (https://ukneqashandi.org.uk/) is one of 16 EPT providers in Europe (https://efi-web.org/fileadmin/Efi_web/Resource_collection/Procedures/List_of_EPT_Providers_May2019.pdf) and has provided an EPT service for clinical laboratories for over 45 years. From its informal beginnings in the 1970s, with some 30 UK laboratories participating in two EPT schemes, it has continued to grow and develop into a professional, dedicated, ISO:17043 accredited service, providing 19 schemes to more than 300 participant laboratories in over 50 countries worldwide. Throughout this time, the Service has maintained its core values of ensuring laboratory testing quality through continual improvement and education for the benefit of patients.

This article provides an overview of how the EPT service has evolved to reflect changes in HLA typing technology over the past 40 years (Figure 1). The evolution of HLA typing techniques has been reviewed extensively by others (e.g., Dunn, 2011; Erlich, 2012), therefore it not our intention to provide an in-depth review of this aspect. Rather we will highlight some of the technological and clinical milestones in HLA typing to show how the continual evolution of the UK NEQAS for H&I EPT schemes contributes to high quality H&I testing in participant laboratories.

The first EPT scheme: HLA phenotyping

This first available methodology for HLA typing was serological phenotyping: examining reactions of sera that led to complement activation and cell lysis to determine the HLA type of the lymphocytes being tested. The origin of the UK NEQAS for H&I schemes can be traced back to 1975, when the National Tissue Typing and Reference Laboratory in Bristol initiated a quality control scheme for HLA phenotyping and crossmatching to help laboratories in the United Kingdom and Ireland compare results. This founded the basis of the HLA phenotyping and cytotoxic crossmatching schemes still in use today. The Service joined the UK NEQAS consortium in 1989 and the first international participants joined in 1994. In line with the expansion of participants, UK NEQAS for H&I became a founding member of the European Federation of Immunogenetics EPT Committee in 1998 and has been represented ever since.

In the early days, prior to commercially available kits and reagents, laboratory tests for phenotyping were developed "in-house" using locally sourced anti-sera and complement. Therefore, the EPT exercises also included technical exercises comparing batches of complement and the sensitivity of different methodologies, to promote standardisation of procedures and comparable results between laboratories. In the following four decades, this scheme has undergone minor changes such as the inclusion of new specificities, but the core concept scheme remains. Laboratory practices changed with the introduction of molecular tests and phenotyping was no longer used in isolation to produce an HLA type and therefore supplementary genotyping to confirm serological HLA specificity assignments was introduced in 2009. Today, HLA genotyping has largely superseded phenotyping, which is reflected by a 38% decrease in the number of laboratories participating in the scheme between 2015 and 2022. Nevertheless, recent performance in this scheme is good: in the 5-year period 2018–2022, the accuracy of reported HLA phenotypes was 96.1%, with most errors due to non-analytical issues; 56% due to laboratories reporting a broad instead of a split specificity, and 38% due to the incorrect use of molecular nomenclature. Unfortunately, some sample mix-ups were identified during this period, which highlights the value of continuous EPT testing.

HLA genotyping schemes

The first UK NEQAS for H&I EPT genotyping scheme was introduced in 1992 after the introduction of DNA based methodology into H&I laboratories (Parham, 1988). Initially the scheme covered Class II (HLA-DRB1 and DQB1) but expanded in 1999 to include Class I. Participants could choose to be assessed at "high" or "low" resolution depending on the level of typing performed. By the mid-1990s most laboratories were using PCR-sequence-specific primer (PCR-SSP) methodology (Olerup and Zetterquist, 1992; Bunce et al., 1995), with the majority using "in-house" developed primers. Indeed, by 1997, 21/27 participants were using PCR-SSP, but only 6 used commercially available primers.

The increasing number of HLA alleles detected during the next decade meant continual development of new primer sets for laboratories using "in-house" methods and increasing complexity in manual interpretation of gel electrophoresis bands. This was reflected in the EPT scheme during the 2000s by a gradual move to commercially available kits and methods [e.g., PCR-sequence-specific oligonucleotide probe (SSOP) and sequence-based typing (SBT)] that included software aided analysis; in 2001 71% of participants were testing using PCR-SSP (41% using "in-house" primers), 26% PCR-SSOP and 3% SBT, compared to 2010 where 29% used PCR-SSP, 41% PCR-SSOP and 30% SBT. Over this time-period there was also an increase in laboratories reporting using multiple techniques (from 16% to 36%), reflecting the increasing complexities of HLA genotyping and limitations of available tests.

With changing HLA genotyping technology came changes to the EPT scheme, e.g., in 2005 HLA-DRB3/4/5, DQA1, DPA1 and

DPB1 were added as options for "high resolution" typing assessment. In 2011, the HLA genotyping scheme was split into two separate schemes to provide different samples for "high" and "low" resolution typing (later to become first field and second field resolution schemes). This was in recognition of laboratories moving to different technology for "low" (e.g., to support solid organ transplantation) and "high" (e.g., for HSCT) resolution typing.

In 2016 an "interpretative" scheme for first field HLA genotypes was introduced. This was in recognition of the fact many laboratories performed HLA typing using molecular techniques but converted the results into serological HLA nomenclature for reporting or assessment of donor specific antibodies. This scheme aimed to detect any post-analytical errors due to the conversion between HLA nomenclature systems which could impact on patient care.

With the introduction of NGS and real time-PCR/quantitative-PCR assays into H&I laboratories and changing clinical practice, the HLA genotyping schemes were further modified. In 2017, the option to report HLA genotypes at the third and fourth field was introduced to the second field resolution scheme. The first laboratory reporting EPT samples using NGS was in 2014, using an "in-house" method, but it was not until commercial solutions were widely available that this level of typing became more common-place, and enough laboratories were typing at the third or fourth field level to make EPT assessment possible. The move to NGS for HLA genotyping is evident, with 64% of laboratories using NGS in 2022 and 25% being assessed at the third field.

In 2018, HLA-DPB1 typing was included into the HLA first field genotyping EPT scheme, as many laboratories were now performing HLA-DP genotyping in support of solid organ transplantation. As these laboratories only require a DP type at the resolution to ascertain if a donor-specific antibody is present, the first field genotyping scheme was altered to allow laboratories to report at the resolution that is applicable to their clinical need, including reporting DPB1 alleles that differ at the first field.

Overall, performance in the molecular HLA typing scheme is excellent; in the 3 years 2020–2022 the accuracy of HLA genotyping at the first field resolution was 99.6%, second field 99.7% and third field 99.5%. Errors are often due to post-analytical errors. Continuous improvement in the quality of HLA typing has been noted by other EPT providers (Bogunia-Kubik et al., 2010; Kekik Cinar et al., 2020).

Educational HLA typing schemes

From the outset the Service has provided educational material to share "interesting" types. In the early days, this was "rare-cell" exchanges and by today's standards, the samples distributed would not be "interesting". However, in the 1980s issues assigning A28 in the presence of A2 or detecting Aw33 (A33) highlights some of the challenges that faced the early "tissue typers" and the important role these exchanges provided for laboratories to compare performance of anti-sera with challenging types. The introduction of molecular typing in the 1990s shifted the focus to the detection of rare HLA alleles, or expression variants such null alleles. Over the years, testing of routine EPT samples has contributed to the identification of novel HLA alleles, including A*23:12 (Hammond et al., 2006), A*11:15 (Bendukidze et al., 2006), DQB1*02:01:04 (Smillie et al., 2011), and A*03:162N (Bengtsson et al., 2014).

This educational ethos is still at the core of the Service. Paper-based clinical scenarios in which participants are asked to provide interpretation of results and clinical advice now forms a key component of the educational provision of the Service. Webinars started in 2021 to discuss the results of these scenarios have provided further opportunity for discussion, learning, and sharing of practice between laboratories. In this way the EPT service has moved from solely covering the technical elements of the laboratory testing, to cover appraisal of result interpretation and clinical advice.

HLA disease association/ pharmacogenetic schemes

In 1990, the first HLA-disease association EPT scheme was introduced. This was for HLA-B27 testing, which aids in diagnosis of Ankylosing Spondylitis. The scheme was initially dominated by cytotoxic methodology, but by the mid 1990s flow-cytometry and molecular based methodology had started to replace phenotyping for HLA-B27 testing; in 1996 50% of laboratories used phenotyping, 38% flow cytometry and 14% a molecular technique. Despite the move to other technologies, even as recently as 2016, some laboratories still reported HLA-B27 results using serological cytotoxic methodology, although the proportions are much reduced; 6% phenotyping, 62% molecular and 33% flow cytometry.

The overall performance of laboratories for HLA-B27 typing is excellent with 99.4% of samples correctly assigned (2018–2022) During the 5 years, 48% of samples distributed by UK NEQAS for H&I were HLA-B27 positive, and there was a greater proportion of false negative (67.5%) than false positive results (32.5%). There was no trend with methodology used.

The next targeted HLA typing scheme was for B*57:01 created in 2008 to support testing for Abacavir hypersensitivity. The accurate detection of B*57:01 is crucial; clinicians rely on negative reports to prescribe Abacavir or withhold it for positive patients to avoid potentially life-threatening hypersensitivity reactions (Cargnin et al., 2014). In terms of accuracy of testing, in the first 6 years (2008–2013) there were four errors (0.46%), all false negative B*57:01 reports (Darke and Corbin, 2014). In the last 6 years, 2017–2022, there was a 40% increase in participants and 13 errors (0.34%) with 9 false negative results, at least one of which stemmed from a pre-analytical error. A low error rate has also been reported by other EPT providers (Turriziani et al., 2016).

The number of HLA genes identified as being of diagnostic use to avoid hypersensitivity reactions has since expanded. These additional HLA-associated pharmacogenetic reactions, e.g., B*15:02 and carbamazepine (Tangamornsuksan et al., 2013), will be combined with the B*57:01 scheme to make a complete HLA pharmacogenetic EPT scheme.

The challenges of HLA typing to aid the diagnosis of coeliac disease

The most recent HLA genotyping scheme was developed in 2010 to aid disease diagnosis. Initially it covered Class II HLA

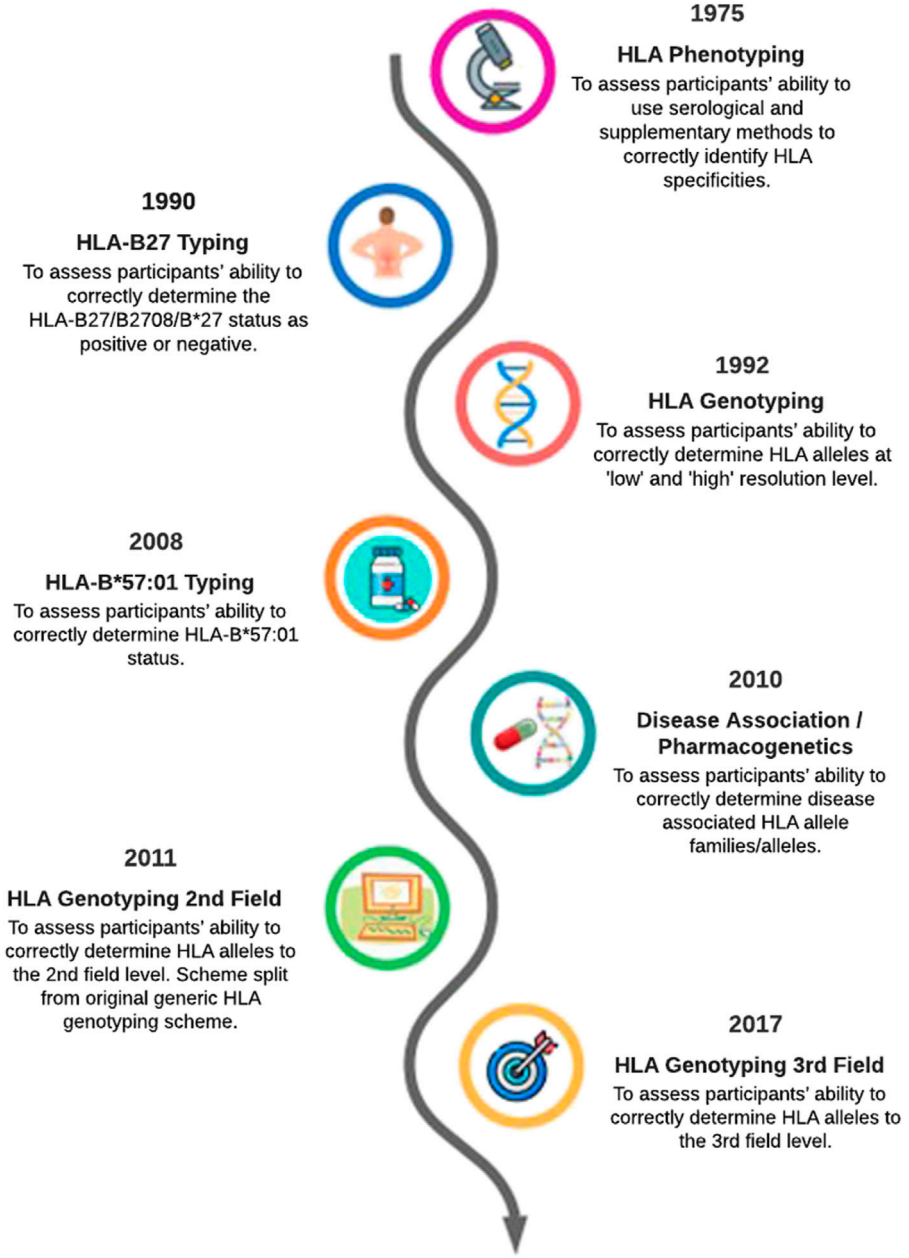

FIGURE 1
Evolution of UK NEQAS for H&I external proficiency testing schemes relating to HLA typing a simplified timeline.

genotyping only, notably for HLA-DQ2/DQ8 for Coeliac Disease (CD) and DQ6 for Narcolepsy but was expanded in 2018 to cover Class I associated diseases. This dedicated HLA disease association scheme is primarily aimed at laboratories that perform partial HLA typing or use commercial kits to detect the presence or absence of specific disease-associated HLA alleles, to support diagnosis of, e.g., CD, Birdshot Retinopathy, Actinic Prurigo, Psoriasis and Narcolepsy. This scheme also allows laboratories to report interpretative comments, but these are not currently assessed.

The scheme is highly flexible; laboratories can register for the diseases relevant to their repertoire and report their results at the resolution that is reported to their clinical users. Results are assessed by comparing participant results to a reference HLA type. The absence of a prescriptive reporting format or resolution allows the scheme to mimic the way a laboratory reports clinically. There have been an uncharacteristically high number of errors in the scheme (De'Ath and Rees, 2019) compared to other UK NEQAS for H&I schemes, with 22%–46% of laboratories making errors in the past 3 years (2020–2022). Performance in relation to HLA typing for CD is especially substandard, likely due to the complexity from multiple genes relating to the specific DQ heterodimers (encoded by DQA1 and DQB1) which confer susceptibility to CD. Suboptimal performance in CD schemes has also been noted by other EPT providers (Horan et al., 2010).

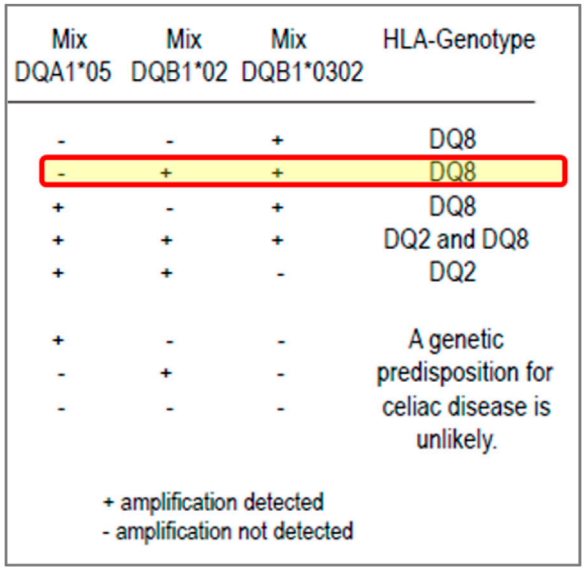

FIGURE 2
Excerpt from a coeliac disease commercial kit package insert: an example of misleading result interpretation guidance. Insert from a commercial kit for result interpretation of coeliac disease testing. The yellow highlighted row shows the interpretation could be misleading, especially for labs with limited H&I experience. The package insert states it can detect DQA1*05, DQB1*02 (DQ2) and DQB1*03:02 (DQ8) with a positive, negative and internal control. The interpretation of results for the kit suggests that if a user notes a positive reaction in the mixes for DQB1*02 and DQB1*03:02 but negative for DQA1*05, they should assign the DQ8 genotype only even though DQB1*02 (DQ2, but not with DQA1*05) has been defined. Although the relevance of this DQ2 heterodimer is less than the higher risk DQ2.5 heterodimer, it is very misleading for laboratories.

There are several issues likely contributing to the high number of errors in this scheme. The availability of "pos/neg" commercial kits for HLA associated diseases has increased the number of non-specialist laboratories performing this testing. Many of the errors appear to be due to a lack of understating of HLA nomenclature, reflected by the finding that 71% of laboratories with HLA genotyping errors for CD over the past 5 years were non-specialist laboratories. For example, a sample with a reference type of HLA-DQB1*03:01, -; DQA1*05:05,—was reported by one participant as "half DQ2 positive." When questioned the laboratory indicated that they wished to convey that they had found DQA1*05 in the sample but not DQB1*02. Laboratories not understanding the detection capabilities or resolution of results provided by commercial kits is another common reason for errors, highlighting that a full understanding of a kit is required to interpret and report the correct results.

Many laboratories with personnel not trained in H&I struggle understanding DQB and DQA subunits and their association. For example, a lab noted that their current guidelines are "*to not report DQ2.2 as DQ2 positive but to report it as "DQ2 negative DQB1*02 positive" and that risk of coeliac disease cannot be excluded based on genotype.*" This type of reporting is contradictory and not informative for the laboratory's service users. UK NEQAS for H&I offers support and expertise and works directly with laboratories that report incorrect results to improve their understanding of HLA and CD.

UK NEQAS for H&I guidelines

The notable lack of standardisation in reporting HLA types in relation to CD (Horan et al., 2010; Tye-Din et al., 2015) and the high proportion of laboratories with errors has prompted UK NEQAS for H&I to develop a set of guidelines on laboratory testing and the clinical interpretation of HLA genotyping results to support a diagnosis of CD (awaiting publication). Assessment of clinical interpretation of results for CD, alongside the reporting of HLA types, using the guidelines as a benchmark for evidence-based responses is planned. The aim is to ultimately harmonise and improve the standard of both testing and reporting of results to clinicians.

Clinical governance

UK NEQAS for H&I work closely with manufacturers and regulatory agencies to alert them to issues and assist in early resolution of problems with assays, analysers, and test kits. The corrective and preventative action investigations submitted by laboratories with errors in EPT, together with the information on testing methodology can help to highlight potential issues.

For example, in 2018, two participants reported several HLA genotypes incorrectly for CD. The investigation found an issue with the package insert of the commercial kit being used. These participants, who were not specialist H&I laboratories, relied on the interpretation of results given in the kit package insert and reported the results in accordance with the result interpretation provided the kit, which was incorrect (Figure 2).

UK NEQAS for H&I provided education and support to the laboratories. The Service also contacted the manufacturer to make them aware of the deficiency in their product insert but the company did not respond. The decision was taken, to report the issue to the UK's Medicines and Healthcare products Regulatory Agency (MHRA). The MHRA contacted the company, and subsequently the manufacturer resolved the issue by redesigning and revising the package insert to add greater clarify on result interpretation. Subsequently, it was noted that laboratories that used this kit were not affected by the same performance issue in subsequent testing. This action resulted in more accurate reporting of the risk of susceptibility to CD for patients.

Discussion

Laboratory participation in EPT schemes and the subsequent comparison of findings with numerous peer laboratories is an important and unique contribution to a laboratory's quality assurance programme. EPT providers are uniquely placed with access to large sets of data continually monitored over time. This distinct perspective is invaluable in the support of national and international organisations, and in the production of guidelines and scientific publications. We believe that a notable feature of UK

NEQAS is its wider support to participants such as offering training and education through scientific meetings and webinars.

EPT must constantly evolve to provide a responsive service able to adapt to changing clinical practice. EPT providers have a duty to provide schemes that are appropriate to the needs of its service users and ensure the quality of laboratory testing for patients. As testing methods improve so must the scheme designed to assess it, especially in terms of the assessment criteria.

Future EPT considerations for HLA typing will focus on the impact of new technologies such as long read Nanopore sequencing and how this impacts transplantation. Ultimately, laboratory testing strategy will be influenced by clinical requirements so there may be increased interest in HLA typing to the third or even fourth field in the future. Consideration is also required to ensure efficient EPT coverage for new methods which can test for multiple genetic systems. This is particularly relevant in the field of pharmacogenomics where crossover between disciplines will become increasingly evident as laboratories take a "whole genome" approach to testing. The rise of point of care testing (POCT) where testing is performed at/near the site of donor/patient care may also extend to the field of transplantation with future advances in technology, and it will be imperative to ensure the quality of such analytical procedures. This may mean additional considerations for provision of EPT specifically for POCT.

In summary, EPT, like all laboratory testing, is a moving target. By continually evolving and developing schemes UK NEQAS for H&I have aimed to keep pace with the changes in laboratory technology and clinical practice to support laboratory quality assurance. We believe that the Service has achieved this by working with its participants, stakeholders and international organisations with aim to continually develop a service that would be high quality and patient focused.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

AD: Writing–original draft, Writing–review and editing. MR: Writing–review and editing. DP: Writing–review and editing.

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Utilizing proficiency testing survey data to create advanced educational content: the virtual crossmatch challenge model

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Proficiency testing (PT) surveys include data from laboratories across the world and are ideal for creating advanced educational content, beyond just consensus grading. Educational challenges provide a unique opportunity to probe common laboratory practices and risk assessment, especially in cases where there is no "analyte" tested. Human leukocyte antigen (HLA) compatibility evaluation between donor and recipient pairs has been traditionally assessed using T-cell and B-cell physical crossmatches. However, advancements in our ability to identify and characterize HLA antibodies using solid phase assays, in combination with changing deceased donor allocation schemes and improved HLA typing, have shifted the paradigm from performing physical crossmatches to the use of the virtual crossmatch (VXM). VXM is a compatibility assessment relying on the interpretation of pre-transplant HLA laboratory data and as such, it is not an "analyte". However, VXM results are used in clinical decision-making. The VXM assessment depends on patient characteristics as well as laboratory and transplant center practices but must ensure safe transplantation outcomes while maintaining equity in access to transplantation. In this manuscript, we describe the American Society for Histocompatibility and Immunogenetics (ASHI) PT Educational VXM Challenge, as a model for creating educational content using PT survey data. We discuss the different components of the VXM Challenge and highlight major findings and learning points acquired from ASHI VXM Challenges performed between 2018–2022, such as the lack of correlation between the VXM and the physical crossmatch in the presence of low level donor-specific antibodies (DSA),

Abbreviations: AC, Antibody and crossmatching; AHG, Anti-human globulin; APHIA, Asia Pacific Histocompatibility and Immunogenetics Association; ASHI, American Society for Histocompatibility and Immunogenetics; CDC, Complement dependent cytotoxicity; CDCXM, Complement dependent cytotoxicity crossmatch; CSV, comma-separated values; DSA, Donor specific antibodies; DTT, Dithiothreitol; EC, Executive Committee; EDTA, Ethylenediaminetetraacetic acid; FCXM, Flow cytometry crossmatch; HI, Heat Inactivation; HLA, Human leukocyte antigen; MFI, Mean fluorescence intensity; NGS, Next-generation sequencing; PT, Proficiency testing; PXM, Physical crossmatch; RT-PCR SABR, Real-time PCR single antigen bead resolution; SAB, Single antigen bead; VXM, Virtual crossmatch.

or when the DSA were aimed against donor alleles that are not present on the antibody panel, and in the presence of an antibody to a shared eplet. Finally, we show that the VXM Educational Challenge serves as a valuable tool to highlight the strengths and pitfalls of the VXM assessment and reveals differences in testing and result interpretation among participating HLA laboratories.

KEYWORDS

proficiency testing, virtual crossmatch, HLA, transplant, HLA antibodies

Introduction

Histocompatibility laboratories around the world perform high complexity testing and must adhere to regulatory requirements by participating in proficiency testing (PT) for each analyte reported clinically. A preference is given for participation in graded external PT programs, however, when this requirement cannot be met, a laboratory can opt to participate in an ungraded PT program or use alternate mechanisms described in the American Society for Histocompatibility and Immunogenetics (ASHI) standards to validate test performance. PT programs provide blinded samples, collect test results, analyze the data, grade participants based on consensus and produce a summary of the results. PT survey results include data from laboratories across the world and are ideal for creating advanced educational content, beyond just consensus grading. This may be achieved by designing educational challenges which provide a unique opportunity to probe common laboratory practices and assessment of risk, especially in cases where there is no "analyte" tested.

Human leukocyte antigen (HLA) compatibility between donor and recipient pairs has been traditionally assessed using T-cell and B-cell physical crossmatches. T-cell and B-cell physical crossmatches are analytes that can be assessed by PT surveys and are often combined with HLA antibody identification testing. PT surveys for detection of anti-HLA antibody and physical crossmatching generally include 2 cell samples that are crossmatched against 4-5 serum samples. Of 156 laboratories that participated in the 2022 ASHI PT antibody and crossmatching (AC) survey, 68% are USA laboratories and the rest are international laboratories. Detailed demographics of the 2022 AC Survey participants can be found in Table 1. Among those participants, the most commonly reported physical crossmatch (PXM) assays are the T-cell and B-cell flow cytometry crossmatch (FCXM). The complement dependent cytotoxicity (CDC) crossmatch, with or without anti-human globulin (AHG), is still utilized by some laboratories due to its ability to detect strong complement binding antibodies, yet its use has decreased over the years (Putheti et al., 2022). Advancements in our ability to identify and characterize HLA antibodies using solid phase assays, in combination with changing deceased donor allocation schemes and improved HLA typing resolution, have shifted the paradigm from performing a PXM towards the use of a virtual crossmatch (VXM) as an organ offer screening tool and *in lieu* of a prospective PXM (Adler et al., 2021; Israni et al., 2021).

The VXM is not a physical laboratory test, but rather a compatibility assessment that relies on an interpretation of pre-transplant HLA laboratory test data. As such, the VXM assessment is not an "analyte" as opposed to a T-and B-cell crossmatch or Class I and Class II HLA antibody test. The VXM has been defined previously in (Morris et al., 2019) as an assessment of HLA compatibility between a donor and recipient based on the recipient's anti-HLA antibody profile and the donor's histocompatibility antigens. This definition will be applied henceforth. In the context of the PT survey, the VXM assessment was compared to the results of the physical crossmatch. There are several advantages to performing a VXM assessment *in lieu* of a prospective PXM. The efficiency of the allocation process is greatly improved due to a decrease in organ cold ischemia time, allowing for matching over a larger geographic area, as well as better access to transplant for highly sensitized patients (Bingaman et al., 2008; Johnson et al., 2016; Rohan et al., 2020; Puttarajappa et al., 2021). The VXM assessment is rapid, sensitive and does not require donor cell incubation with a recipient serum. Another important advantage is the absence of actual bench work, reduced on-call time, and reagent use, which decreases the overall operational cost of the HLA laboratory. Despite these many advantages, the VXM assessment has some limitations, which may restrict its utilization as a final compatibility assessment (i.e., without a prospective or retrospective PXM) in some cases. These limitations include the availability of a current serum, as defined by agreement between the laboratory and the transplant program, the presence of allele-specific antibodies in the absence of donor high-resolution HLA typing, donor HLA alleles that are not covered by the single antigen bead (SAB) panels, and limitations of the SAB assays themselves. For example, antibodies to shared eplets, multiple low level donor-specific antibodies (DSA) that may have an additive effect, inhibitory factors, false positive reactions due to denatured antigens, etc. In such cases, the VXM assessment may not provide accurate results and a PXM should be performed (Jani et al., 2017; Guidicelli et al., 2018; Greenshields and Liwski, 2019; Garcia-Sanchez et al., 2020; Sullivan et al., 2020; Kumar et al., 2021).

HLA laboratories may assess or predict compatibility differently based on their experience and practices. Laboratories differ in MFI cut-offs, approaches for analysis of antibody patterns and reactivity, physical crossmatch methodologies, servicing different transplant programs with different organ types, immunosuppression regimens and so on. This makes this compatibility assessment patient- and center-specific (Puttarajappa et al., 2023). Notably, the accuracy of the VXM assessment relies heavily on the quality of antibody test results. Given that the VXM assessment is used for clinical decision making, HLA laboratories must follow best practice guidelines to appropriately evaluate the immunologic risk to the patient (Tambur et al., 2018). Therefore, it was desirable to create an educational challenge that would help our global HLA community to compare detection of DSA, to understand how HLA laboratories are interpreting donor HLA typing and anti-HLA antibody profiles

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TABLE 1 Demographics of AC Survey participants in 2022.

Country	2022 AC-1	2022 AC-2
Argentina	2	2
Australia	4	4
Brazil	4	4
Canada	16	16
Chile	1	1
China	2	1
Colombia	2	2
Costa Rica	—	1
Guatemala	1	1
Hong Kong Special Administrative Region of China	1	1
Italy	1	1
Mexico	6	6
New Zealand	1	1
Panama	1	1
Peru	1	1
Poland	1	1
Puerto Rico	1	1
Qatar	1	1
Saudi Arabia	1	1
Singapore	1	1
Thailand	1	1
United States	106	107
Total	155	156

to predict a physical crossmatch result and assess transplant compatibility. These elements were missing from the ASHI PT surveys that only grade based on results of the physical crossmatch and antibody tests.

In this manuscript, we describe the ASHI PT Educational VXM Challenge as a model for creating educational content using PT survey data. We discuss the different components of the VXM challenge and highlight major findings and learning points acquired from the ASHI VXM challenges.

Materials and methods

Designing a VXM challenge

The PT Committee oversees the ASHI PT program with the goal of collaborating with HLA laboratories around the world to achieve the highest standards and continuous quality improvement in clinical testing and patient care. The committee strives to identify new PT challenge opportunities and to provide customized and comprehensive educational content. Given the increased use of the VXM assessment throughout our HLA community, the ASHI PT Executive Committee (EC) identified the need for a VXM Educational Challenge and launched the first challenge in 2018. This challenge was conducted in conjunction with the ASHI AC survey that is offered twice per year. The survey was built based on the grouping assignment for participants on the ASHI AC survey (Groups, A, B and C), where laboratories from each group received a different set of cells for crossmatch testing. The VXM Challenge participants were given a low/intermediate resolution HLA-A, B, Bw, C, DRB1, DRB345, DQA1, DQB1, DPA1 and DPB1 donor typing. Participants were required to use their own anti-HLA antibody data from the AC survey to perform a VXM assessment without knowledge of the PXM result. Serum/cell combinations for inclusion in the challenge were selected by the ASHI PT EC based on data from the AC survey. When possible, some of these combinations included challenging samples such as weak DSA, non-consensus physical crossmatch results, etc. Participant responses were collected in Google Forms after AC survey results were submitted but before AC Summary Reports

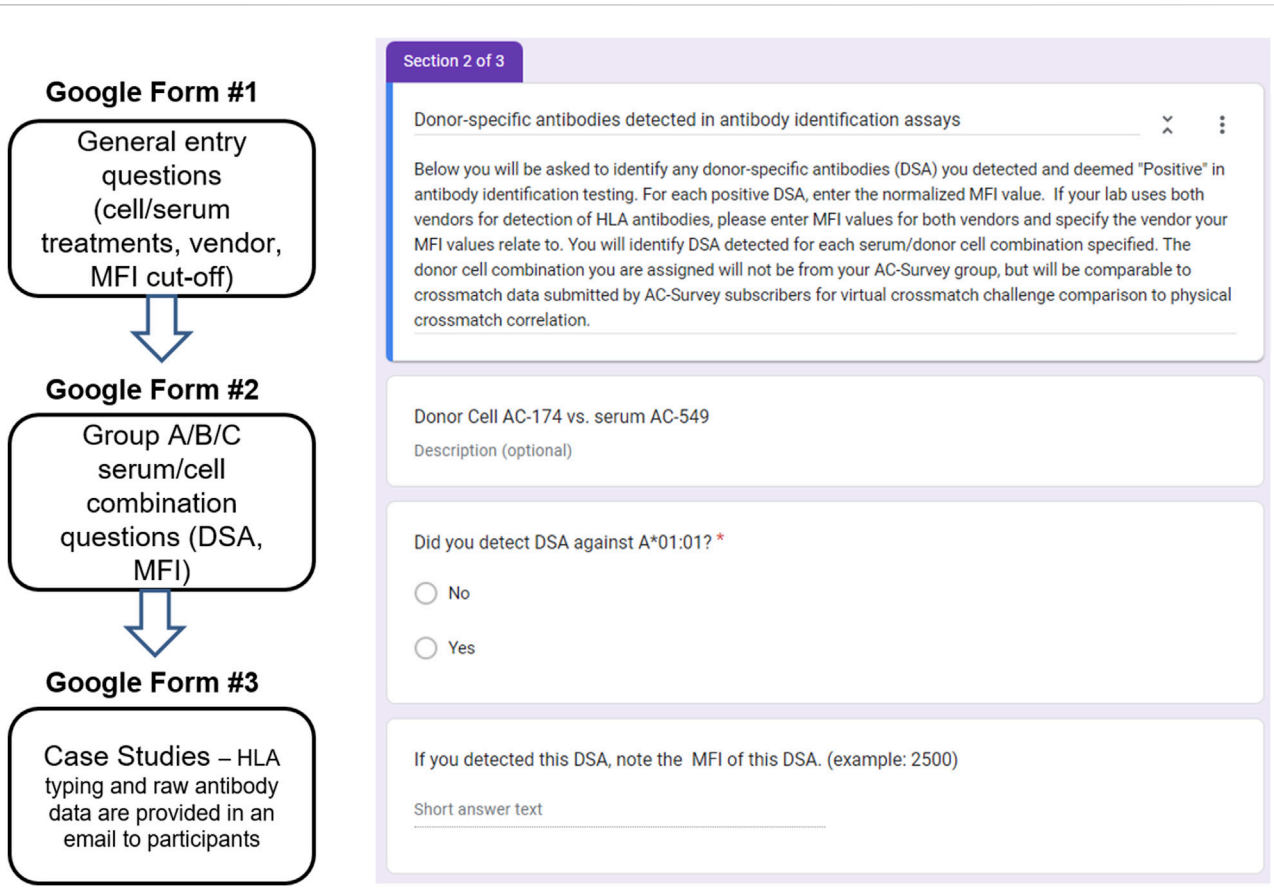


FIGURE 1
All VXM data were collected in Google Forms, exported, and analyzed in Microsoft Excel and/or GraphPad Prism. This challenge used PT data from the AC survey for educational purposes and was designed to have several components: General Entry questions, Cell/Serum Combination questions, and Case Studies. Right: An example for a Cell/Serum Combination Google Form.

TABLE 2 Number of 2022 VXM-1 Participants and cell assignments.

# Of responses per group	Cells used in AC-1 survey	Cells provided for VXM-1 challenge
Group A (N = 13)	AC-165, AC-166	AC-167 and AC-168 vs. AC-540
Group B (N = 10)	AC-167, AC-168	AC-165 and AC-170 vs. AC-540
Group C (N = 15)	AC-169, AC-170	AC-167 and AC-168 vs. AC-540
All Groups (N = 38)	n/a	APHIA C-05 and C-06 vs. Ser-07 and Ser-08

The 2022 VXM-1 challenge consisted of four AC-1 donor cells (AC-165, AC-167, AC-A68, AC-170), each virtually crossmatched with one serum (AC-540) tested in the 2022 AC-1 survey. Each group was assigned a donor cell that was not tested by this group for their cell-based crossmatch in the 2022 AC-1 survey.

with PXM results were published. The responses were exported and analyzed in Microsoft Excel and/or GraphPad Prism and comprehensive summary reports of analyzed data were written and reviewed by members of the ASHI PT EC. The challenge included three components (Figure 1), that contained questions, each allowing submission of information on different aspects of VXM assessment and/or laboratory practices:

1) General Entry questions: These questions were selected for the purpose of probing participants' use of SAB panels for HLA antibody detection and identification, serum treatment for solid-phase assays, cell treatment with pronase for FCXM and mean fluorescence intensity (MFI) cut-off values for positive DSA. This section provided valuable information regarding common practices and trends across HLA laboratories.
2) Cell/Serum Combination questions: Participants were asked to identify DSA using data from the ASHI AC survey and to predict the outcome of a physical FCXM or complement dependent cytotoxic crossmatch (CDCXM). An example for participant groups and cell assignment is found in Table 2. HLA typing for donor cells is performed using real-time PCR single antigen bead resolution (RT-PCR SABR) trays and the results are provided to all participants of the AC survey, while the antibody data comes from each participating

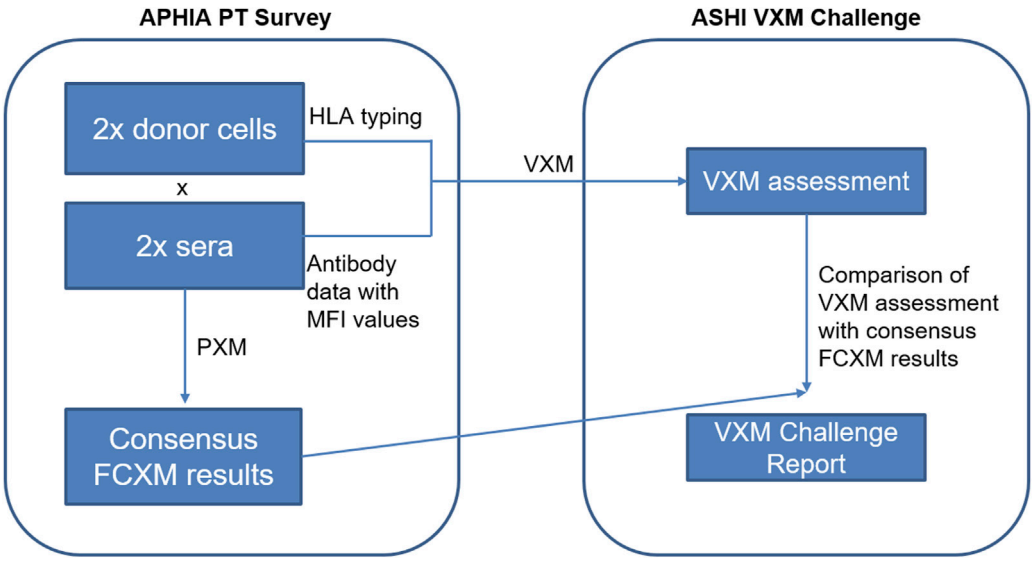


FIGURE 2
The International Case Study data consisted of two donor cells, each virtually crossmatched with two sera tested in the APHIA PT survey. Participants were provided with raw MFI data for the two serum samples and donor next-generation sequencing (NGS) typing for the two cell samples, and were asked to predict results of the FCXM for T- and B-cells based on the detected DSA for each serum/donor cell combination. Consensus PXM results were provided by APHIA retrospectively.

laboratory. MFI data for detected DSA is collected and analyzed to determine the variability in MFI values between different laboratories. VXM assessments from the three groups are compared to the physical CDCXM and FCXM results obtained from the AC survey. This section of the VXM Challenge also includes questions related to risk stratification (low, moderate or high risk), as well as offer acceptance criteria and provides information on whether participants would accept an organ offer based on their VXM assessment for different organ types. In later versions, participants were asked if they would recommend a PXM based on their VXM assessment.

3) Case Studies: Case studies may include real patient scenarios provided by an ASHI accredited HLA laboratory or include cases which are a result of collaboration between ASHI and a sister society, (e.g., Asia Pacific Histocompatibility and Immunogenetics Association - APHIA). For each case study, participants receive donor and recipient HLA typing data as well as recipient's antibody identification data, and are asked to predict the results of the PXM. An example of a case study is illustrated in Figure 2 and was a result of a collaboration between ASHI and APHIA. The selected case studies emphasized the importance of evaluating current and historical antibody data, antibodies with low signal yet spread over multiple HLA specificities, donor specific anti-HLA antibodies with borderline strength, as well as detection of inhibitory factors in sera, to mimic the level of complexity that HLA laboratories face in real clinical situations. The goal of these case studies is to emphasize key VXM learning points from real cases and highlight them in the summary reports for participants.

Correlation between VXM assessment and PXM

The ASHI VXM Challenge remains educational and therefore participants do not receive a grade for this effort. However, the results from the VXM assessments are compared to PXM results collected from the AC survey which is graded. In addition, reported DSA are compared to the consensus antibody results from the AC survey. Grading for the AC survey was performed as follows: The physical T-cell and B-cell FCXM and CDCXM were graded based on 80% consensus among participating laboratories. HLA class I and class II antibody specificities were graded separately. An antibody specificity reported by ≥ 90% of participants, either positive or negative, reached consensus. A minimum of 10 laboratories were required for grading. When less than 10 laboratories participated and when consensus requirements were not met the, results were not graded.

Results

Over a 5-year period, 2975 virtual crossmatch assessments were reported. The median number of labs that participated in this non-graded challenge was 54 laboratories per challenge. Over time, as challenges have become more focused on "interesting" or educational cell/serum combinations, the number of VXM performed and reported per challenge by each laboratory decreased, and the participation rate was around 35% of the AC survey membership (Table 3). Serum treatment protocols were monitored overtime using the General Entry Question section of the VXM Challenge. Figure 3 depicts those trends and shows that the percentage of HLA laboratories that are not treating their sera has

TABLE 3 Characteristics of the VXM challenges from 2018 to 2022.

	2018		2019		2020		2021		2022	
Challenge	VXM-1	VXM-2	VXM-1	VXM-2	VXM-1	VXM-2	VXM-1	VXM-2	VXM-1	VXM-2
Laboratories participating, n (% of ASHI AC Survey laboratories)	84 (56%)	63 (41%)	65 (43%)	49 (32%)	54 (37%)	51 (43%)	58 (38%)	44 (30%)	38 (25%)	53[a] (34%)
VXM cell/serum combinations assessed by each participant[b]	each lab had 5 sera/2 cells (840 VXMs)	each lab had 5 sera/1 cell (315 VXMs)	each lab had 5 sera/1 cell (325 VXMs)	each lab had 5 sera/1 cell (245 VXMs)	each lab had 5 sera/1 cell (270 VXMs)	each lab had 5 sera/1 cell (255 VXMs)	each lab had 3 sera/1 cell (174 VXMs)	each lab had 3 sera/1 cell (132 VXMs)	each lab had 1 serum/ 2 cells (76 VXMs)	each lab had 1 serum/1 cell (50 VXMs)
Case Studies	none	none	none	none	none	none	none	none	2 sera/2 cells (152 VXMs)	Case 1. 1 serum/ 1 cell
										Case 2. 2 sera/ 1 cell (141 VXMs)

[a]53 laboratories responded to the Data Entry Questions section of the VXM challenge. Of those 50 laboratories completed the cell/serum combination section and 47 laboratories completed the Case Study section of the VXM challenge.
[b]The number of total VXM does not include CDCXM prediction due to the variability in CDC XM results availability.

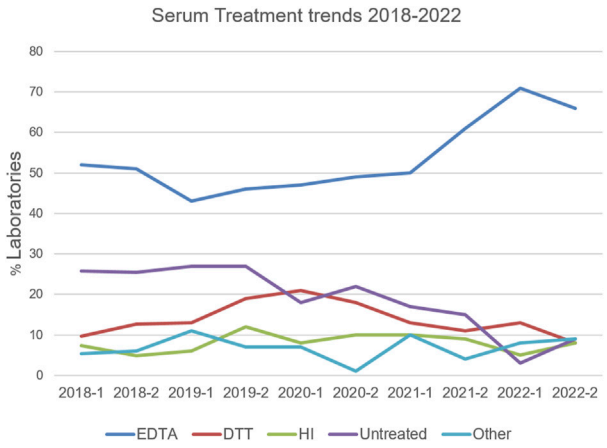


FIGURE 3
Serum treatment trends reported in the VXM Challenges between 2018 and 2022. Percentage of HLA laboratories using EDTA (blue), DTT (red), Heat Inactivation (HI; green), Untreated (purple) or Other (light blue) is depicted. "Other" included a combination of two treatments, dilutions, AdsorbOut beads, and Fetal Calf Serum treatment.

decreased over time (26% in 2018 versus 9% in 2022), while the use of Ethylenediaminetetraacetic acid (EDTA) as serum treatment has become more popular (52% of laboratories have reported the use of EDTA in 2018 versus 71% in 2022). The use of other treatments such as Heat Inactivation (HI) and Dithiothreitol (DTT) has fluctuated over time, although did not exhibit a specific trend. Most laboratories (>80%) reported the routine use of one SAB panel, which is distributed more heavily towards one particular vendor. Up to 16% reported the use of SAB panels from two vendors (Figure 4). Most laboratories reported using a cut-off for antibody detection. In 2022, for example, 66% of participating laboratories reported using cut-offs of 500–1,000 MFI to define positive specificities, while 34% reported using cut-offs of 1001–2000 MFI (Figure 4). Several laboratories reported locus specific cut-offs indicating higher cut-offs for HLA-C, HLA-DQ and HLA-DP loci, and some laboratories reported using different cut-offs based on organ type and urgency. Laboratories that did not use a hard cut-off mentioned that their analysis was based on antibody patterns and eplets.

Detection of DSA and prediction of PXM

The participants of the VXM Challenge were asked to identify any DSA detected for each serum/donor cell combination with MFI values deemed "Positive" based on their established MFI cutoff. The percentage of laboratories reporting a specific DSA, mean MFI, Standard deviation (SD) and Coefficient of variation (CV) for cell/serum combinations from the 2021 ASHI VXM-1 Challenge can be seen in Table 4. In general, there were 2 types of predictions: 1) In the presence of consensus positive DSA, typically with a high MFI, the prediction of a positive PXM is fairly straight forward (e.g., cell AC-153 VS. serum AC-532); 2) In the presence of low level DSA, typically <3000 MFI (single or combined), there was a lack of concordance between the VXM and PXM (e.g., cell AC-153 VS. serum AC-534, T-cell FCXM and cell AC-153 VS. serum AC-531). A detailed explanation for specific discrepancies between VXM and PXM results is provided in the highlights section below.

Highlights from ASHI VXM challenges

Each VXM Challenge was unique and provided an opportunity to look at factors contributing to the prediction of a PXM. More recent challenges incorporated fewer AC Survey cell/serum combinations but included additional educational case studies. A few highlights from past ASHI VXM challenges are provided below.

Example 1: Low level DSA

The 2022 VXM-1 challenge included three donors typed as HLA-A*02, each donor carrying a different HLA-A*02 allele: AC-

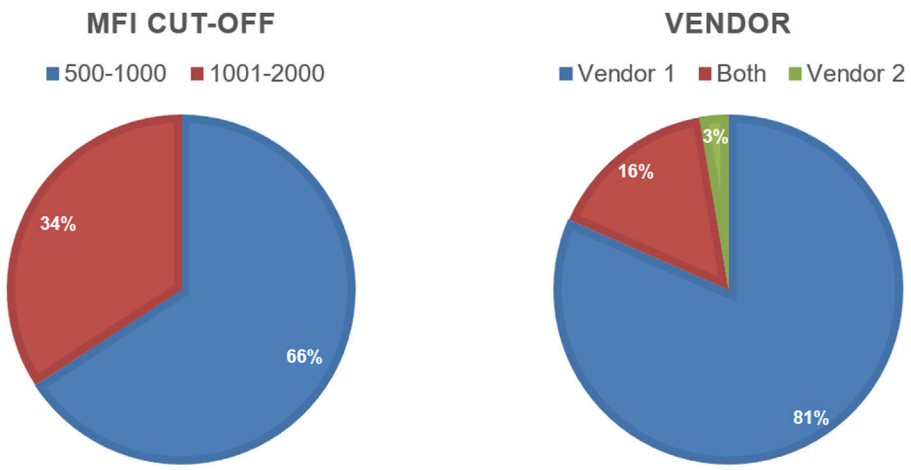


FIGURE 4
Pie charts showing MFI cut-off and Vendor use reported by the 2022 VXM-1 Challenge participants.

TABLE 4 DSA reported for cell AC-153 with sera AC-531, 532, and 534.

Pair	DSA	%Labs reporting this DSA in the VXM Challenge	Mean MFI	SD	% CV	Consensus on AC survey	%Lab predicting a pos T-cell VXM	%Lab predicting a pos B-cell VXM	%Labs reporting a pos T-cell FCXM	%Labs reporting a pos B-cell FCXM
AC-153 VS. AC-531	B*14:02	36	918	218	24	No (35%)	32	28	81	79
	B*18:01	75	1820	524	29	No (71%)				
AC-153 VS. AC-532	DRB1*12:01	100	2560	735	29	Yes DR12 (99%)	0	100	3	100
	DRB1*13:04	93	4577	1881	41	Yes DR13 (100%)				
	DRB3*01:01	46	1135	697	61	Yes DR52 (94%)				
	DRB3*02:02	82	1390	562	40	Yes DR52 (94%)				
	DQA1*01:04/ DQB1*05:01	82	10939	2104	19	Yes DQ5 (99%)				
AC-153 VS. AC-534	C*02:02	75	2122	934	44	No (68%)	17	86	7	100
	DQA1*01:04/ DQB1*05:01	86	15164	6603	44	Yes DQ5 (100%)				

168 A*02:05, AC-165 A*02:02 and AC-170 A*02:01. The three donors were crossmatched against the serum AC-540. Participants were asked if they identified antibodies in serum AC-540, directed against each of the three HLA-A*02 donor alleles (A*02:05, A*02:02 and A*02:01). A total of 52 responses for all 3 donors were collected and of those, 42 laboratories (81%) indicated that they did not detect DSA against the corresponding A*02 donor allele in serum AC-540, some due to the donor allele not being present on the SAB panel, whereas 10 responses (19%) indicated that this serum was positive for DSA against HLA-A*02 with a mean MFI = 1377, SD = 600 and CV = 44. The MFI range using one vendor was 682–2441 and two laboratories also reported lower MFI values using a second vendor. Laboratories that reported MFI values commented that they used the HLA-A*02 beads present on their SAB panel to estimate the presence of antibody against donor A*02 alleles that were not represented on their single antigen bead panels (i.e., A*02:02 and A*02:05). A few laboratories also commented on stacking of the A*02 beads around the cut-off, or mentioned reactivity against the A2 CREG beads. Based on the low MFI value for the DSA, the majority of participants predicted that the T-cell FCXM would be negative with all three donors. The physical T-cell FCXM was consensus negative only for cells AC-165 (A*02:02) and AC-168 (A*02:05), while 42% of AC-1 survey participants reported a positive T-cell FCXM for cell AC-170

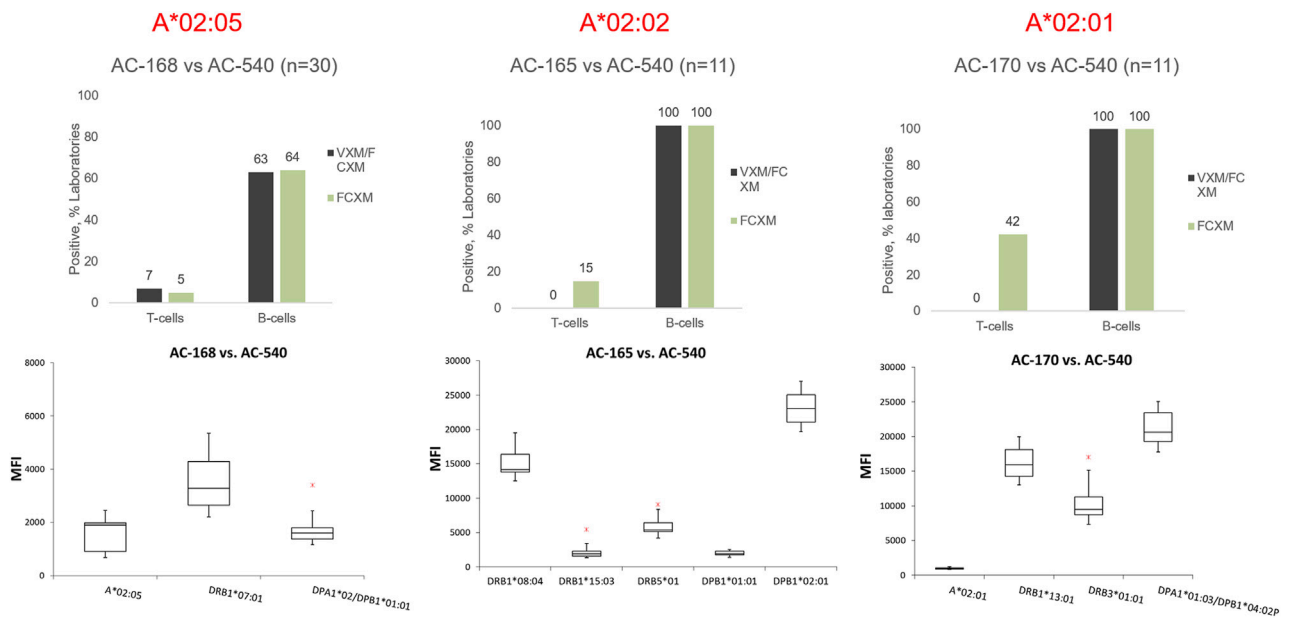


FIGURE 5
Top panel: Predicted (VXM/FCXM) and physical (FCXM) results for cells AC-168 (right), AC-165 (middle), and AC-170 (left) against serum AC-540. Cell AC-168 was typed as HLA-A*02:05, AC-165 was typed as HLA-A*02:02 and AC-170 was typed as HLA-A*02:01. Bottom panel: boxplots displaying the distribution of normalized MFI values reported by VXM Challenge participants against each cell based on a five number summary ("minimum" whisker, first quartile, median, third quartile, and "maximum" whisker). The * indicates the outliers. DSA reported by a single laboratory were not plotted. Only One lab reported an MFI value for A*02:02 (AC-165 vs. AC-540) and therefore, A*02:02 was not included in the graph.

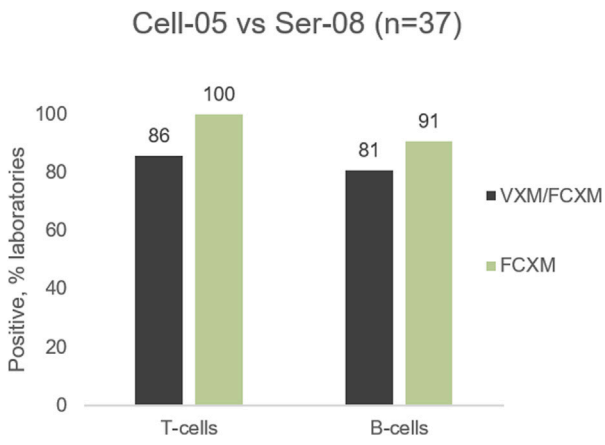


FIGURE 6
Predicted (VXM/FCXM) and physical (FCXM) results for Cell-05 against serum Ser-08.

(A*02:01) (Figure 5). Of note, HLA-A2 antibody specificity was reported by 34% of AC survey participants and did not reach consensus. This example illustrates several points: first, VXM prediction lacks correlation with PXM in the presence of low level DSA. Second, when donor alleles are not represented on the SAB panel most laboratories use beads within the same antigenic group as surrogates. Since SAB panels from different vendors have different HLA allele coverage, it is recommended to tailor the panel to the donor alleles in order to accurately assess the presence of DSA. In ethnically diverse donor pools, utilizing an extended SAB panel that includes alleles commonly found in specific ethnic groups may be warranted. Another option is to test more than one panel/platform. In this example, a phenotype panel has representation of all three donor HLA-A*02 alleles and could be used to confirm reactivity.

Example 2: Impact of HLA-C antibodies

The 2022 VXM-1 International Case Study included a cell/serum combination with DSA to HLA-Cw5 (MFI = 10,024), and Cw6 (MFI = 8924). Most laboratories accurately predicted a positive T- and B-cell FCXM. However, several laboratories seemed to underestimate the ability of anti-HLA-C antibodies to cause a positive FCXM, with 86% (32/37) of laboratories predicting a positive T-cell FCXM versus 100% (11/11) of laboratories reporting a positive physical T-cell FCXM and 81% (30/37) of laboratories predicting a positive B-cell FCXM versus 91% (10/11) of laboratories reporting a positive physical B-cell FCXM (Figure 6). Several laboratories commented that the prediction in this case is challenging due to the presence of C-locus antibodies only, as HLA-C has lower expression on the cell surface than HLA-A and HLA-B (Apps et al., 2015). As previously noted, data from the VXM Challenge indicates that laboratories tend to have higher cut-offs for calling C-locus antibodies. Despite the lower expression and the association of some C-locus antibodies with "non-specific" antibody patterns, this case highlights that these antibodies can be clinically relevant and therefore, a prospective PXM may be warranted to assess their ability to cause a positive crossmatch. On the other hand, Table 4 shows an example of lack of correlation between the VXM and T-cell FCXM in the presence of a DSA to HLA-C (cell AC-153 VS. serum AC-534). 75% of the VXM

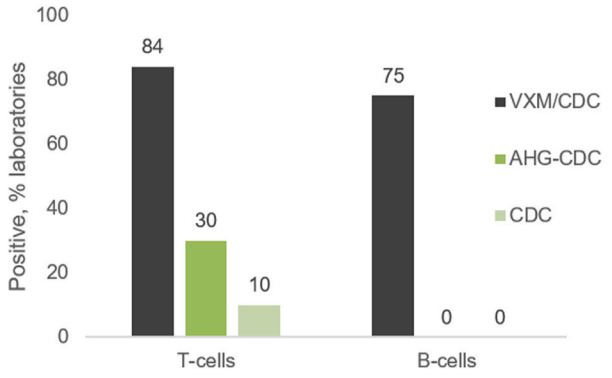


FIGURE 7
Predicted (VXM/CDC) and physical (AHG-CDC, CDC) results for cell AC-174 against serum AC-549.

Challenge participants detected DSA against C*02:02 with an average MFI of 2122 in serum AC-534, and 83% of the participants correctly predicted a negative PXM in the presence of this low-level DSA, in correlation with the AC-1 survey negative consensus for the FCXM with AC-153 cells and AC-534 serum. However, 17% predicted a positive T-cell FCXM.

Example 3: Prediction of CDCXM

The 2022 VXM-2 included a serum/cell combination, serum AC-549 against cell AC-174, with a consensus DSA to HLA-B8. A high mean MFI value of 12,235 was reported by the participants of the VXM challenge and may explain the positive prediction of T-cell and B-cell FCXM which agreed with the physical FCXM results obtained in the AC-2 survey. However, 84% and 75% of the VXM Challenge participants predicted positive T-cell and B-cell CDCXM, respectively, while consensus physical CDCXM was negative for both T-cells and B-cells (Figure 7). Only 10% of laboratories reported a positive T-cell CDCXM and only 30% of participants reported a positive T-cell AHG-CDCXM, even though 85% of the 2022 AC-2 Survey participants who utilized the C1q assay reported anti-HLA-B8 as complement fixing. This indicates that although C1q binding is often considered to be associated with antibody strength and a positive CDCXM (Zeevi et al., 2013; Tambur et al., 2015), predicting CDCXM results remains a challenge. Of note, the participants of the VXM Challenge did not have the C1q information at the time of VXM assessment, unless their laboratory performed the C1q assay. This information was available to participants only after the Challenge has been concluded. The high percentage of participants predicting a positive CDCXM in this case suggests that CDCXM results should not be predicted based on MFI values alone.

Example 4: Antibody against a shared eplet

One of the 2022 VXM-2 Case Studies included a donor typed as DRB1*13:02, an allele which is not present on the single antigen bead panel that was provided to the participants with this case study. Other DRB1*13 alleles had low normalized MFI values (DRB1*13:01 = 1,201; DRB1*13:03 = 1,336). 98% of participants correctly predicted a negative T-cell FCXM in the absence of HLA class I DSA. 26 out of 47 participants (55%) predicted a negative B-cell FCXM, 8 (17%) predicted a positive B-cell FCXM and 13 (28%) responded "Other" and commented that further testing would be required to determine compatibility in this case, either by running this sample on a different panel that covers the DRB1*13:02 allele, or by running a PXM. Several laboratories responded that the B-cell FCXM is likely to be negative assuming that the reactivity of the antibody to DRB1*13:02 is similar to the reactivity of the other DRB1*13 antibodies. Other laboratories noted that an antibody against a shared eplet might be present based on a reactivity pattern that included all the DR52 associated DRB1 beads stacking with low level MFI (Figure 8), and therefore the B-cell FCXM might be positive, and a few laboratories commented that the B-cell FCXM prediction is indeterminate. Based on their prediction most laboratories (64%) responded that a physical FCXM would be requested in this scenario. 17% of the participants responded that they would not request a physical FCXM and 19% responded "Other" indicating that a crossmatch would or would not be requested depending on the specific scenario. Interestingly, the physical FCXM for this case study was B-cell positive, despite the low MFI value of the surrogate DRB1*13 single antigen beads. As mentioned by several participants this B-cell FCXM positivity is likely due to the presence of an antibody against a shared eplet, although this phenomenon has been questioned by other groups and there may be additional or other factors contributing to the lack of correlation between SAB assays and FCXM results (Claisse et al., 2022). This reactivity could be explained by eplet 96HK which is present on DR8,11,12,13,14,17,18 but not on DR52 or by a combination of eplet 11STS which is present on DR11,13,14,17,18 but not the DR52 and eplet 16Y, which is present on DR8 and specific alleles of DR12 and DR14. Another example of this phenomenon can be seen in Table 4. For serum AC-531 vs. cell AC-153, a DSA against B*14:02 (B65) was reported by 36% of the VXM Challenge participants with a mean MFI value of 918, and a DSA against B*18:01 (B18) was reported by 75% of the VXM Challenge participants with a mean MFI value of 1820. Based on these results, 32% of laboratories predicted a positive T-cell FCXM and 28% of participants predicted a positive B-cell FCXM. However, the consensus result for the physical FCXM was T-cell positive. B-cell FCXM results were not graded due to lack of consensus, but 79% of laboratories reported a positive B-cell FCXM. This discrepancy is most likely due to the presence of an antibody against the Bw6 motif, which is expressed by both B65 and B18. These examples indicate that MFI cannot be trusted as accurate in cases of a shared eplet, and that weak shared eplets should trigger a PXM.

Example 5: Assessment of risk

In the 2020 VXM-2 challenge, participating laboratories were asked to use the data from the VXM to provide a risk assessment in the case of a kidney, heart, lung, pancreas, kidney/pancreas and kidney/liver transplant. For all sera/cell combinations, variability in risk assessment was mostly observed for kidney/liver transplantation (Figure 9). Specifically, serum AC-525 demonstrated DSA against cell AC-149: anti-HLA-A1 with mean MFI = 18,705, and anti-HLA-B38 with mean MFI = 2,040. All VXM Challenge participants

	A		B		C		DRB1		DRB3,4,5		DQA1	DQB1	DPA1	DPB1
Recipient	2	-	44	62	5	9	04:01	07:01	-	53	02:01, 03:01P	02:02, 03:01	n/t	n/t
Donor	2	-	51	60	10	15	04:04	13:02	52	53	01:02, 03:01P	03:02, 06:04	01:03, -	04:01, -

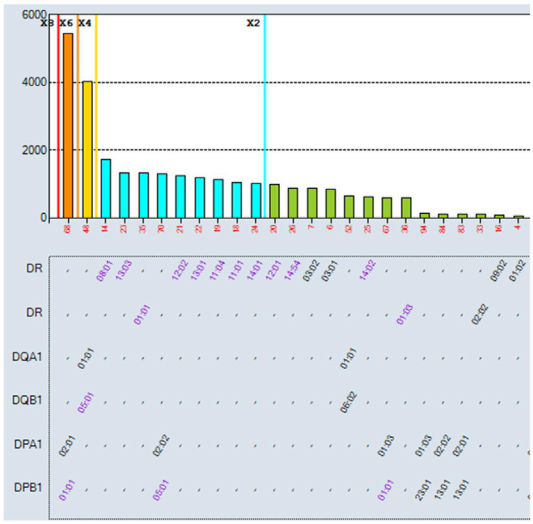
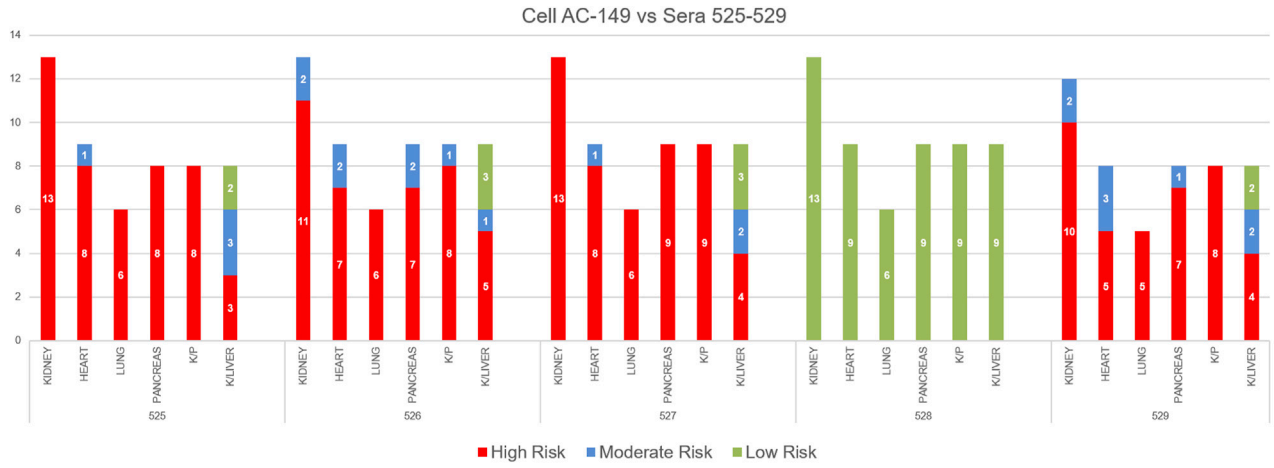


FIGURE 8
Participants of the 2022 VXM-2 Challenge were provided with recipient's raw MFI antibody data (one serum sample) and donor HLA typing, and were asked to predict results of a FCXM for T- and B-cells based on detected DSA for the serum/donor cell combination. A total of 47 participants responded to this section of the challenge. Top: Recipient and donor HLA typing results. Bottom: HLA Class II SAB results.



AC-525	Mean MFI	SD	AC-526	Mean MFI	SD	AC-527	Mean MFI	SD	AC-528	Mean MFI	SD	AC-529	Mean MFI	SD
A*01:01	18705	4681	A*01:01	1841	1261	B*08:01	9368	5755	NO DSA	-	-	C*12:03	1335	229
B*38:01	2040	415	DRB1*03:01	8392	2912	B*38:01	5601	5908				DQB1*02:01	5145	1025
			DRB1*13:01	8690	2995							DPB1*04:01	7176	2206
			DRB3*01:01	1974	1125							DPB1*04:02	11350	2850

FIGURE 9
In the 2020 VXM-2 challenge, participating laboratories were asked to use the data from the VXM to provide a risk assessment in the case of kidney, heart, lung, pancreas, kidney/pancreas and kidney/liver transplantation. The responses are depicted in a bar graph; Red = High Risk, Blue = Moderate Risk, Green = Low Risk. For all sera/cell combinations, variability in risk assessment was mostly observed for kidney/liver transplantation. The bottom table is showing mean MFI values and standard deviation (SD) for DSA against cell AC-149 in sera AC-525-529.

assessed this donor/recipient pair as "High Risk" for kidney, heart, lung, pancreas and kidney/pancreas transplantation with the exception of one participant assessing this pair as "Moderate Risk" for kidney transplantation. In contrast, of the 8 participants assessing this pair for a kidney/liver transplantation, 3 scored this pair "High Risk," 3 "Moderate Risk" and 2 "Low Risk," indicating

that VXM may vary based on center's risk tolerance. Of note, the PXM for this serum/cell combination was reported T- and B-cell positive by 100% of laboratories using FCXM and T-cell positive by 55% laboratories using AHG-CDC, while, CDC T- and B-cell crossmatches were consensus negative.

Discussion

The ASHI VXM Educational Challenge collects data from HLA laboratories across a broad geographical area on which assays laboratories are using for performing VXM assessment (i.e., SAB antibody panels), how they treat serum and cells, and what criteria they use for calling positive DSA. In addition, the challenge collects DSA MFI data and provides information on participants' assessment of risk and criteria for accepting organ offers for renal versus non-renal transplant candidates. DSA MFI data reported by VXM Challenge participants demonstrated variability consistent with previously published data (Reed et al., 2013). These data could be used by laboratories to assess performance and determine whether any changes to their testing protocol are needed.

Despite the increased use of VXM in clinical practice, the participation in the VXM Educational Challenge has slightly decreased overtime, with a typical response rate of 30%–40% of laboratories enrolled in the ASHI AC Survey. This decrease could be due to the fact that the participation in this challenge is optional and not graded, or due to the length of the survey, which factored into the decision to reduce the number of cell/serum combinations per challenge. Each challenge was unique and varied in the number of cell/serum combinations and Case Studies given to participants and was designed to address different clinical scenarios encountered by HLA laboratories in their everyday practice. Our data shows that the percentage of HLA laboratories using serum treatments has increased from 2018 to 2022, with EDTA being the most used serum treatment. These data allow the PT EC to monitor changes in HLA laboratory practices over time. Overall, the data from VXM challenges demonstrate that in the presence of antibodies which are consensus-positive by SAB, most laboratories can predict a FCXM result more accurately than a CDCXM result. This could be due to CDCXM requiring additional crosslinking by multiple DSA of high titer for effective complement mediated cell death (Diebolder et al., 2014; Tambur et al., 2015). Virtual assessment guidelines may need to consider the antibody titers and correlation with C1q assay results to increase the accuracy of physical CDCXM result prediction. Although prediction of FCXM was generally more accurate, it became more difficult in the presence of low level DSA which often did not reach consensus on the ASHI AC Survey, when the DSA was aimed against donor antigens that are not present on the SAB panel (Kumar et al., 2021), and when an antibody against a shared eplet was suspected (Garcia-Sanchez et al., 2020). These limitations, for example, lack of antigen/allele representation on the single antigen bead assay, may result in unintentional exclusion from transplantation. In addition, the reverse may also occur (i.e., proceeding with a transplant in the presence of an undetected DSA). Tools such as the HLA eplet registry and HLA MatchMaker can be used to identify eplets present on alleles that are not included in SAB panels and common antigens that share these eplets can be used as surrogates when analyzing SAB reactivity patterns (eplet-based analysis). Alternatively, the use of expanded panels may help resolve some of these issues.

Some laboratories prefer to use a VXM assessment-only approach to select transplant candidates. Considerations for the exclusion of patients as a result of conservative evaluation of a VXM due to low or unclear reactivity on the SAB may disenfranchise a category of patients. Limitations of the SAB assay include false negative and false positive results and inability to accurately predict results of PXM in the presence of more than one weak DSA or DSA directed against shared eplets. Therefore, laboratories are encouraged to perform VXM assessment based on comprehensive evaluation of different test methods and analyses, including screening and phenotype assays and/or surrogate crossmatches, as well as eplet analysis. Laboratories should define which cases require a PXM in their transplant agreements, such as in highly sensitized patients.

Our VXM Assessment Challenge with follow-up PXM allows participants to determine, using their center practice guidelines, if their positive VXM assessment interpretation of the potential DSA would have led to a negative PXM. These VXM challenges are essential to educate our Histocompatibility community to increase awareness and understanding on how to fine-tune the correlation between the VXM assessment and PXM, which is critical for transplant equity.

FCXM sensitivity can be impacted by multiple factors including test protocols which are variable across laboratories (e.g., cell to serum ratio especially impacts samples showing weak DSA reactivity, cell treatment, etc.) (Jaramillo et al., 2018), cell source (blood vs. spleen vs. lymph nodes, as well as deceased vs. living donors) (Badders et al., 2015), and HLA expression level on the cell surface, which can be assessed either at the protein level or at the transcript level. New technologies for the assessment of HLA allele transcript levels, in combination with patients' SAB data and donor HLA typing, could provide more granular data for VXM assessment and risk stratification (Cornaby et al., 2022). Importantly, participants of the ASHI VXM Challenge are instructed to respond based on their center practices. HLA laboratories utilize different DSA cut-offs, cell and serum treatments, and antibody SAB panels, all of which impact the prediction of a PXM. The VXM Challenge provides opportunity for participating laboratories to assess the analytic validity of their solid phase assay, improve their prediction capabilities, make VXM assessment more accurate and put policies in place for when a VXM can be used *in lieu* of a PXM.

The VXM Challenge has some limitations: 1) Transplant centers vary in the risk they are willing to take based on organ type, transplant volumes and patient clinical characteristics. The VXM Challenge does not collect data on transplant volumes and organ type, however, the risk stratification section includes separate questions for renal versus non-renal transplant candidates and some challenges included organ-specific risk assessment questions. 2) It is important to remember that VXM Challenge participants do not have clinical information such as history of sensitizing events (e.g., pregnancies, prior transplants, transfusions, etc.) and "patient" typing, with the exception of Case Studies, which may limit their ability to accurately predict PXM results. Therefore, for the purpose of this challenge, participants were instructed to assume that there is no matching between HLA typings of donor and recipient pairs, each recipient has had no significant clinical events to consider, the participants have tested current serum samples and the patient's anti-HLA antibody testing history is consistent. In addition, while the participants of the VXM utilize their own raw antibody data to

perform VXM assessment for the cell/serum combinations, the antibody data for the Case Studies is provided in excel spreadsheets, which may limit their ability to perform eplet analysis. In the future, comma-separated values (CSV) files may be used to address this issue. 3) The VXM Challenge does not collect raw data on all single antigen beads from participants, it only collects DSA bead data, and therefore the analysis of antibody and eplet patterns relies on comments provided the participants. The participants are encouraged to report pertinent observations such as eplet reactivity in the Google Form. 4) Response rate is about a third of all AC Survey participants. However, even with this response rate the challenge had a median of more than 50 participants per challenge, which translates into more than 100 VXMs per challenge.

In conclusion, the ASHI VXM Educational Challenge serves as a valuable tool that highlights the strengths and pitfalls of the VXM assessment and reveals differences in testing and results interpretation in participating HLA laboratories. This is particularly important since understanding the collective mindset of HLA laboratories during a challenging or borderline case can help all members of the HLA community when they are faced with a difficult assessment and are unable to perform a PXM. In an era where 26% of HLA laboratories use VXM followed by a retrospective PXM, and 8% rely solely on VXM to determine donor-recipient compatibility for deceased donor kidney transplants (Puttarajappa et al., 2023), it is imperative that we collect data that can shed light on when it is most advantageous and safe to utilize VXM and when a PXM is still preferable.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

RH-D: Conceptualization, Data curation, Formal Analysis, Methodology, Writing–original draft. MP: Data curation, Formal Analysis, Methodology, Writing–review and editing. OT: Data curation, Formal Analysis, Methodology, Writing–review and editing. IG: Data curation, Formal Analysis, Methodology, Writing–review and editing. HD: Data curation, Writing–review and editing. AG: Writing–review and editing. PJ: Conceptualization, Data curation, Formal Analysis, Methodology, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Novel alleles in the era of next-generation sequencing-based HLA typing calls for standardization and policy

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Next-Generation Sequencing (NGS) has transformed clinical histocompatibility laboratories through its capacity to provide accurate, high-throughput, high-resolution typing of Human Leukocyte Antigen (HLA) genes, which is critical for transplant safety and success. As this technology becomes widely used for clinical genotyping, histocompatibility laboratories now have an increased capability to identify novel HLA alleles that previously would not be detected using traditional genotyping methods. Standard guidelines for the clinical verification and reporting of novelties in the era of NGS are greatly needed. Here, we describe the experience of a clinical histocompatibility laboratory's use of NGS for HLA genotyping and its management of novel alleles detected in an ethnically-diverse population of British Columbia, Canada. Over a period of 18 months, 3,450 clinical samples collected for the purpose of solid organ or hematopoietic stem cell transplantation were sequenced using NGS. Overall, 29 unique novel alleles were identified at a rate of ~1.6 per month. The majority of novelties (52%) were detected in the alpha chains of class II (HLA-DQA1 and -DPA1). Novelties were found in all 11 HLA classical genes except for HLA-DRB3, -DRB4, and -DQB1. All novelties were single nucleotide polymorphisms, where more than half led to an amino acid change, and one resulted in a premature stop codon. Missense mutations were evaluated for changes in their amino acid properties to assess the potential effect on the novel HLA protein. All novelties identified were confirmed independently at another accredited HLA laboratory using a different NGS assay and platform to ensure validity in the reporting of novelties. The novel alleles were submitted to the Immuno Polymorphism Database-Immunogenetics/HLA (IPD-IMGT/HLA) for official allele name designation and inclusion in future database releases. A nationwide survey involving all Canadian HLA laboratories confirmed the common occurrence of novel allele detection but identified a wide variability in the assessment and reporting of novelties. In summary, a considerable proportion of novel alleles were identified in routine clinical testing. We

propose a framework for the standardization of policies on the clinical management of novel alleles and inclusion in proficiency testing programs in the era of NGS-based HLA genotyping.

KEYWORDS

next-generation sequencing, HLA, novel alleles, standardization, proficiency testing

1 Introduction

The history of HLA typing in transplantation began with serology-based techniques such as complement-dependent cytotoxicity assays (Middleton, 2005). This was followed by molecular platforms such as the Reverse Sequence-Specific Oligonucleotides typing (RSSO) and Sanger sequencing which continue to be the main typing methods in many HLA laboratories. In recent years, Next-Generation Sequencing (NGS) has emerged as an effective and powerful HLA typing platform. One of the major advantages of NGS is its ability to generate high-resolution genotypes across the 11 classical HLA genes at high throughput and low cost (Hosomichi et al., 2015), which enables many HLA genes to be sequenced at full length. Depending on the NGS assay, many discriminating single nucleotide polymorphisms (SNPs) between allele combinations can now be phased, thereby reducing the occurrence of cis/trans ambiguities. These collective improvements have enabled NGS to identify novel sequences at a greater capacity compared to traditional molecular methods. New alleles are discovered at a record pace in recent years, with approximately 6,000 novel alleles on average being added each year to the Immuno Polymorphism Database-Immunogenetics/HLA (IPD-IMGT/HLA Database), the official database for HLA alleles (Barker et al., 2023; IPD-IMGT/HLA Database). Much of this phenomenon can be accredited to the widespread use of NGS for HLA genotyping (Barker et al., 2023).

Currently, there is limited guidance on the best practice for the evaluation and verification of novel alleles. In addition, how to report novel alleles clinically and the level of information that should be conveyed to the end-user is unclear. The American Society for Histocompatibility and Immunogenetics (ASHI), the governing body for accredited clinical transplant laboratories, also does not currently have a Proficiency Testing (PT) Program to evaluate the ability of accredited laboratories to identify novel alleles accurately (Proficiency Testing Program).

In addition to clinical reporting, histocompatibility laboratories also play an important role in contributing to the IPD-IMGT/HLA database. While this official catalogue for documented HLA alleles is essential for the function of clinical histocompatibility by ensuring accurate and comprehensive interpretation of patient data, it is unknown the proportion of clinical HLA laboratories that routinely submit novel alleles to IPD-IMGT/HLA and potential barriers that might impede this process.

In this study, we describe the experience of an ASHI-accredited laboratory's use of NGS and its management of novel alleles in an ethnically diverse population. In addition, we summarize the results of a nationwide survey that describes the local practice of all Canadian histocompatibility laboratories that utilize NGS for clinical HLA genotyping. Based on these results, we advocate for the standardization of practice and propose a practical framework to facilitate the management and reporting of novel alleles and a streamlined mechanism to incorporate these alleles into established international HLA databases.

2 Materials and methods

2.1 Study cohort

The study cohort included all solid organ (kidney, heart, lung, liver, and pancreas) and hematopoietic stem cell transplant candidates and donors who underwent HLA genotyping at the Vancouver General Hospital Immunology Laboratory, the provincial reference laboratory for transplantation in British Columbia, Canada. All patients and donors were sequenced by Next-Generation Sequencing (NGS) as part of the routine workflow for transplant assessment. This study was approved by the UBC Research Ethics Board (#H22-03627).

2.2 DNA extraction

Whole blood was extracted using the EZ1 DNA Blood 350 µL Kit (Catalog 951054) or QIAsymphony DSP DNA Mini Kit (192) (Catalog 937255) (Qiagen, Germany). Both methods use magnetic beads to isolate DNA from leukocytes where the resulting DNA is eluted in water or a buffer. DNA was quantified using the Qubit Fluorometer (ThermoFisher, United States) and diluted to 10–35 ng/µL for sequencing.

2.3 NGS

Samples were sequenced with the Holotype HLA Kit version 2 (Omixon, Budapest). DNA underwent PCR amplification of the 11 classical HLA genes (HLA-A, -B, and -C; DRB1, -DRB3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1). HLA-A, -B, -C, -DPA1, -DQA1, and–DQB1 were sequenced in their entirety (i.e. 5′UTR–3′UTR), HLA-DRB1, -DRB3, and -DRB4 were sequenced from partial intron 1 to partial intron 4, and HLA-DRB5 and -DPB1 were sequenced from partial intron 1 to partial 3′-UTR. Amplicons were prepared for sequencing by enzymatic fragmentation, end-repair, and ligation of adaptors provided by the kit. Prepared libraries were sequenced using MiSeq Sequencer with the 300-cycle MiSeq Reagent Cartridge (Illumina, California, United States).

Sequence data were first routinely analyzed using HLA Twin (Omixon) (Omixon, Budapest, Hungary) versions (v) 4.3.0 and 4.4.1 with IPD-IMGT/HLA data versions 3.39, 3.43, and 3.45. All

samples with novel mutations were re-analyzed on HLA Twin v4.8.1 using IPD-IMGT/HLA v3.51 prior to submission into the IPD-IMGT/HLA database.

2.4 Identification of novel alleles

Under our current clinical practice, novel alleles are identified by the NGS analysis software HLA Twin (Omixon) (Omixon, Budapest, Hungary). The software aligns the sequenced data (i.e., consensus sequence) against the reference sequences of HLA alleles in the IPD-IMGT/HLA database to identify the allele with the most similarity to the consensus sequence. When there are exon mismatch(es) between the reference sequence and the consensus sequence, this is highlighted as a novelty by the software. A novel allele is indicated by the most related reference sequence followed by a "#1" at the end of the allele name. For example, the A*03:452 novelty was identified as A*03:05:01#1 at the point of routine analysis.

This study focused on the identification of alleles with novelties in exons. In alleles where intronic novelties co-occurred with exonic novelties, the intronic SNPs were also flagged and reported as part of the submission to the IPD-IMGT/HLA database. All samples with novel alleles passed the quality control (QC) metrics on the HLA Twin Software. As per our laboratory's standard operating procedure, all novel alleles were further verified by another ASHI-accredited HLA laboratory (HLA Typing Laboratory, Halifax, Nova Scotia, Canada) using an alternate NGS HLA kit, AllType FASTplex NGS (OneLambda, United States) and NGS platform, Ion Torrent (Thermofisher, United States).

2.5 RSSO and real-time PCR (RT-PCR)

In addition to confirmatory testing using an alternative NGS-based assay, RSSO, and RT-PCR were also performed for a proportion of samples during routine testing. RSSO was performed with the LABType SSO and XR kits (OneLambda, United States), where DNA was amplified at key regions of the HLA genes. Amplicons were then denatured and hybridized to sequence-specific oligonucleotides on beads. The bound amplicon was detected using PE-conjugated streptavidin and beads were ran through the LABScan3D (OneLambda, United States) flow analyzer. Reaction patterns to determine HLA typing were performed in HLA Fusion (Onelambda, United States). RT-PCR was performed using the LinkSeq PCR Typing Kits (OneLambda, United States) where sequence-specific primers amplified regions of DNA, and fluorescent reaction patterns were detected using the LightCycler 480 System (Roche, Canada) to determine HLA typing at low to intermediate resolution. Reaction patterns were analyzed on SureTyper for HLA (OneLambda, United States).

2.6 Submission to GenBank and IPD-IMGT/HLA

As per IPD-IMGT/HLA submission guidelines, novel sequences were first submitted to GenBank using BankIt (BankIt). Once an accession number was assigned, novel alleles were then submitted to IPD-IMGT/HLA for confirmation and documentation. The novel alleles were officially assigned by the WHO Nomenclature Committee for Factors of the HLA System in September–October 2022. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature Report (Marsh et al., 2010), names will be assigned to new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature Report.

2.7 Ethnicity determination

The majority of ethnicities (76%) of the study cohort were self-reported. When self-reported ethnicity was not available (24%), it was inferred based on the most probable predicted ethnicity using Haplostats, based on HLA-A, -C, -B, -DRB3/4/5, -DRB1, and -DQB1 associations (National Marrow Donor Program).

2.8 Analysis of non-synonymous/missense mutations

To assess the potential impact of non-synonymous mutations on the HLA protein phenotype, changes in the type and location of the modified amino acid were analyzed and assigned a score. First, variables that were used for the analysis included whether the mutation resulted in an amino acid change. As this was true for all non-synonymous mutations, all novelties with a missense mutation were assigned a "+". Next, the basic characteristics of the original and novel amino acids were compared based on the properties listed in Supplementary Table S1 (Sanvictores and Farci, 2022). If there was a change in the basic properties (non-polar (aromatic/aliphatic) versus polar (basic, acidic, uncharged) of the original residue, an additional "+" was assigned. If the novel mutation led to an altered amino acid in an antigen-binding site (i.e., exons 2 and 3 for class I and exon 2 for class II), another "+" was assigned. A "+" was further assigned if the mutation affected an HLA eplet as defined by the HLA Eplet Registry (HLA Eplet Registry). An eplet was defined as the critical amino acids residing within 3.0–3.5 Angstrom radius that constitute an HLA epitope considered essential for antibody-binding and specificity (Duquesnoy, 2006). To assess for potential changes to eplets, we evaluated whether the novel mutation occurred at a position of a known eplet that is expressed by the original allele or if the mutation introduced a new eplet. Combining all of the scores assigned above, each missense novelty can have a minimum of one "+" designation to four (i.e., "++++").

3 Results

3.1 Description of novel sequences

A total of n = 3,450 clinical samples were sequenced using NGS to derive high-resolution HLA genotypes from 1 January 2021–1 July 2022 (18 months), averaging approximately 48 samples per week. During this timeframe, 29 unique novel alleles (Table 1) were identified in

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TABLE 1 The unique novel HLA alleles identified in the study. Details of the novel mutations identified in exons are described here, including the type of mutation, exon location, the corresponding location on the protein, the nucleotide change, and the IPD-IMGT/HLA genomic position of the mutation.

Novel allele	Type of mutation	Exon	Location on protein	Nucleotide change	IMGT genomic position
A*03:452	Missense	Exon 3	Antigen-binding site, α2	A > C	873
A*26:203*	Missense	Exon 1	Leader peptide	G > C	28
B*15:675	Missense	Exon 1	Leader peptide	C > T	47
B*48:01:12	Silent	Exon 3	Antigen-binding site, α2	C > T	736
B*48:55	Missense	Exon 4	α3 extracellular arm	A > G	1701
B*56:88*	Missense	Exon 3	Antigen-binding site, α2	A > C	793
C*05:277	Missense	Exon 7	Cytoplasmic tail	G > C	2721
C*07:1041	Missense	Exon 2	Antigen-binding site, α1	C > A	256
C*07:1043	Missense	Exon 2	Antigen-binding site, α1	T > G	385
DPA1*01:03:38:02*	Silent	Exon 1	Leader peptide	C > T	51
DPA1*01:03:45	Silent	Exon 3	α2 extracellular arm	C > T	4506
DPA1*01:106*	Missense	Exon 1	Leader peptide	G > A	5
DPA1*01:136	Missense	Exon 3	α2 extracellular arm	A > G	4463
DPA1*01:137N	Nonsense	Exon 1	Leader peptide	C > T	79
DPA1*01:60*	Missense	Exon 4	Transmembrane/cytoplasmic tail	C > T	4901
DPA1*02:02:13	Silent	Exon 4	Transmembrane/cytoplasmic tail	T > C	4876
DPA1*02:96	Missense	Exon 4	Transmembrane/cytoplasmic tail	A > T	4850
DPB1*1088:01*	Missense	Exon 4	Transmembrane	A > G	9701
DQA1*01:01:09:02*	Silent	Exon 1	Leader peptide	C > G	36
DQA1*01:02:15	Silent	Exon 1	Leader peptide	C > T	48
DQA1*01:02:16	Silent	Exon 2	Antigen-binding site, α1	C > A	3976
DQA1*01:04:08	Silent	Exon 3	α2 extracellular arm	C > T	4548
DQA1*02:01:15Q	Silent	Exon 2	Antigen-binding site, α1	C > T	3809
DQA1*04:01:07	Silent	Exon 1	Leader peptide	C > T	48
DQA1*05:05:16	Silent	Exon 1	Leader peptide	T > A	69
DRB1*14:249	Missense	Exon 2	Antigen-binding site, β1	C > T	8091
DRB1*14:54:12*	Silent	Exon 3	β2 extracellular arm	A > G	10752
DRB5*01:130	Missense	Exon 3	β2 extracellular arm	G > T	10808
DRB5*02:37	Missense	Exon 3	β2 extracellular arm	A > G	10732

An asterisk (*) indicates an allele that was detected as novel at the time of analysis but upon submission into IPD-IMGT/HLA, had already received an official name by an independent laboratory.

41 samples (Supplementary Table S2). Six of the novel alleles were detected in multiple patient samples due to the sequencing of related donors and patients, which resulted in the same novel mutation being identified in related samples. For example, the novelty DQA1*05:05:16 was identified in two samples, one from a kidney patient and one from their related offspring donor. In another case, the novel allele DQA1*01:01:09:02 was identified in seven samples, where one was from a patient candidate for a bone marrow transplant, and three were from related siblings. Interestingly, the remainder three samples were not related to the patient where two were derived from matched unrelated donors and another was from an unrelated kidney patient. On average, two samples with a novel mutation were identified per month. When analyzed by unique novel mutations, 1.6 novelties were identified per month.

Among the 41 samples identified with a novel mutation in this study, almost half of the samples (n = 20, 49%) were detected in patients (n = 11, 27%) or donors (n = 9, 22%) considered for solid organ transplantation. Another 20 samples were derived from patients (n = 3, 7%) considered for a bone marrow transplant and their potential donors (n = 17, 41%). One additional sample with a novelty was identified in a patient tested for genetic disease association of ankylosing spondylitis.

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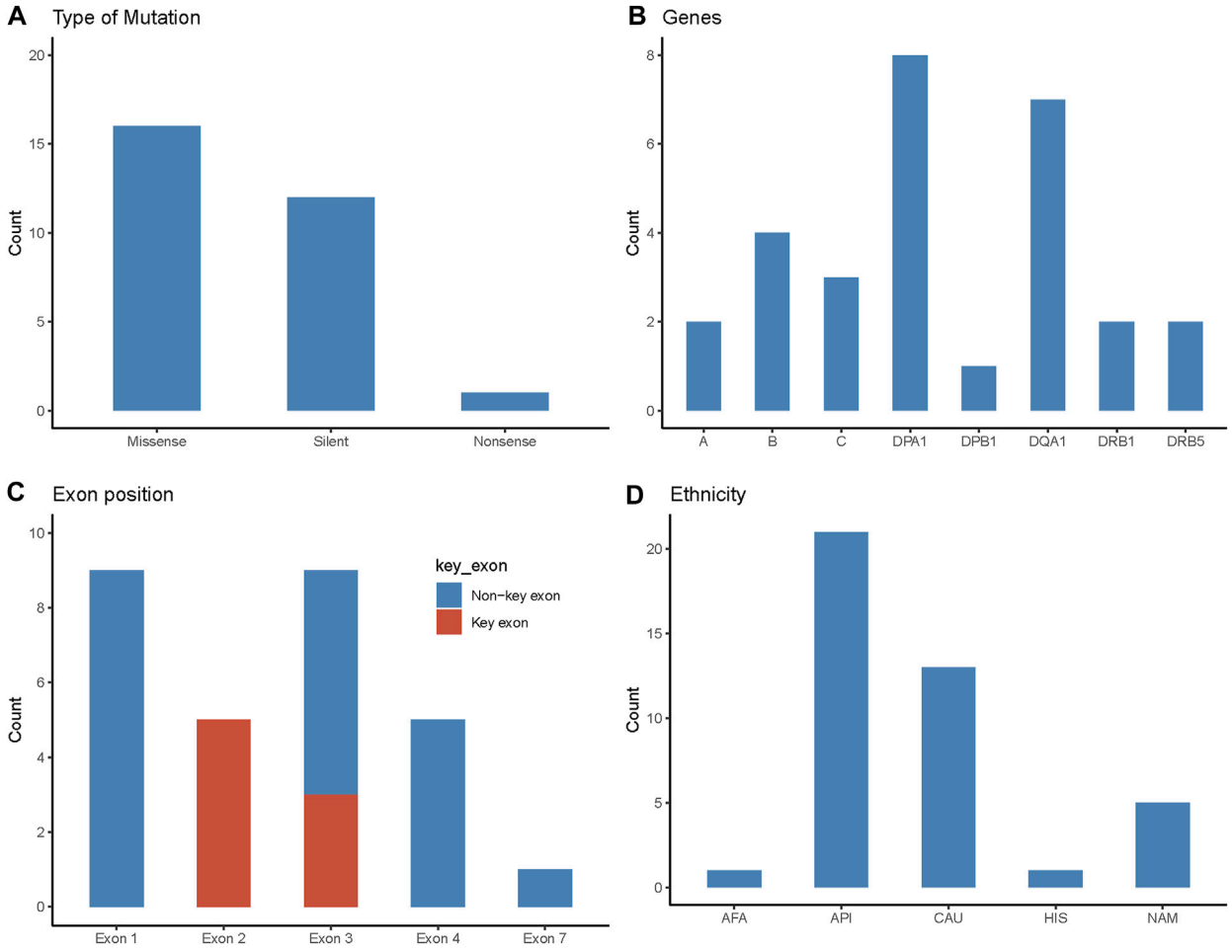

FIGURE 1
Characteristics of novel mutations identified in the study cohort. **(A)** Type of the novel mutations identified in the study and their frequency. **(B)** The HLA gene locus where the novel alleles were identified. **(C)** The exon position of novel mutations. Key exon represents the region that encodes the antigen-binding site of the HLA protein. Exons 2 and 3 are key exons for class I HLA proteins and exon 2 is the key exon for class II proteins. **(D)** Ethnicity of patients with novel mutations (n = 41). AFA, African American; API, Asian Pacific Islander; CAU, Caucasian; HIS, Hispanic; NAM, Native American.

All novelties reported in this study were single nucleotide polymorphisms (SNPs): 16 (55%) were missense mutations resulting in a non-synonymous amino acid change; 12 (41%) were silent mutations with no amino acid change; and one novelty (3%) resulted in a stop codon (i.e., nonsense) (Figure 1A).

In this study cohort, a novel allele was identified in all 11 HLA classical genes, except for HLA-DRB3, -DRB4, and -DQB1 (Figure 1B). Of the 29 novel alleles, HLA-DPA1 had the most novelties with eight sequences, followed by HLA-DQA1 with seven. HLA-B had four novel sequences, followed by HLA-C with three novelties. HLA-A, -DRB1, and -DRB5 had two novel sequences each. Lastly, HLA-DPB1 had one novel allele.

Eight novelties (28%) identified in this study occurred in the key exons that encode the antigen-binding site (i.e., exons 2 and 3 for class I and exon 2 for class II) (Figure 1C). Nine (31%) of the mutations occurred in exon 1 which encodes the leader peptide. Another nine novelties (31%) occurred in exon 3, where three were considered key exon changes (as stated above for class I) and six were non-key exons encoding the alpha-2 or beta-2 region in HLA class II. Five (17%) novel alleles were due to exon 4 mutations, where one was found in HLA class I (HLA-B*48:55) which encodes alpha-3, and the other four belonged to HLA class II, encoding the transmembrane/cytoplasmic regions. Lastly, there was one novelty (3%) in exon 7 (HLA-C*05:277), encoding for the cytoplasmic region of the protein.

The ethnic makeup of patients/donors with novelties was predominantly Asian or Pacific Islander (51%), followed by Caucasian (32%), Native American (12%), Hispanic (2%), and African American (2%). These proportions were similar to patients/donors of only self-reported ethnicities as well, where the proportions were Asian or Pacific Islander (52%), Caucasian (32%), Native American (13%), and African American (3%). These compositions were noticeably different from the general Canadian and British Columbian population, in which European origins comprise 53% and 60%, respectively, of self-reported ethnicity (compared to 32% in this cohort) with smaller proportions of minorities (Statistics Canada).

Non-synonymous/missense mutations where a single base pair alteration resulted in an amino acid change were present in 16 novel alleles. We studied the potential effect of the missense

TABLE 2 Novel alleles resulting in non-synonymous/missense mutations. The potential effect of missense mutations was assessed by comparing the novel amino acid to the original amino acid through differences in location, amino acid characteristics (Supplementary Table S1), and impact on the associated eplet (HLA Eplet Registry).

Novel allele	Location on protein	IMGT-IPD/ HLA codon position	Original a.a.	Basic properties of original a.a.	Novel a.a.	Basic properties of novel a.a.	Potential effect on protein	Variables that constitute "Potential effect on HLA protein"			
								A.a. change	Change in a.a. basic properties	Occurs in antigen-binding site	Affects an eplet
A*03:452	Antigen-binding site, α2	144	Lys	Basic	Gln	Polar uncharged	++++	+	+	+	+
A*26:203	Leader peptide	−15	Val	Nonpolar aliphatic	Leu	Nonpolar aliphatic	+	+			
B*15:675	Leader peptide	−9	Ala	Nonpolar aliphatic	Val	Nonpolar aliphatic	+	+			
B*48:55	Extracellular arm, α3	228	Thr	Polar uncharged	Ala	Nonpolar aliphatic	++	+	+		
B*56:88	Antigen-binding site, α2	116	Leu	Nonpolar aliphatic	Phe	Nonpolar aromatic	++++	+	+	+	+
C*05:277	Cytoplasmic tail	340	Cys	Polar uncharged	Ser	Polar uncharged	+	+			
C*07:1041	Antigen binding site, α1	17	Arg	Basic	Ser	Polar uncharged	++++	+	+	+	+
C*07:1043	Antigen binding site, α1	60	Trp	Nonpolar aromatic	Gly	Nonpolar aliphatic	+++	+	+	+	
DPA1*01:106	Leader peptide	−30	Arg	Basic	His	Basic	+	+			
DPA1*01:136	Extracellular arm, α2	149	His	Basic	Arg	Basic	+	+			
DPA1*01:60	Transmembrane/ cytoplasmic tail	224	Arg	Basic	Trp	Nonpolar aromatic	++	+	+		
DPA1*02:96	Transmembrane/ cytoplasmic tail	213	Ile	Nonpolar aliphatic	Phe	Nonpolar aromatic	++	+	+		
DPB1*1088:01	Transmembrane	194	Gln	Polar uncharged	Arg	Basic	++	+	+		
DRB1*14:249	Antigen-binding site, β1	6	Arg	Basic	Cys	Polar uncharged	++++	+	+	+	+
DRB5*01:130	Extracellular arm, β2	160	Met	Nonpolar aliphatic	Ile	Nonpolar aliphatic	+	+			
DRB5*02:37	Extracellular arm, β2	150	Asn	Polar uncharged	Ser	Polar uncharged	+	+			

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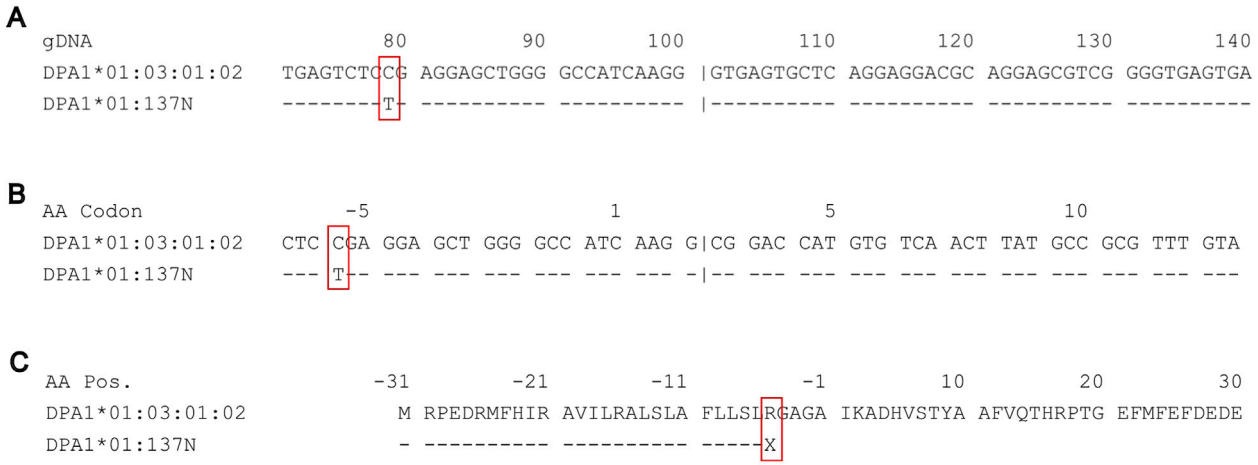

FIGURE 2
Sequence alignments on IPD-IMGT/HLA of DPA1*01:03:01:02 against the novel nonsense mutation DPA1*01:137N. **(A)** Genomic sequence alignment of partial exon 1 and intron 1 of DPA1*01:03:01:02 (top) against the novel allele DPA1*01:137N (bottom). A novel mutation at IMGT genomic position 79 from C (cytosine) to T (thymine). **(B)** Coding sequence alignment of partial exon 1 and exon 2, demonstrating a change in the −5 codon CGA to TGA. **(C)** Amino acid sequence alignment of the leader peptide and partial antigen-binding site, demonstrating the nonsense mutation from R (arginine) to X (stop codon) occurring in the leader peptide.

mutation on the protein's phenotype by the location and change in the basic properties of the original and new amino acid (Table 2). In addition, we assessed if the mutation had a potential effect on antibody-binding by evaluating if it occurred in the same position as a known eplet. For example, the B*56:88 novelty was designated "++++" due to the mutation resulting in a change in amino acid ("+") between residues of different basic chemical properties (original leucine was nonpolar aliphatic and the novel phenylalanine was nonpolar aromatic) ("+"), and that this mutation occurred in the antigen-binding site ("+") and also affected a known eplet ("+"). This is in contrast to the C*05:277 novelty where a polar uncharged cysteine was changed to a serine which is also polar uncharged. This mutation occurred in the cytoplasmic tail and did not affect antibody binding (i.e., no eplet). Based on this analysis, the C*05:277 was marked with just one "+" as a potential effect on the protein's phenotype. The amino acid alteration score of other non-synonymous missense mutations identified in this study are shown in Table 2.

In one sample, we identified a nonsense mutation in exon 1 of DPA1 (HLA-DPA1*01:137N) (Figure 2). The mutation led to a change from cytosine (IMGT genomic position 79) to thymine (Figure 2A), resulting in the codon change of CGA to TGA (IMGT codon position −5) (Figure 2B), which encodes a stop codon at the point of the 5′ leader peptide (Figure 2C).

Many novelties included nucleotide changes that did not result in an amino acid change (12 unique silent novel sequences). However, for DQA1*02:01:15Q, although the exon novelty was a silent mutation, there was also an additional SNP in intron 2 (IMGT genomic position 4027) that may affect splicing, resulting in questionable (Q) expression.

3.2 Discrepant results for patients with hematological malignancies

We identified two patients diagnosed with acute myeloid leukemia that had discrepant HLA typing between DNA collected from different specimens (Supplementary Table S2). The first patient (VGH050), based on DNA extracted from peripheral blood, was found to have a guanine insertion in exon 2 of B*15:01:01:01, resulting in a novel frameshift mutation when sequenced using NGS. When re-testing on RSSO and RT-PCR, both assays yielded a "normal" B*15:01:01:01 allele. A buccal swab was then collected to investigate the possibility that the novelty was the result of cancerous mutation in the malignant cell line. Indeed, the buccal-derived DNA when sequenced using NGS yielded a "normal" B*15:01:01:01 allele without reads to support the previously observed guanine insertion in exon 2.

In a similar pattern, a second patient's (VGH049) initial DNA sample extracted from peripheral blood resulted in a novel SNP mutation (G > T) in exon 3 of A*02:06:01:01. However, a buccal swab was then collected which resulted in a "normal" A*02:06:01:01 without the novel mutation. In both cases, novel alleles were only observed in DNA extracted from patients' peripheral blood and not the buccal cells, indicating the typing discrepancy was most likely attributed to abnormal mutations derived from circulating malignant cells. Thus, a typing based on germline cells is always required for patients with hematological malignancies to account for cancer cells yielding a different HLA genotype from the patient's actual typing, especially if a novel mutation was detected. This is also a requirement for submission into the IPD-IMGT/HLA database.

3.3 Confirmation of novel alleles by different molecular assays

As described in the examples above, RSSO and RT-PCR did not confirm any of the novelties identified by NGS in our cohort. In most cases, these molecular methods yielded the "normal" allele type most related to the novel sequence but were not able to identify the mutation. However, in one sample (VGH029), the confirmatory RSSO resulted in a completely inaccurate result (Supplementary Figure S1). NGS identified a novel missense mutation for HLA-B*56:02:01:01 due to a nucleotide change of adenine to cytosine (IMGT genomic position 793). This resulted in the amino acid change of leucine to phenylalanine (IMGT codon position 116). At the time, the NGS analysis software assigned a typing of B*40:01 + B*56:02:01:01#1. Re-testing the sample on RSSO yielded a discrepant result for both normal and novel alleles: B*40:36 + B*55:08. Comparing the sample reaction pattern to what was expected of B*40:01 + B*56:02, two beads were unexpectedly positive and one was negative. Further investigation showed that all three probes bound to the region in which the novel mutation occurred, and the presence of the mutation resulted in unexpected reaction patterns to yield inaccurate typing. The new allele name for this novel sequence was B*56:88.

To ensure the accuracy of the novel sequences, all samples were re-tested using an alternate NGS sequencing platform in an external ASHI-accredited sister laboratory. All samples were concordant in identifying the same novel sequence, in addition to having complete concordance at the remaining HLA loci.

3.4 Investigation of novel alleles and reporting to the clinical team

During routine testing, all alleles with novel exon mutations underwent further investigation prior to clinical sign out. The mutations were evaluated for their effect(s) on the coding amino acids (e.g., missense/non-synonymous, silent/synonymous, nonsense/truncated protein, insertion/frameshift) as well as the exon location and if the mutation occurred in the antigen-binding site.

Novel alleles were communicated to the clinical team through a custom comment in the HLA typing case reports. The comments were written by the laboratory director and conveyed the presence of a novel mutation and relevant details of the mutation, including the nucleotide and amino acid changes, exon location, and if it occurred in the antigen-binding site.

3.5 Submission to IPD-IMGT/HLA

At the point of submitting the novel sequences to the IPD-IMGT/HLA database (i.e., September 2022), eight of the novelties were already registered and given an official name (alleles ending with an asterisk in Table 1). The remaining 21 novel sequences were submitted together. Many of the key requirements for novel allele submission outlined by IPD-IMGT/HLA, such as bi-directional sequencing; typing at HLA-A, B, and DR loci; and a minimum of key exon sequencing (IPD-IMGT/HLA Database), were fulfilled by the commercial NGS kit and software used in our laboratory. However, there were additional requirements beyond the typical scope of HLA genotyping in the clinical laboratory. One main criterion included obtaining an accession number for every novel allele identified. To do this, the novel sequences require submission first to any of the following three public sequence repositories: DDBJ (DNA Data Bank of Japan) (DDBJ), ENA (European Nucleotide Archive) (ENA Browser), or GenBank (BankIt).

GenBank's submission tool BankIt was used to obtain accession numbers for the submitted samples with novelties. A wizard guided the submission process where multiple sequences were included in one submission. Information required for this process included the sequences of the novel alleles. In this study, we uploaded a single master FASTA file that combined all novel allele FASTA files exported from the analysis software. One entry consisted of a "SeqID" acting as a sample identifier, where the organism was included with a descriptor "*Homo sapiens*", followed by a brief description of the sequence (Figure 3A). This was immediately followed by the actual sequence containing the novel mutation in the next line.

The sequences were annotated using a "feature table file" (Figure 3B). The feature table was a plain text file that corresponded to each sequence in the FASTA file using the "Sequence identifier". The table annotates "features" of the FASTA, including the sequenced gene, mRNA, and coding sequence (CDS) relevant to the submitted sequence. Within each feature are annotations of nucleotide positions of intervals relevant to the feature. For example, the mRNA feature was divided into the UTRs and exons. Each FASTA corresponding to a novel sequence had its own feature table. Upon submission of the FASTA and feature table files, GenBank provided accession numbers for the sequences.

The novel sequences were then submitted to IPD-IMGT/HLA to request an official naming into the database. This process required a written description of the mutation observed, including its position, type of mutation, and any phenotypic changes. An example was "VGH002 has 1 nt change from B*48:01:01:01 at nt 1,701 where A > G (codon 228 ACT > GCT), resulting in a coding change 228 Thr is changed to Ala." The IPD-IMGT/HLA website provided a tool that can help annotate sequence features for class I sequences (but not for class II). However, as a feature table of annotations was required to obtain an accession number from GenBank, this information was already determined at the point of IPD-IMGT/HLA submission. The submission form also included sample demographics if available, such as ethnicity and sex. Successful submissions resulted in an official report provided by IPD-IMGT/HLA and a new allele name for each reported novelty.

There was one submission for a DRB3 allele that could not be officially named because the novel sequence contained a silent variant that required complete coding sequence data to differentiate it from other possible proteins. The sequencing kit used in our laboratory covered only exon 2 to exon 4 and thus could not rule out potential variants in exons 1, 5, 6 for DRB3 variants. This sample (VGH025) was excluded from the final study cohort (Supplementary Table S2).

A

```
>VGH002 [organism= Homo sapiens] HLA-B*48:01:01:01 exon 4 variant position 1701 A>G
ACCCACCCGGACTCAGAGTCTCCTCAGACGCCGAGATGCTGGTCATGGCGCCCCGAACCGTCCTCCTGCTGCTCTCGGCGGCCCTGGCCCTGACCGAGACCTC
CTGGTACCAGGGGCAGTGGGGAGCCTTCCCCATCTCCTATAGGTCGCCGGGGATGGCCTCCCACGAGAAGAGGAGGAAAATGGGATCAGCGCTAGAATGTCGC
CTGTGGTCGCTGCTGTGATGTGTAGGAGGAAGAGTTCAGGTAGGGAAGGGGTGAGGGGTGGGGTCTGGGTTTTCTTGTCCCACTGGGGGTTTCAAGCCCCAGC

>VGH003 [organism= Homo sapiens] HLA-B*48:01:01:01 exon 4 variant position 1701 A>G
ACCCACCCGGACTCAGAGTCTCCTCAGACGCCGAGATGCTGGTCATGGCGCCCCGAACCGTCCTCCTGCTGCTCTCGGCGGCCCTGGCCCTGACCGAGACCTC
CTGGTACCAGGGGCAGTGGGGAGCCTTCCCCATCTCCTATAGGTCGCCGGGGATGGCCTCCCACGAGAAGAGGAGGAAAATGGGATCAGCGCTAGAATGTCGC
CTGTGGTCGCTGCTGTGATGTGTAGGAGGAAGAGTTCAGGTAGGGAAGGGGTGAGGGGTGGGGTCTGGGTTTTCTTGTCCCACTGGGGGTTTCAAGCCCCAGC

>VGH004 [organism= Homo sapiens] HLA-A*03:05:01 exon 3 variant position 873 A>C
CCAATCAGTGTCGTCGCGGTCGCTGTTCTAAAGCCCGCACGCACCCACCGGGACTCAGATTCTCCCCAGACGCCGAGGATGGCCGTCATGGCGCCCCGAACCC
CCGCAGATACCTGGAGAACGGGAAGGAGACGCTGCAGCGCACGGGTACCAGGGGCCACGGGGCGCCTCCCTGATCGCCTGTAGATCTCCCGGGCTGGCCTCCC
CCATCGTGGGCATCATTGCTGGCCTGGTTCTCCTTGGAGCTGTGATCACTGGAGCTGTGGTCGCTGCCGTGATGTGGAGGAGGAAGAGCTCAGGTGGAGAAGC
CCTGCATGTGTCTGTGTTCGTGTAGGCATAATGTGAGGAGGTGGGGAGACCACCCCACCCCCATGTCCACCATGACCCTCTTCCCA
```

B

```
>Feature VGH010 <-- Sequence identifier corresponding to FASTA
<1      >5626   gene                                                    Feature: gene
                        gene    HLA-DPA1
                        allele  HLA-DPA1*02:02:02:01 exon 4 variant position 4876 T>C
Intervals
5'UTR   <1      66      mRNA                                            Feature: mRNA
Exon 1  67      166
Exon 2  3733    3978
Exon 3  4319    4600
Exon 4  4815    4969
3'UTR   4970    >5626
                        product MHC class II antigen
Exon 1  67      166     CDS                                             Feature: CDS
Exon 2  3733    3978
Exon 3  4319    4600
Exon 4  4815    4969
                        product MHC class II antigen
<1      66      5'UTR                                                   Feature: UTR
4970    >5626   3'UTR
Start position of feature   End position of feature
```

FIGURE 3
Examples of the FASTA and Feature Table File required for IPD-IMGT/HLA submission. **(A)** A screenshot of three novel allele entries submitted to GenBank as a FASTA file. Each entry begins with a ">", followed by the "SeqID" which acts as the identifier (ex. VGH002). This is followed by the organism the DNA was extracted from in brackets, and then a descriptor of the entry. In the next line is the sequence of the entry, which includes the novel mutation. **(B)** Example of a Feature Table File used to submit into GenBank. Each entry in a Feature Table File began with a ">", followed by "Feature" and the sequence identifier that corresponded to the FASTA file. The corresponding text described details of features regarding the submitted sequence, such as the gene, mRNA, or coding sequence (CDS). For every feature, the position of when the feature begins and ends within the corresponding FASTA file is indicated. For the "gene" feature, the gene begins at nucleotide 1 of the FASTA file and ends at nucleotide 5626. The "mRNA" feature, the UTR and exon regions are indicated. The "CDS" feature indicates only exon positions. The 5′UTR and 3′UTR represent the untranslated regions.

3.6 Canadian survey

We conducted a nationwide survey including all 16 Canadian HLA Laboratories serving hematopoietic stem cell transplant and/or solid organ transplant programs to evaluate their experience with novel allele detection and reporting using NGS (Table 3). Overall, 10/16 (63%) laboratories employed NGS technology as their primary genotyping tool. A variety of kits were used to prepare sequencing libraries: the top two were AllType FastPlex (OneLambda) (n = 3, 30%) and NGSgo 11 (GenDx) (n = 3, 30%), followed by Holotype (Omixon) (n = 1, 10%), Nanotype (Omixon) (n = 1, 10%), Mflex 11 Typing Kit (Mia Fora) (n = 1, 10%), and in-house reagents (n = 1, 10%). The leading sequencing platform was the MiSeq (n = 6, 60%), followed by the MiniSeq (n = 2, 20%). One laboratory used the Ion Torrent system while another adopted the emerging Nanopore Minion which delivered real-time rapid sequencing with long reads.

All but one laboratory routinely detected novel HLA alleles with their sequencing platform. One other laboratory also reported never detecting novelties but this was likely attributed to a lack of primer coverage resulting in the inability to rule out novelties occurring in the unsequenced regions. Of the remaining, four laboratories (40%) reported a novel allele detection rate at less than two per month, while another four (40%) report detecting two or more per month.

There was a mixed response from the laboratories in their protocol for the verification of novel alleles and the method(s) utilized. The majority of the laboratories would only selectively retest patient samples depending on the novelty or patient/donor type. For example, one surveyed laboratory reported only confirming novelties for patients requiring a bone marrow transplant or when submitting to IPD-IMGT/HLA. Others reported confirmatory testing only if the mutation arose in a clinically meaningful location, such as in coding region or key exons. Three laboratories routinely confirmed

TABLE 3 Canadian survey on novel allele detection. The results of the ten Canadian HLA laboratories that use NGS for as the main sequencing platform.

	Count	Proportion (%)
Commercial Kit		
AllType FastPlex	3	30
Omixon Holotype	1	10
Omixon Nanotype	1	10
NGSgo 11 loci from GenDx	3	30
In-house reagents with GenDx reflex kit	1	10
Mia Fora Mflex 11 Typing Kit	1	10
Sequencing Platform		
MiniSeq	2	20
Nanopore Minion	1	10
MiSeq	6	60
Ion Torrent	1	10
Approximate number of novelties detected		
None	1	10
Less than two a month	4	40
More than two a month	4	40
Other	1	10
Other reason	• Unknown as there are regions not sequenced due to a lack of primer coverage	
The following applies to only laboratories that have detected novel alleles		
Confirm novel alleles		
Yes	3	33
No	1	11
Selective confirmation	5	56
	• Depends on mutation/recombination	
	• Depends on patient category, such as for patients with leukemia or when submitting to IPD-IMGT/HLA	
	• Depends on the position of the mutation	
	• Occasionally (no criteria listed by respondent)	
Novel allele investigation		
No additional investigations	0	0
Location of novelty (ex. exon, intron, antigen binding site)	9	100
Type of mutation (ex. missense, silent, insertion, deletion)	8	89
Splicing	6	67
Other	1	11
Other reasons	• Identify parent alleles for recombination	
Reporting of novel alleles		
The most similar allele or G/P group without additional comments	0	0

(Continued on following page)

TABLE 3 (*Continued*) Canadian survey on novel allele detection. The results of the ten Canadian HLA laboratories that use NGS for as the main sequencing platform.

	Count	Proportion (%)
The most similar allele or its G/P Group with comment	3	33
The most similar allele or its G/P group with comment only if it has a reasonable effect on the protein	4	44
The custom allele with novelty noted	1	11
Antigen equivalent if allele cannot be reported as a G/P group	1	11
Does your laboratory submit novel alleles to the IPD-IMGT/HLA database?		
Yes	7	78
No	2	22

novel alleles with a sister laboratory using a different NGS kit. One repeated NGS typing in addition to SSP (Olerup SSP by CareDx) within their own laboratory. One center did not engage in confirmation of novel alleles when detected. Despite this heterogeneity in practice, all centers performed additional analysis to investigate the location of the mutation to inform the predicted impact of the mutation on the expressed protein product. In addition, 67% of the laboratories reported routine evaluation of the effect of nucleotide change on RNA splicing.

Further heterogeneity was observed in the reporting of novel alleles. Some centers reported the most similar allele (ex. the allele with the same sequence as the novelty except for the mutation) or the novel allele's G/P group if it belonged to one. If the mutation arose in a key exon, one center reported the novel allele's antigen equivalent. One laboratory reported a custom allele, where the term "NEW" was added to the second field (ex. DQA1*01:NEW). A proportion of laboratories (n = 3, 33%) included an additional comment on the report describing the novelty, whereas some only included a clinical comment if the novelty had a clinically meaningful effect on the protein (n = 4, 44%). Only two laboratories consistently submitted novel alleles into the IPD-IMGT/HLA database and five laboratories only submitted their alleles once or a limited number of times, or were just beginning to do so. Many survey respondents reported challenges in submitting novelties, including a lack of resources and time, the perceived requirement to re-sequence a second sample, and limitations of gene coverage resulting in complications in reporting.

4 Discussion

Next-Generation Sequencing has transformed HLA genotyping in the clinical histocompatibility laboratory with its capacity to provide high-throughput and high-resolution genotypes at the 11 classical HLA genes. One of the advantages of NGS is the ability to discover novel sequences through routine testing. Unfortunately, there is limited societal guidance on how to manage novel alleles in the clinical laboratory. Herein, we described the experience of an ASHI-accredited laboratory that uses NGS as a routine testing technology and performed a nationwide survey including the HLA laboratories in Canada to highlight important laboratory and clinical considerations in the detection and reporting of novel alleles. Based on our findings, we advocate for consensus-building and the development of best practices on novel allele management.

In the multi-ethnic population of 5 million in British Columbia, Canada, the overall rate of detecting novel mutations was approximately 1.6 per month. This frequency is comparable to the frequency observed in other Canadian laboratories and may even be considered "common" based on the standards of the Common-Intermediate and Well-Documented (CIWD) catalog (Hurley et al., 2020). Indeed, other groups have also reported on novel alleles after the adoption of NGS in clinical HLA laboratories (Shen et al., 2022; Dhuyser et al., 2023; Kouniaki et al., 2023; Liacini et al., 2023; Zhong et al., 2023). In one study, the DKMS Life Science Laboratory reported the detection of 1,919 unique novel allele sequences out of 1.4 million donors sequenced, but this frequency only represents the discovery rate in a genetically homogenous stem cell donor population (Schöfl et al., 2017).

While these data highlight the common occurrence of novel HLA alleles encountered in a HLA laboratory, there are limited guidelines on how to handle these novel sequences clinically. The World Marrow Donor Association recently published recommendations on novel allele reporting for hematopoietic stem cell transplants (Hofmann et al., 2023). The publication describes how to best communicate novel alleles between registries for unrelated donor searches, taking into consideration the mutation type (non-synonymous vs. synonymous) and location (antigen-binding site vs. outside this region). However, additional considerations are required when reporting novel alleles in the clinical setting, which might include the assessment of mutations outside the antigen-binding site (ex. splice site variants) and their potential impact on antibody-binding (ex. effect on eplet), as well as the physiochemical changes of non-synonymous mutations. To our knowledge, the only ASHI policy according to the ASHI Standards (2022) is D.5.2.5.8: "Laboratories must determine the sequences of both sense and anti-sense DNA strands if a sequence suggests a novel allele" (American Society for Histocompatibility and Immunogenetics, 2023). For IPD-IMGT/HLA, there are further requirements for allele submission, such as novel sequences identified by NGS must be completely phased, but the remaining standards may pertain more to molecular techniques that are not NGS-based (e.g., Sanger Sequencing) (IPD-IMGT/HLA Database). This lack of standardization has resulted in a wide variation of practice and uncertainty in the discovery and documentation of novel alleles by histocompatibility laboratories.

There is no official requirement by ASHI to verify novel sequences identified by NGS. Additionally, there is ambiguity on whether a novel sequence should be confirmed and if so, what method is most appropriate. For example, RSSO and RT-PCR were not able to detect the novel sequences accurately at our center. Instead, the results were either the most similar allele or even a completely inaccurate typing due to probes binding to the novel sequence. Other studies have also shown the ineffectiveness of RSSO in confirming NGS novelties (Smith et al., 2019). These data support that the only current reliable method of confirming novel alleles is on another sequence-based platform. To this end, all novel alleles detected at our site were confirmed by an external ASHI-accredited HLA laboratory using a different commercial kit and NGS platform to avoid assay-related biases. All novelties were in complete concordance between the two laboratories, supporting the use of two independent NGS-based technologies to add validity to the detection of novel sequences.

The accuracy of novel sequences is highly dependent on sequencing quality as well as knowledge of the patient's disease and specimen type. For example, we observed the presence of a false positive novel allele in two cancer patients due to sequencing DNA extracted from peripheral blood containing leukemic cells. Considering this risk, IPD-IMGT/HLA and National Marrow Donor Program (NMDP) require confirmation of germline DNA for any sequences identified in an individual with hematological malignancy (IPD-IMGT/HLA Database; Be The Match). Inaccurate novel mutations may also be identified due to poor sequencing quality flagged by QC metrics. Indicators of inaccurate novelty identification include poor read coverage, high mismatch count, and multiple novel mutations identified in one sample. In this study, all novel alleles passed relevant QC metrics and only one exonic novelty was observed in each patient. Given the common occurrence of novel alleles and numerous potential assay-related, bioinformatics, and interpretation pitfalls in detection and reporting, the HLA community might consider establishing a formal Proficiency Testing (PT) program to standardize and ensure accuracy in the identification and reporting of novel sequences by accredited laboratories. For example, DNA samples with confirmed novelties using the most updated IPD-IMGT/HLA database can be circulated to accredited laboratories that perform routine NGS typing. In addition to sequence identification, the PT program may further evaluate the laboratory's ability to describe the mutation (ex. missense, silent, or nonsense point mutations, insertions) and provide a standardized assessment of its effect on the overall protein.

The location and type of a novel mutation are important in determining their effect(s) on the translated HLA protein. In our study, 28% of novelties occurred in key exons and all were single point mutations, which is consistent with other studies and confirms the heterogeneity of these regions (Robinson et al., 2020). The key exons encode the antigen-binding site, which is arguably the most vital genetic region as it directly affects the functional portion of the HLA protein. In addition, the key exons express many of the epitopes bound by HLA antibodies (Duquesnoy, 2006). Less clear are mutations occurring outside the key exons. For example, many novelties were located in exon 1, which encodes the leader peptide. The leader peptide flags newly synthesized HLA proteins to the cell surface and a mutation in exon 1 may affect the protein's successful translocation (Wu et al., 2020). However, this hypothesis would require experimental cell expression studies to draw any actionable conclusions and the extent to which a clinical HLA laboratory is responsible for this is not defined. Furthermore, this study only included exon novelties and intron variants may have additional implications for protein expression (Alexander et al., 2010). Currently, our center does not routinely report intronic novelties because their clinical relevance in transplantation remains unclear. However, it is known that certain intronic SNPs which affect splicing can greatly alter protein expression. Indeed, the common DRB4*01:03:01:02N allele null variant is caused by an SNP in intron 1 affecting splicing and thus protein expression (Sutton and Knowles, 1990). As the HLA community moves towards sequencing and cataloguing intronic sequences, it may be beneficial to have a standardized approach to the evaluation of intronic SNPs on RNA splicing to improve the understanding of their clinical significance.

In contrast to mutations that lead to null protein expression (i.e., DPA1*01:137N in this study), the clinical significance of missense and silent mutations are less clear. The majority of novelties were missense mutations resulting in an amino acid change. Studies have found that differences in physiochemical structures were associated with adverse outcomes in a transplant rejection setting (Kosmoliaptsis et al., 2016; Wiebe et al., 2018). To investigate the potential effects of this change, the basic properties as defined by the polarity and charge of the original and altered amino acids were compared. Using this method, we observed that four novelties resulted in potentially significant changes to the protein due to a change in the basic properties of the amino acid occurring in an antigen-binding site, which also resulted in an eplet change that may affect antibody binding. The remaining missense novelties did not affect an eplet, however, they included mutations in key exons that resulted in a change of amino acid of different physiochemical properties (one novel allele). Some novelties were presumed to not affect the protein significantly due to a substitution of a similar residue in a non-key exon (seven novel alleles). This approach may serve as an initial strategy to assess the potential clinical significance of amino acid substitution caused by a missense mutation, but will require confirmation by experimental data and correlation with clinical outcomes.

Results of the pan-Canadian survey also highlight the lack of consensus on how to report and communicate novel alleles to the clinical team. Reporting the most related sequence, calling G/P groups, creating a custom allele designation, or reporting the antigen equivalent were methods used for the communication of novel allele results. Furthermore, labs report that the type of mutation and its possible effect on the protein plays a role in how to report. This wide variation in practice demonstrates that there is still a great need from the community for guidance on how to communicate novel alleles to the physician and clinical teams. Future guidelines may standardize a list of minimal assessment criteria (ex. mutation type and location) in the clinical reporting of novel alleles.

The ethnicity of patients with the novel sequences was more diverse than the general Canadian population. This emphasizes the need to encourage and facilitate novel allele submissions by clinical histocompatibility laboratories to ensure IPD-IMGT/HLA continues to maintain a diverse and representative dataset. Indeed, in a recent IPD-IMGT/HLA report, the major

contributors to the database were large companies that provide HLA typing services to registries such as for bone marrow donation (Barker et al., 2023). As NGS becomes more widely used in clinical histocompatibility laboratories, this is an invaluable opportunity for local centers to contribute to the community and submit novel sequences that are representative of their population. As highlighted in our survey, there are certain complexities involved when submitting novel sequences. Survey respondents reported a lack of resources and time; the perceived need to re-sequence a second sample; and limitations of gene coverage resulting in complications in reporting as major barriers which hinder their ability to submit novel alleles. Establishing clear requirements on the submission of novel sequences detected specifically by NGS, coupled with improvement and familiarity of tools provided by the NGS analysis software to aid in the creation of required files (ex. FASTA, feature tables), can greatly advance this endeavor.

In this new era of high-throughput NGS technology, the identification of novel sequences is more feasible than ever and will likely accelerate in the future. To ensure that clinical histocompatibility laboratories remain poised to manage this changing landscape, we advocate for developing standards for the verification of novelties, building consensus on the minimum criteria required for clinical evaluation of novel alleles, and updating protocols on the submission of NGS novel sequences to the official database. The HLA community has always adapted to the evolution of laboratory technologies, and we are called upon again to set the standard in the appropriate management of novel sequences in the clinical histocompatibility laboratory.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by the University of British Columbia Research Ethics Board (H22-03627). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because samples were sequenced for clinical purposes, where a proportion of patients/donors are no longer reachable due to reasons including being out of the primary care of the transplant programs, do not live in BC, Canada, or are deceased.

Author contributions

JT: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. KS: Conceptualization, Methodology, Resources, Writing–review and editing. AM: Writing–review and editing. RB: Writing–review and editing, Investigation, Validation. AE: Investigation, Validation, Writing–review and editing. AG: Writing–review and editing, Validation. PK: Conceptualization, Writing–review and editing. RL: Conceptualization, Validation, Writing–review and editing. JL: Resources, Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.1282834/full#supplementary-material

SUPPLEMENTARY FIGURE S1
The Reverse Sequence-Specific Oligonucleotide (RSSO) reaction pattern for VGH029 that yielded discrepant results from the Next-Generation Sequencing results. The top two panels depict the reaction pattern of the sample followed by the expected reaction patterns for B*56:02:01:01, B*40:01:01, B*40:36, and B*55:08. The red boxes highlight three beads (527, 558, 589) that had an unexpected reaction due to the novel mutation at this position (IMGT codon 116). The novel allele name was B*56:88.

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Validation of next-generation sequencing-based chimerism testing for accurate detection and monitoring of engraftment in hematopoietic stem cell transplantation

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Allogenic hematopoietic stem cell transplantation (allo-HSCT) is a life-saving treatment for various hematological disorders. The success of allo-HSCT depends on the engraftment of donor cells and the elimination of recipient cells monitored through chimerism testing. We aimed to validate a next-generation sequencing (NGS)-based chimerism assay for engraftment monitoring and to emphasize the importance of including the most prevalent cell subsets in proficiency testing (PT) programs. We evaluated the analytical performance of NGS-based chimerism testing (AlloSeq-HCT and CareDx) with a panel of targeted 202 informative single-nucleotide polymorphisms (SNPs) (i.e., linearity and precision, analytical sensitivity and specificity, system accuracy, and reproducibility). We further compared the performance of our NGS panel with conventional short tandem repeat (STR) analysis in unfractionated whole blood and cell-subset-enriched CD3 and CD66. Our NGS-based chimerism monitoring assay has an impressive detection limit (0.3% host DNA) for minor alleles and analytical specificity (99.9%). Pearson's correlation between NGS- and STR-based chimerism monitoring showed a linear relationship with a slope of 0.8 and r = 0.973. The concordance of allo-HSCT patients using unfractionated whole blood, CD3, and CD66 was 0.95, 0.96, and 0.54, respectively. Utilization of $CD3^+$ cell subsets for mixed chimerism detection yielded an average of 7.3 $\pm$ 7-fold higher donor percentage detection compared to their corresponding unfractionated whole blood samples. The accuracy of the NGS assay achieved a concordance of 98.6% on blinded external quality control STR samples. The reproducibility series showed near 100% concordance with respect to inter-assay, inter-tech, inter-instrument, cell flow kits, and AlloSeq-HCT software versions. Our study provided robust validation of NGS-based chimerism testing for accurate detection and monitoring of engraftment in

allo-HSCT patients. By incorporating the cell subsets (CD3 and CD66), the sensitivity and accuracy of engraftment monitoring are significantly improved, making them an essential component of any PT program. Furthermore, the implementation of NGS-based chimerism testing shows potential to streamline high-volume transplant services and improve clinical outcomes by enabling early relapse detection and guiding timely interventions.

KEYWORDS

next-generation sequencing, chimerism, cell subsets, standardization, proficiency testing

1 Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a form of curative treatment for a variety of hematological malignancies, such as acute leukemias, lymphomas, myelodysplastic syndromes, plasma cell disorders, myeloproliferative neoplasms, and other genetic disorders. The annual allo-HSCT rate in Canada from 2008 to 2019 has been 926 ± 107 transplants (CTTC. Cell, 2021). This prevalence stems from the impact of allo-HSCT in concatenating the positive rates for remission and overall survival in both pediatric and adult cohorts (Svenberg et al., 2016; Appelbaum, 2017; Döhner et al., 2017). Despite this success, allo-HSCT can cause a myriad of complications, such as treatment-associated toxicity, relapse, graft-versus-host disease (GvHD), and death. Therefore, clinicians need to utilize newer tools post-allo-HSCT to monitor complications, minimal residual disease status, and propensity for rejection.

The success of allo-HSCT is measured by engraftment, where the donor cells initiate the production of healthy hematopoietic stem cells against a background of complete eradication of pre-transplant hematological/hemato-oncological disorders. Engraftment at a cellular level can be substantiated by chimerism, which refers to the ratio of the genetically distinct donor and recipient cell populations. The delineation of the ratio of these cell populations is dependent on factors such as the intensity of the conditioning regimen, GvHD prophylaxis, the recipient's prior chemotherapy regime, and graft composition. Mixed chimerism is defined as either the persistence or relapse of the host non-neoplastic cells or, in the worst-case scenario, the re-emergence or repopulation of the neoplastic cells. Allo-HSCT patients with mixed chimerism (MC) show an increased proclivity for graft rejection and disease recurrence (Busque et al., 2020). Chimerism testing can be instrumental in the longitudinal monitoring of the patient's immune convalescence and cellular reconstitution post-allo-HSCT, monitoring engraftment kinetics and trends in donor engraftment in the follow-up period.

Next-generation sequencing (NGS) has emerged as a promising tool for chimerism monitoring due to its high sensitivity, accuracy, and multiplexing capacity. In comparison to already established methods such as short tandem repeats (STRs), quantitative polymerase chain reaction (qPCR), or fluorescence *in situ* hybridization (FISH), NGS reigns supreme in the detection of minuscule fractions of donor cells within the recipient's blood or bone marrow. Chimerism monitoring with NGS depends on either sequencing informative single-nucleotide polymorphisms (SNPs) or InDel panels from the donor and recipient DNA samples. These results are then analyzed via specialized software to quantify the relative ratio of donor and recipient alleles. NGS allows for the detection of low-level chimerism with a sensitivity of ~0.1–0.5%, compared to ~1–5% with these conventional methods (Blouin and Askar, 2022). This could be useful in predicting graft failure after full engraftment and early detection of potential complications, influencing clinical decision-making, and improving patient outcomes in allo-HSCT and other cellular therapies. A previous study reported that NGS-based chimerism monitoring could predict relapse with high accuracy across adult patients (n = 75) undergoing allo-HSCT with low, intermediate, and high MC (Pettersson et al., 2021).

Cell subset analysis plays a crucial role in NGS-based chimerism testing. Isolating specific cell subsets such as CD3-positive T lymphocytes, CD19-positive B cells, and CD66-positive myeloid cells provides more accurate representation of the post-allo-HSCT dynamics than a whole blood analysis (Lion et al., 2012). Distinct chimerism patterns can be gleaned, and these can provide a comprehensive understanding of the immune reconstitution process or temporization of engraftment failure.

The current literature reveals a gap in incorporating lineage-specific cell subset-based chimerism analysis into proficiency testing. The large-scale adaptation of PT will enable laboratories to assess their proficiency metrics, such as accuracy, consistency, reliability, and comparability of results across different laboratories. Incorporating these quality assurances will result in accurate and standardized assessments of engraftment outcomes via cell subset testing, potentially revolutionizing and facilitating clinical decision-making.

In this study, we validated the NGS-based assay with a panel of the target 202 loci of known biallelic SNPs. These biallelic SNPs were selected from the 1000 Genomes Project due to their high heterozygosity and lack of linkage disequilibrium, ensuring that each SNP provides independent information about chimerism (Zhang et al., 2015). After sequencing, the resulting data were analyzed to determine the percentage of donor and recipient alleles at each SNP locus. The analytical performance and clinical utility of NGS-based chimerism monitoring were summarized.

2 Methods

2.1 Samples

A total of 196 post-transplant samples and 54 genomic samples were subjected to analysis using the NGS-based chimerism assay (CareDx, Stockholm, Sweden), and the results were compared with those from the STR assay (AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit, Applied Biosystems). These samples were

obtained from 27 patient/donor pairs. Among them, 22 pairs were obtained from allo-HSCT cases, while the remaining 32 pairs were obtained from unrelated allo-HSCT cases. Prior to method validation, approval was obtained from the Saskatchewan Cancer Agency (SCA) Privacy Office to utilize de-identified residual samples for method validation and scientific research.

As part of the external quality control (EQC), we received 22 blind post-transplant samples and 10 genomic samples from HLA Laboratory, Cancer Care Manitoba, Winnipeg, MB, Canada. Samples were obtained as whole blood (n = 13) and bone marrow (n = 3). Enriched samples were obtained as CD3-positive T cells (n = 2), CD19-positive neoplastic B cells (n = 2), and CD66-positive myeloid cells (n = 2). In addition, 15 whole blood post-transplant samples and six genomic samples were supplied as part of the American Society of Histocompatibility and Immunogenetics (ASHI) proficiency testing program.

2.2 Cell enrichments

To increase the assay sensitivity for minor cell fractions, cell enrichment was conducted from the whole blood prior to DNA extraction using the EasySep™ Human Whole Blood Positive Selection Kit (STEMCELL Technologies, Vancouver, Canada) for CD3-positive T cells (Catalog #18081) and CD66b/33-positive myeloid cells (Catalog #18683), followed by DNA extraction, according to manufacturer's recommendation. Cells were eluted in 300 µL of EasySep buffer (STEMCELL Technologies, Vancouver, Canada) and then counted using the Countess cell counter (Thermo Fisher Scientific, Canada).

2.3 DNA extraction

DNA extraction was performed, as previously described by Kakodkar et al. (2023). Briefly, DNA extractions were prepared by QIAGEN, using the BioRobot® EZ1 system (QIAGEN, Toronto, Canada) and EZ1 DNA Blood 350 µL Kit (Catalog 951,054), using whole blood collected in acid citrate dextrose tubes and isolated cell fractions. The DNA concentration and purity were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Canada), and samples with a 260/280 ratio >1.8 were processed. Extracted DNA samples were normalized to 0.625 ng/µL using PCR-grade water (Thermo Fisher Scientific, Canada) to fulfill the 10-ng input requirement in a 16 µL volume. DNA was stored at a temperature range of 2°C to 8°C for up to 1 week and was subsequently frozen (−20°C).

2.4 NGS chimerism assay

The targeted NGS-based assay (AlloSeq HCT) was performed in accordance with the manufacturer's instructions (CareDx, Stockholm, Sweden). Briefly, one PCR amplification cycle was performed, using the target DNA (0.625 ng/µL), PCR master mix (PCR Mix, SNP primer pool, and PCR enzyme), dual sample-specific indices, and flow-cell adapters. Following the PCR amplification, the products were pooled and cleaned using the AlloSeq HCT purification beads. The final library concentration was measured using the Qubit Flex Fluorometer (Thermo Fisher Scientific, Canada), followed by dilution and denaturation using 2 N NaOH (supplied in the kit). The final library was diluted to 2 pm and spiked with 1% PhiX (Illumina, Canada). Depending on the total number of samples, the final library was loaded into either the mid-output (Illumina, Canada Cat# FC-420-1001) or the high-output (Illumina, Canada Cat# FC-420-1002) flow cell and sequenced on the MiniSeq instruments (Illumina, Canada). FASTQ files generated using MiniSeq were imported into AlloSeq HCT software versions 1 and 2.1.2 (CareDx, Stockholm, Sweden).

2.5 Short tandem repeat assay

The STR assay was performed at HLA Laboratory, Cancer Care Manitoba, Winnipeg, Canada, according to the manufacturer's recommendation (AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit, Applied Biosystems). The STR assay was performed on a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA) using one injection per sample (four-color, 16-plex detection). Analysis was conducted on GeneMapper v.4.1 (Applied Biosystems), and the corresponding electropherograms were printed for each sample and interpreted manually.

3 Results

3.1 Linearity and precision

To validate linearity and precision, seven artificial DNA mixtures were created. These mixtures were prepared by diluting DNA samples within the range of 0.3%–50%, adhering to the predetermined proportions outlined in Supplementary Table S1. The primary objective was to maintain precise and accurate measurements throughout the experiment. Each of these samples underwent triplicate runs utilizing the AlloSeq HCT Kit. Subsequently, they were sequenced using the MiniSeq instruments (Illumina, Canada).

There was a strong linear relationship between the observed and expected outcomes, with a Pearson correlation coefficient of 0.99 ($p < 0.001$). The standard deviation (SD) ranges from 0.3 to 0.004, indicating a high level of precision. This precision is further supported by fixed SD among the replicates, as shown in Figure 1A. Additionally, Figure 1B, the Bland–Altman plot, demonstrates the assay's high precision, with the triplicates tightly clustered across the dilution series.

3.2 Analytical sensitivity and specificity

In order to simplify the analysis, we created two graphs to compare the observed donor DNA percentages with the expected values. Figure 2A represents the results for the lower fractions of the chimerism mixture, which range from 0.05% to 0.35%. Figure 2B, on the other hand, shows the corresponding results for the chimerism mixture, with the higher fractions ranging from 0.3% to 50%.

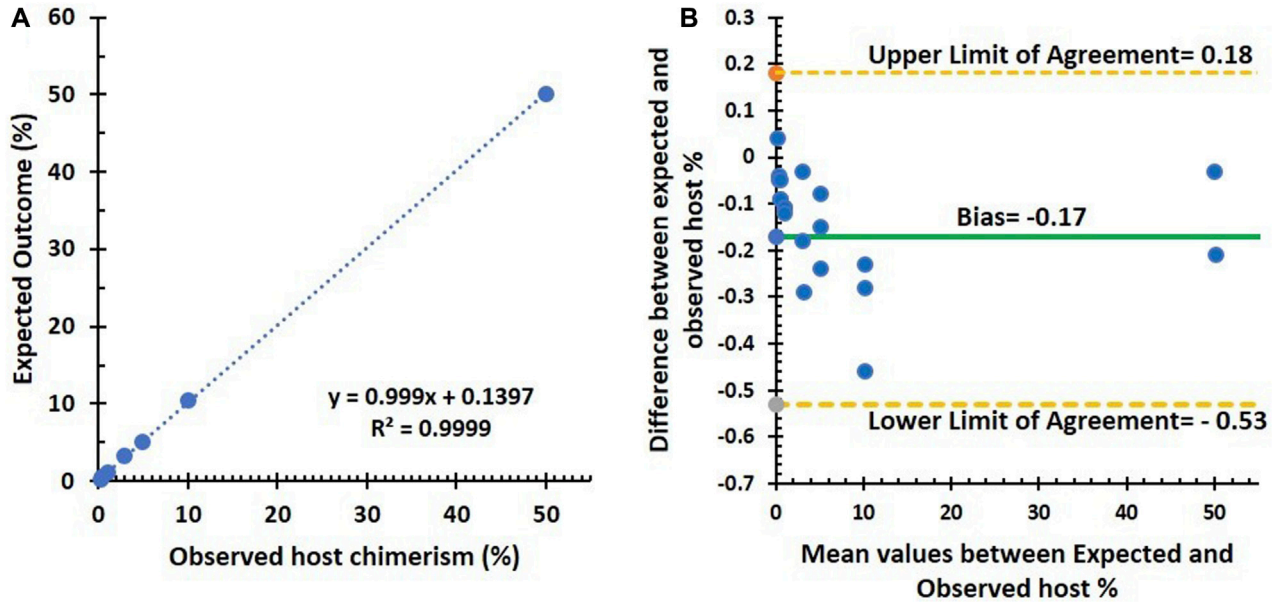

FIGURE 1
Summary of linearity and precision testing results. **(A)** Correlation (Pearson's correlation coefficient = 0.99, $p < 0.001$) between observed and expected host % using the NGS assay in seven artificial DNA samples diluted from 0.3% to 50%. **(B)** Bland–Altman plot between observed and expected host %: an average difference of −0.17% host chimerism (green line), with 95% limits of agreement [−0.53 to 0.18] (dashed lines).

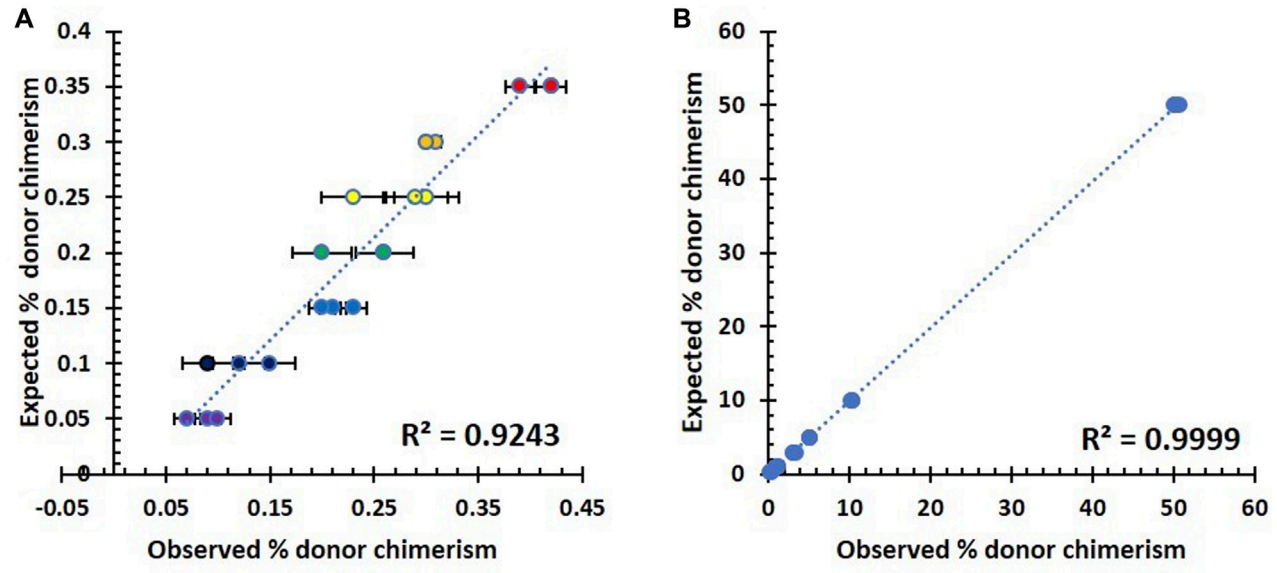

FIGURE 2
Summary of analytical sensitivity testing in seven triplicates of artificial DNA samples. **(A)** Correlation between observed and expected % host chimerism using the NGS assay in the lower fraction (0.05–0.35%). **(B)** Correlation between observed and expected % host chimerism using the NGS assay in the higher fraction (0.3–50%).

As shown in Figure 2, there was a direct proportional relationship between the observed and expected percentage of host chimerism by the NGS assay across the lower (Figure 2A) and higher fractions (Figure 2B) of mixed chimerism samples. The lower fraction has a slightly higher variation within its repeats, and the resultant correlation is 0.9243. Contrastingly, the higher fraction showed a constrained variation with a significantly higher correlation of 0.9999. The accurate measurement of the 0.3% minority fraction proved to be reproducible (standard deviation 0.005). Therefore, the lowest detection limit for our NGS assay was chosen to be 0.3%.

TABLE 1 Summary of two replicates of the four DNA samples. Expected (%) and observed (%) DNA output from the four input DNA samples (1.25 ng, 2.5 ng, 5 ng, and 10 ng) and their respective mixtures: neat (100%), mixture 1 (15%: 85%), and mixture 2 (50%:50%). Data variability between the expected (%) and observed (%) DNA output is measured with the coefficient of variance statistical test.

Expected output (%)	Sample input	Replicate	Neat		Mixture 1		Mixture 2	
			100%		15% and 85%		50% and 50%	
Observed output (%)	**10 ng DNA**	**Replicate 1**	0.08	99.92	14.4	85.6	49.33	50.67
		Replicate 2	0.07	99.93	14.6	85.41	49.37	50.63
	5 ng DNA	**Replicate 1**	0.09	99.91	14.1	85.87	49.74	50.26
		Replicate 2	0.06	99.94	15.1	84.87	49.36	50.64
	2.5 ng DNA	**Replicate 1**	0.07	99.93	13.6	86.37	49.03	50.97
		Replicate 2	0.06	99.94	14.7	85.13	49.92	50.08
	1.25 ng DNA	**Replicate 1**	0.05	99.95	13.5	86.51	49.27	50.73
		Replicate 2	0.05	99.95	13.5	86.51	49.27	50.73
	Mean			99.93375	14.19375	85.78375	49.41125	50.58875
	Standard deviation			0.01317	0.573889	0.596006	0.265303	0.265303
	Coefficient of variance			0.013178	4.043251	0.694778	0.536929	0.524432

The utilization of bold titles serves to enhance the differentiation between the section headings and the presented results.

To determine analytical specificity, we utilized two distinct DNA samples, and these were further split into two aliquots. One aliquot from each DNA sample was intentionally labeled as Recipient (Ref1) and Donor (Ref2) to serve as genomic reference DNA. The remaining two aliquots were labeled as Recipient (post-transplant) and Donor (post-transplant), representing complete host or complete donor chimerism after the transplant (Supplementary Table S2). All DNA samples were processed using the AlloSeq HCT Kit and subsequently sequenced using the MiniSeq instruments (Illumina, Canada). The background noise levels detected in Ref1 and Ref2 were 0.05% and 0.06%, respectively. Additionally, the measured background noise levels in Recipient (post-transplant) and Donor (post-transplant) were 0.05% and 0.04%, respectively. By comparing the specificity of the post-transplant samples with their respective reference genomic DNA, we concluded that the background signal in our NGS assay ranged from 0.04% to 0.06%. We approximated the background signal to be 0.1%. Therefore, the specificity of our NGS assay is 99.9%.

3.3 Limit of the sample input

Two duplicates were prepared for each DNA mixture using a serial dilution of DNA ranging from 10 ng to 1.25 ng. An artificial chimerism mixture was prepared from each DNA concentration for an expected concentration of 100% (neat); 15% and 85% (mixture 1); and 50% and 50% (mixture 2) and run on the NGS assay. The overall summary of the aforementioned mixture schema and coefficients of variance are shown in Table 1. The concordance between the observed and expected DNA concentrations was recorded. The coefficients of variance (CV) for the neat, mixture 1, and mixture 2 groups were 0.013%, 0.69%, and 0.53% respectively. These miniscule CVs indicate a near homogenous dataset, and the comparisons between the expected and observed DNA input are repeatable and can detect DNA fractions in DNA sample inputs as low as 1.25 ng.

3.4 Reproducibility

Inter-assay reproducibility was performed on four replicates of five different samples, where a single NGS assay was performed by the same technologist on the same run for each sample (Figure 3A). Similarly, 11 samples were tested in duplicate by the same technologist on two different runs (Figure 3B). The reproducibility of our NGS-based MC monitoring assay showed a concordance of almost 100% when testing the same samples four times in the same run and when repeated in separate runs. To observe the concordance in inter-tech variance, eight samples were tested in duplicate by two different technologists on a different run (Figure 3C). Similarly, the output from five samples was analyzed using Alloseq-HCT software versions 1 and 2.1 (Figure 3D). The concordance for the donor % with both the two laboratory technologists and the two different software versions was 99.99%. Moreover, five samples were tested in duplicate by the same technologist on a different instrument to validate the inter-instrumental variance in reporting the donor % between Illumina MiniSeq1 and MiniSeq2 (Figure 3E). Similarly, 14 samples were tested in duplicate to compare the variance in detecting donor % between the Illumina mid-output and the high-output cell flow kits (Figure 3F). The concordance for these comparisons remained near 100%. The overall findings from the reproducibility showed direct proportional comparative outcomes, with all the aforementioned variables highlighting 99.99% concordance throughout.

3.5 Method comparison (STR vs. NGS assay)

To detect the accuracy of the NGS assay, we used 196 post-transplant clinical samples that were previously analyzed using the STR assay, as our parallel sample testing. Figure 5 shows the

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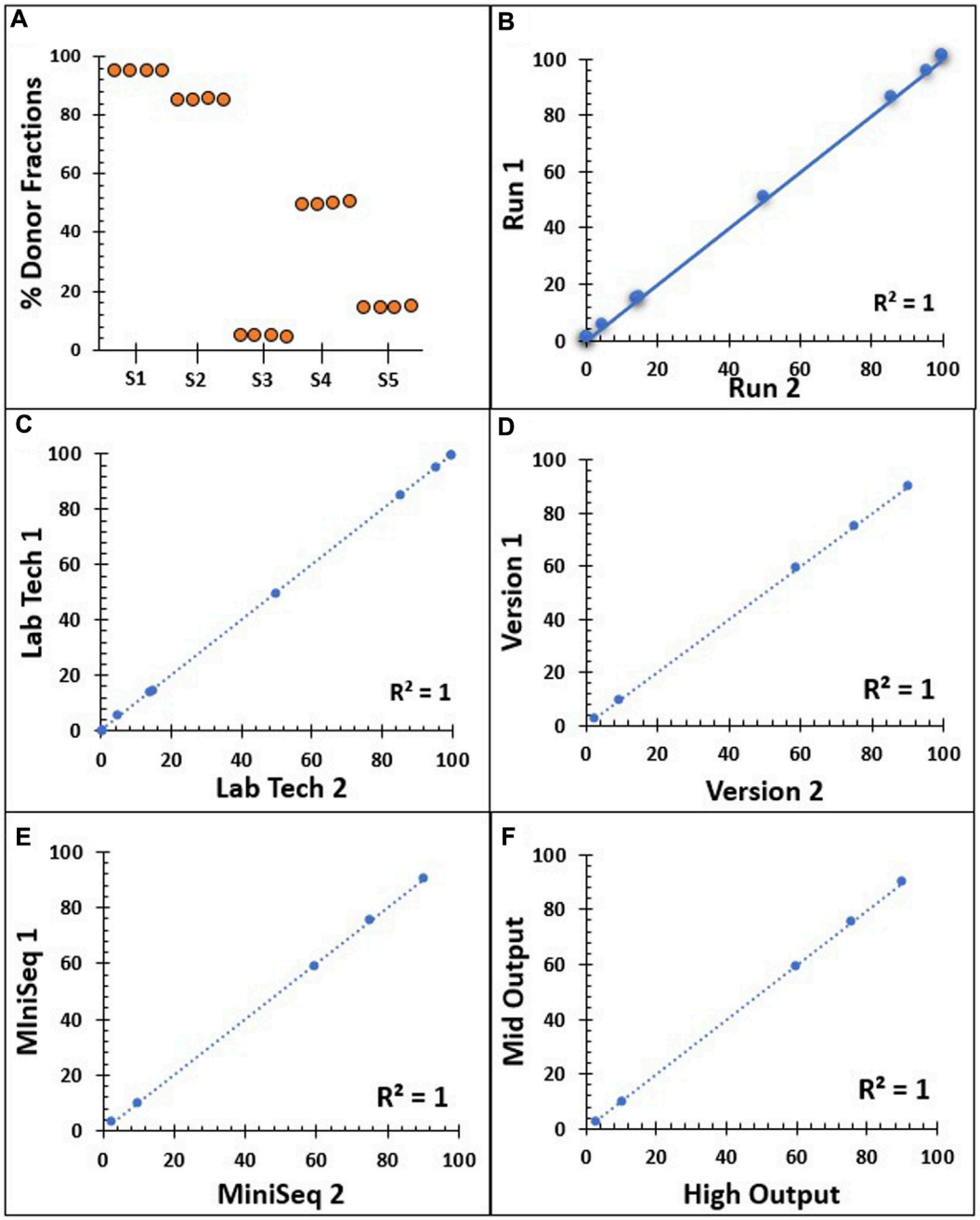

FIGURE 3
Reproducibility series summarizing the NGS assay donor % results. **(A)** Inter-assay reproducibility on the same run, conducted on four repeats with five different donor % samples (S1–S5) by a single technician. **(B)** Inter-assay reproducibility on two different runs on 11 samples with different donor % samples by a single technician. **(C)** Inter-tech reproducibility by two different laboratory technologists on eight duplicate samples. **(D)** Output from five different donor % samples analyzed using Alloseq-HCT software versions 1 and 2.1. **(E)** Correlation between two NSG instruments, Illumina MiniSeq1 and MiniSeq2 on five different donor % samples. **(F)** Reproducibility comparison between mid-output and high-output cell flow kits on 14 duplicate samples from different donor % samples. All dashed and solid lines in B–F indicate the trend lines, and all five Pearson's correlation coefficient values are 0.999.

comparison of % donor chimerism between our NGS chimerism assay and parallel sample testing (STR assay) There is a positive linear correlation between the NGS and STR assays in unfractionated (Figure 4A), $CD3^+$ (Figure 4C), and $CD66^+$ cells (Figure 4E). Pearson's correlation was higher in the comparison of % donor chimerism between the NGS and STR assays within unfractionated cells (0.973) and $CD3^+$ cells (0.979) relative to the $CD66^+$ (0.73) input sample. The Bland–Altman plot shows that the bias line is near 0 for unfractionated (0.32) (Figure 4B), $CD3^+$ (−0.13) (Figure 4D), and $CD66^+$ cells (−0.09) (Figure 4F), which indicates a high level of agreement between the NGS and STR assay methods. Similarly, the Bland–Altman plot shows that a low R^2 value

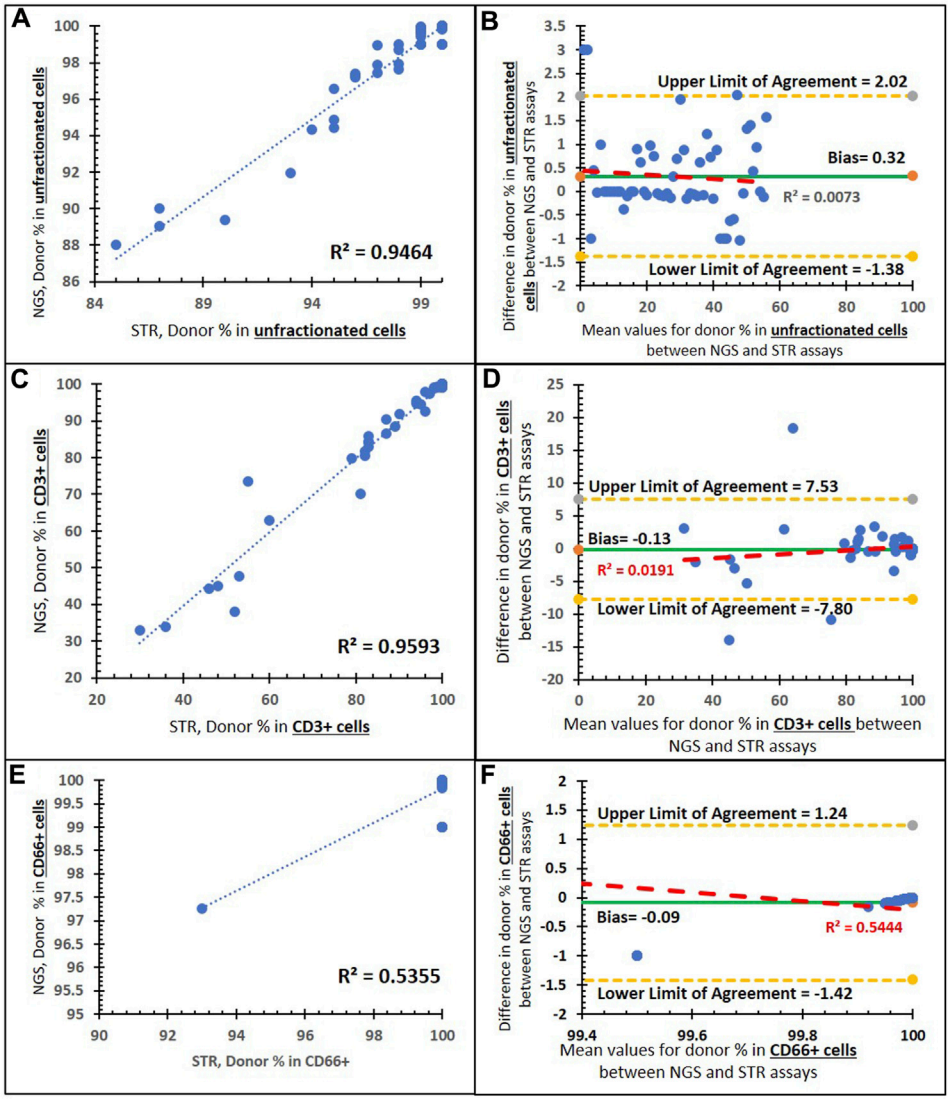

FIGURE 4
Method comparison of the NGS and STR assays. **(A)** Correlation between the two assays in unfractionated cells with a trend line (dashed blue line), an R^2 value of 0.9464, and a Pearson's correlation coefficient of 0.973 ($p < 0.001$). **(B)** Bland–Altman plot of the two assays in unfractionated cells: an average difference of 0.32% host chimerism (green solid line) is observed, with 95% limits of agreement [-1.38 to 2.02] (yellow dashed lines). **(C)** Correlation between the two assays in $CD3^+$-enriched cells with a trend line (blue dashed), an R^2 value of 0.959,3 and a Pearson's correlation coefficient of 0.979 ($p < 0.001$). **(D)** Bland–Altman plot of the two assays in $CD3^+$-enriched cells: an average difference of −0.13% host chimerism (green solid line) is observed, with 95% limits of agreement [−7.80 to 7.53] (yellow dashed lines). **(E)** Correlation between the two assays in $CD66^+$-enriched cells with a trend line (blue dashed), an R^2 value of 0.535, and a Pearson's correlation coefficient of 0.73 ($p < 0.001$). **(F)** Bland–Altman plot of the two assays in $CD66^+$-enriched cells: an average difference of −0.09% host chimerism (green solid line) is observed, with 95% limits of agreement [−1.42 to 1.42] (yellow dashed lines).

for unfractionated cells (0.007) (Figure 5B) and $CD3^+$ cells (0.019) (Figure 4D) indicates a lack of systematic bias. Contrastingly, $CD66^+$ shows an R^2 value of 0.54 (Figure 4F), which indicates some minor underlying systematic bias.

Due to the limited number of overall markers in the STR-based MC assay, it is inherently restrictive to find multiple informative loci between donor and recipient pairs. This phenomenon is exaggerated when these are related donor–recipient pairs. Therefore, we compared the number of informative markers utilized between related and unrelated donor–recipient pairs' runs on our NGS-based MC assay and the corresponding STR-based MC assay. Figure 5A shows the difference between the frequency distribution of NGS informative alleles in related (n = 22) and unrelated (n = 32) donors. In the NGS assay, there was a statistically significant difference (Mann–Whitney U test, $p < 0.001$) between the mean frequency of informative alleles within the unrelated (120.10 ± 1.61) and related (72.35 ± 2.45) donors. Figure 5B shows the difference between the frequency distribution of STR informative alleles in related (n = 20) and unrelated (n = 39) donors. In the STR assay, there was a statistically significant difference (Mann–Whitney U test, $p < 0.001$) between the mean frequency of informative alleles within the unrelated (6 ± 0.0) and related (5.2 ± 0.19) donors. In conclusion, although the number of informative loci in both comparisons was significant ($p < 0.001$), we observe that the STR mean difference is only 0.8, whereas in NGS, it is much larger at

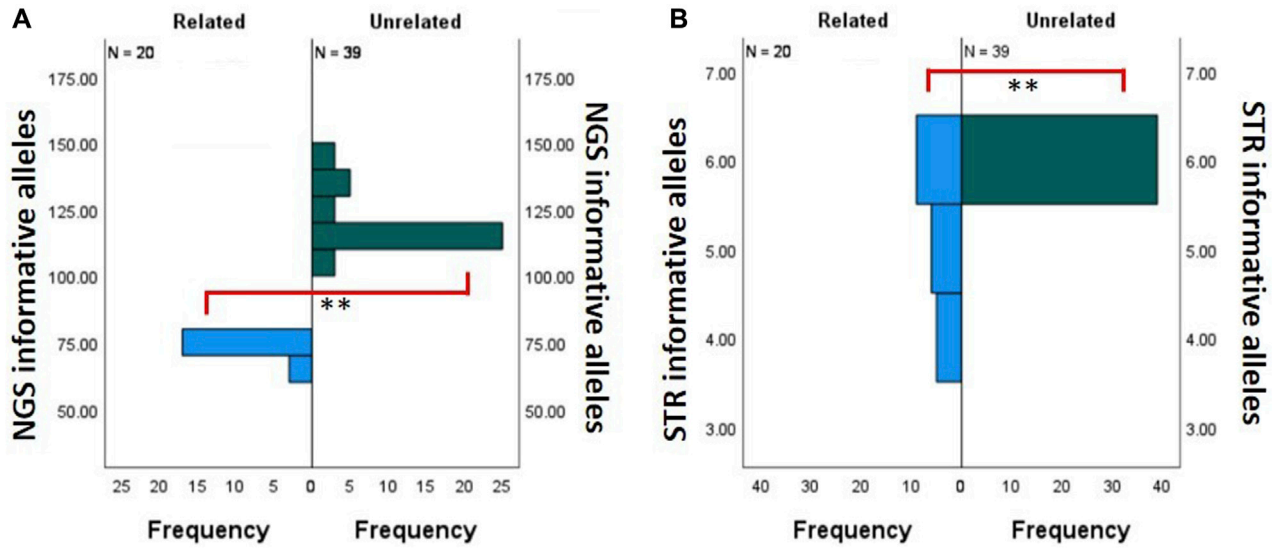

FIGURE 5
Comparison of the number of informative alleles. Related (n = 20, blue) and unrelated (n = 39, green) donor–recipient pairs. **(A)** Comparison bar graph for the NGS assay. **(B)** Comparison bar graph for the STR assay. ** Statistically significant with Mann–Whitney *U* test with $p < 0.001$.

47.75. The overall summary of the independent sample Mann–Whitney *U* test for related and unrelated donor populations in NGS- and STR-based MC monitoring is shown in Supplementary Table S3.

3.6 Comparison between unfractionated blood and CD3$^+$-enriched input in the NGS assay

To identify the significance of cell subsets on NGS-based mixed chimerism monitoring, the concordance and intensity of mixed chimerism in the CD3-enriched cells were compared to the unfractionated blood samples. Mixed chimerism was identified in 36 patient samples. Of these cases of mixed chimerism, 94.4% (n = 34/36) had a concurrent increase in host % chimerism. The remaining two samples showed either a CD3$^+$ (n = 1/36) increase without any unfractionated increase or an unfractionated increase (n = 1/36) without any CD3$^+$ increase. Figure 6 shows NGS results for mixed chimerism represented as host % for unfractionated and CD3-enriched input samples. Mixed chimerism was detected with a higher intensity on CD3-enriched cells compared to unfractionated cells, as shown by the mean 7.1 ± 7.0-fold higher host % detected on CD3-enriched cells compared to the unfractionated samples. This fold change of host % for CD3-enriched to unfractionated input samples ranged from 38.9 to 1.

3.7 Comparison between the NGS assay and external quality control

Twenty-two blind samples were utilized to compare the donor % results between our laboratory's NGS assay and the STR assay from Manitoba provincial HLA laboratory. Similarly, we compared 15 blind samples between our laboratory's NGS assay to the ASHI PT samples, including 65 participated laboratories.

As shown in Figure 7, the comparison of % donor chimerism between our NGS chimerism assay and blind samples that was previously analyzed by STR assay by the Manitoba provincial HLA laboratory (n = 22) and ASHI Proficiency Testing samples (EMO) (n = 15). There is a positive linear correlation between our NGS and both blind samples. The Pearson correlation is nearly 100%. Figures 7B, D show the Bland–Altman plots with their bias line at near 0 for comparisons of % donor chimerism between STR and EQC for the Manitoba HLA laboratory (−0.84%) and ASHI participated laboratories (0.49%), which indicates a high level of agreement between NGS and the PT samples. Similarly, the Bland–Altman plot shows that the low R^2 value for the Manitoba HLA laboratory (0.187) indicates the lack of systematic bias, and moderate R^2 for ASHI-participated laboratories (0.537), which indicates some minor underlying systematic bias. Supplementary Table S4 shows that the mean donor % of the STR based MC assay from the ASHI PT samples compared to our NGS-based MC monitoring assay were consistently around the mean donor % of the ASHI PT.

4 Discussion

Chimerism monitoring remains instrumental in the management of post-allo-HSCT patients by detecting the status of engraftment, early graft failure, and disease relapse. We are the first Canadian clinical laboratory to validate and implement NGS-based cell subset chimerism monitoring in allo-HSCT patients. The guidance in the scientific literature for PT in NGS-based chimerism testing with lineage-specific cell-subsets remains nearly non-

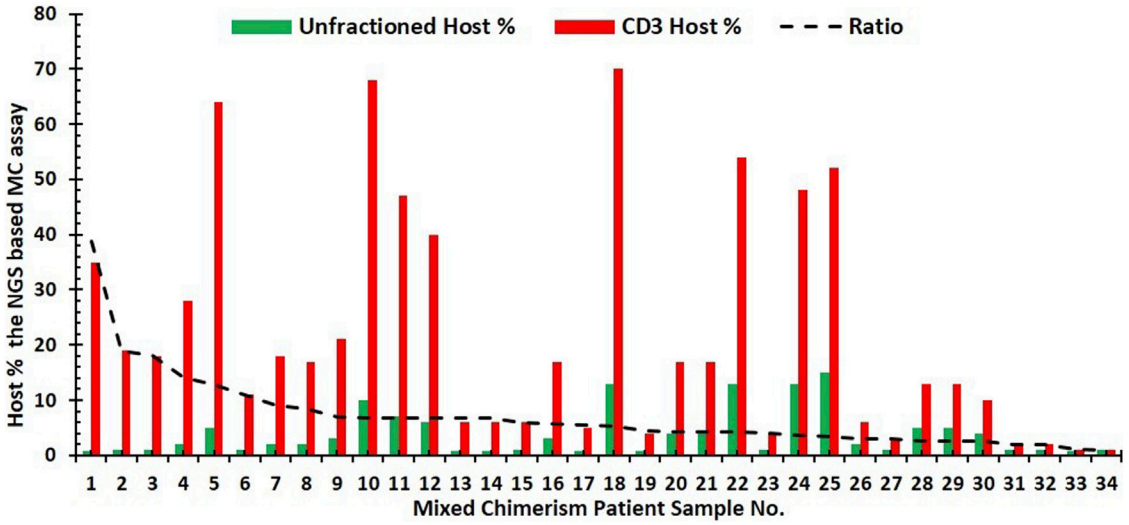

FIGURE 6
Bar graph of NGS results for the mixed chimerism case. Patient's (n = 34) represented by the host % for unfractionated (green) and corresponding CD3-enriched (red) input samples. Trend line (dashed black line) for the ratio of host% in CD3-enriched cells to that of the unfractionated cells.

existent. We have, therefore, included our comparative inter-laboratory lineage-specific cell subset chimerism testing as a proxy for PT in the absence of commercially available PT samples. Herein, we highlight the technical and logistical limitations to this practical solution and promote the commercial development of lineage-specific cell subset chimerism testing PT samples.

Overall, this NGS-based MC assay showed robustness across the battery of proficiency testing performed as none of the samples required repetition due to insufficient yield post-preparation of the DNA library or insufficient DNA read numbers hindering interpretation. This is evident in the high concordance (99.9%) achieved in measuring mixed chimerism across a concentration of 0.3%–50% and the reproducibility testing series showing a nearly 100% concordance for inter-assay reproducibility on the same and multiple runs, amongst our technologists, interpretation across different software versions, with different NGS instruments and cell flow kits. Therefore, the NGS-based chimerism monitoring assay showed high precision across the aforementioned concentration range, indicating high assay consistency and reproducibility across various variables.

Our NGS-based assay's analytical limit of detection is 0.3% and allows for extremely miniscule levels of host DNA detection, which is critical for early detection of relapse or donor failure. Contrastingly, the lowest reported limit of detection in the literature for STR-based MC monitoring is 1% (Kreyenberg et al., 2003; Schraml et al., 2003; Lion et al., 2012; Faraci et al., 2018). Additionally, the specificity of this NGS-based MC assay of 99.9% will enable accurate distinction of the donor and host DNA and thereby minimize the false-positive or false-negative rates. These analytical metrics can also be used to establish clinical guidance for the timing and intensity of immune modulation therapy, such as immune suppression or donor lymphocyte infusions. This NGS-based MC assay had the lowest limit of DNA input (1.25 ng) compared to other chimerism monitoring assays in the literature, such as variable-number tandem repeat PCR (100–250 ng), short tandem repeat PCR (1–5 ng), real-time quantitative PCR (20–300 ng), digital droplet PCR (20–100 ng), and other NGS assays (5–50 ng) (Sreenan et al., 1997; Lion et al., 2001; Alizadeh et al., 2002; Acquaviva et al., 2003; Chalandon et al., 2003; Thiede et al., 2004; Lassaletta et al., 2005; Karlen et al., 2007; George et al., 2013; Kim et al., 2014; Qin et al., 2014; Willasch et al., 2014; Stahl et al., 2015; Aloisio et al., 2016; Roloff et al., 2017; Waterhouse et al., 2017; Chen et al., 2018; Kim et al., 2018; Kliman et al., 2018; Mika et al., 2019; Pedini et al., 2019; Tyler et al., 2019; Valero-Garcia et al., 2019; Tripathi et al., 2020; Pettersson et al., 2021). Our assay's low input DNA limit, high sensitivity, and high specificity make it suitable for implementation for accurate and reliable chimerism monitoring in our large allo-HSCT population. Furthermore, with the emergence of microtransplantation, our assay operating parameters will enable seamless integration of micro-chimerism monitoring.

A comprehensive understanding of the timing of lineage-specific cell-subset immune reconstitution post-allo-HSCT is crucial for discerning lineage-specific engraftment dynamics. The neutrophils reconstitute early (14–30 days), followed by NK cells (30–100 days), T cells (100 days), and finally, B cells (1–2 years) (Ogonek et al., 2016). The MC correlation was slightly higher in the $CD3^+$ (0.959)-enriched samples compared to the unfractionated blood cells (0.946). Contrastingly, $CD66^+$-enriched cells (0.535) did not attain a high correlation. Interestingly, the most common causes for engraftment failure are graft-versus host and its treatment (30–50%), relapse from premorbid hemato-oncologic disease (20–50%), and host T-cell-mediated rejection of donor HSC (Barrett and Battiwalla, 2010; Zeiser and Blazar, 2017). All these entities are driven by T cells, and therefore, $CD3^+$-enriched cells showed a higher linear correlation when comparing NGS and STR. The literature shows that MC analysis with lineage-specific cell subsets has a higher sensitivity compared to unfractionated blood samples (Antin et al., 2001; Horn et al., 2009). This increased sensitivity bolsters chimerism

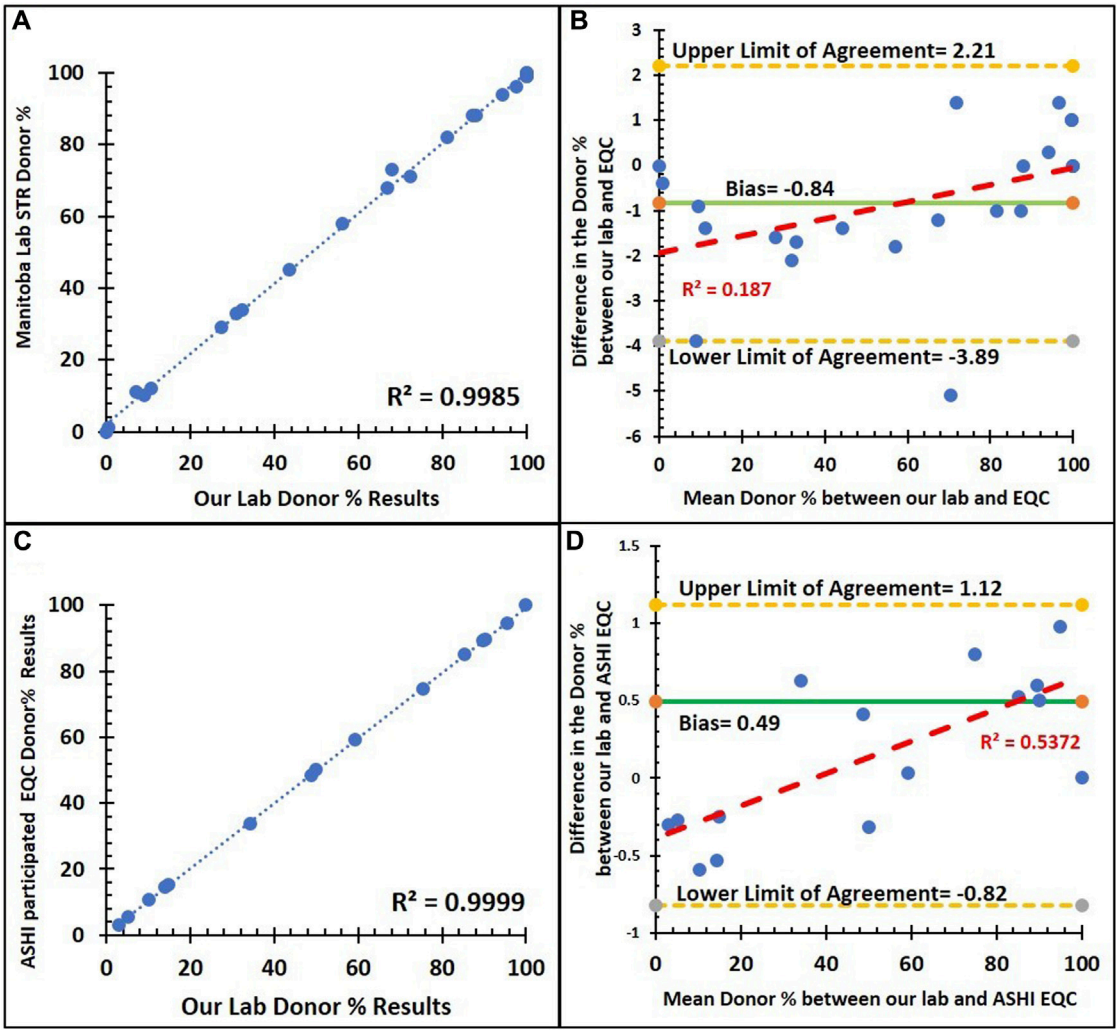

FIGURE 7
Method comparison of our institution NGS assay and external quality control with the STR assay. **(A)** Correlation between the donor % of the NGS and STR assays from the Manitoba provincial HLA laboratory (n = 22) with a trend line (dashed blue line), an R^2 value of 0.9985, and a Pearson's correlation coefficient of 0.999 ($p < 0.001$). **(B)** Bland–Altman plot for donor % between the NGS and STR assays from the Manitoba provincial HLA laboratory: an average difference of −0.84% host chimerism (green solid line) is observed, with 95% limits of agreement [−3.89 to 2.21] (yellow dashed lines). **(C)** Correlation between the donor % of NGS and STR assays from ASHI PT samples (n = 15) with a trend line (dashed blue line), an R^2 value of 0.9999, and a Pearson's correlation coefficient of 0.999 ($p < 0.001$). **(D)** Bland–Altman plot for donor % between the NGS and STR assays from ASHI PT samples: an average difference of 0.49% host chimerism (green solid line) is observed, with 95% limits of agreement [−0.82 to 1.12] (yellow dashed lines).

analysis in lineage-specific cells when compared to unfractionated cells. Additionally, the fold change in MC detection for CD3-enriched compared to unfractionated input samples ranged as high as 38.9-fold, which would clinically translate to an earlier trigger for intervention. Furthermore, $CD66^+$-enriched cells showed no congruent mixed chimerism compared to unfractionated or $CD3^+$-enriched cells. Our dataset for $CD66^+$-enriched cells showed a host % of nearly 100%. Since neutrophils reconstitute early post-allo-HSCT, we observe nearly 100% of these positively selected myeloid cells. Additionally, myeloid cells are not the predominant initiators of engraftment failure, and the $CD66^+$ cell subset can be utilized to monitor the relapse of myeloid lineage malignancies.

Important technical aspects for the implementation of cell subset isolation and MC monitoring in allo-HSCT patient samples are the interplay between cell purity, cell isolate yield, and specimen processing time. We utilized a positive selection method via a cell lineage-specific isolation, leading to higher cell purity with a lower cell subset yield. Contrastingly, negative selection-based isolation of cell subsets may be advantageous in specimens with a larger number of unwanted cells compared to the target-enriched cells (Hanson et al., 2013). Therefore, cell purity and yield are dependent on the frequency of the enrichment target and total cell count. The amalgamation of cell subset isolation and high-sensitivity NGS-based chimerism monitoring capable of detecting MC necessitates a pressing need for standardization of cell purity cut-offs. Other technical considerations include the time and cost of specimen processing for lineage-specific chimerism testing. In our experiments, the additional cost for each lineage-specific cell subset

enrichment was approximately 50 USD. Our automated cell sorter system has high-throughput capabilities as it can run multiple samples in the same run. This automated platform successfully isolated four cell subsets sequentially from the same whole blood sample within 2 h. Due to the reliable automated cell sorting, cross-contamination between samples was mitigated, which is critical to the success of downstream chimerism analyses. The high upstream cost for the automated instrument and the additional time for sample processing are compensated by the high sample purity, low cross-contamination, and reduced additional full-time equivalent laboratory staff required to upkeep large specimen volume demands.

The current Canadian gold standard for MC monitoring is an STR-based assay. This assay has many limitations such as the co-localization of peaks presenting as stutter peaks, susceptibility to preferential amplification, labor-intensive assay setup, and data analysis requiring specialized expertise. Furthermore, a large proportion of allo-HSCT donor–recipient pairs tend to be related, making many of the loci non-informative. The STR-based MC monitoring dataset in our study showed that the average number of informative loci in unrelated (6 ± 0.0) and related (5.2 ± 0.19) donors was separated by a single locus. Conversely, our NGS-based MC assay has a larger distinction between the informative alleles within the unrelated (120.10 ± 1.61) and related (72.35 ± 2.45) donors. This wider range of informative loci can also detect incorrect pre-transplant genomic samples as it compares and creates a genetic profile of the samples by assessing genome similarity and relatedness, and identifying any discrepancies between the expected donor and recipient profiles. Although pre-transplant recipient and donor genome profiles serve as references in most MC monitoring assays, the NGS-based MC assay can be performed in the absence of either the pre-transplant recipient or donor samples as the software application can extrapolate based on any one of the reference genomes. Additionally, NGS is also adaptable when the host has multiple donor transplants, a situation we often encounter in our transplant service. All these factors favor NGS-based assays over STR-based assays for the implementation of MC monitoring in moderate to high volume allo-HSCT centers.

A recent web-based survey by Blouin et al. revealed that lineage-specific chimerism testing was as high as 70% (n = 38) in the respondent laboratory (Blouin et al., 2021). This study showed that most laboratories used lineage-specific cell subsets with CD3-positive T cells (68%, n = 37 laboratories), CD33/CD66B-positive myeloid cells (52%, n = 28 laboratories), and CD19-positive B cells (28%, n = 15 laboratories) (Blouin et al., 2021). Additional candidates for cell subsets included CD56/CD16-positive NK cells (22%, n = 12 laboratories), CD34-positive hematopoietic stem cells (18%, n = 10 laboratories), CD14-positive cells (9%, n = 5 laboratories), and CD71-positive erythroid precursors (n = 1 laboratory) (Blouin et al., 2021). Despite this traction in utilizing lineage-specific cell sorting for MC monitoring, there is no known commercially available PT provider offering proficiency testing for MC in cell subset isolates. Currently, PT is performed on unfractionated blood samples in these laboratories, which do not truly represent the laboratory's performance metrics regarding cell isolation yield, purity, and chimerism detection. Other logistical and technical limitations in the implementation of PT testing for cell subset isolates will be the increase in cost of obtaining commercially developed PT samples, the additional blood volume requirement to attain an adequate yield, and the urgency in shipping these samples at room temperature to avoid cell surface immunomarker loss, which will affect cell isolation. To circumvent these limitations, we utilized a proxy PT for cell subset MC testing in the form of an interprovincial comparative study between our NGS-based assay and the current Canadian gold standard assay (STR-based MC assay).

One of the limitations to this study is that all these unfractionated blood samples or cell subset isolates were collected in patients within their 1st year post-allo-HSCT at various time points. The next iteration of this study will aim to collect these samples at 1 month, 3 months, 6 months, and 12 months post-allo-HSCT to assess the temporal MC profile of lineage-specific cell subsets. In the absence of commercially available proficiency testing of chimerism in lineage-specific subset isolates, we can utilize inter-laboratory comparison with our study partner at the Cancer Care Manitoba HLA Laboratory and lean on our external quality assessment (EQA) programs via ASHI.

In conclusion, chimerism monitoring by NGS on cell subset isolates is highly accurate compared to STR-based assays and can provide early triggers for intervention through early detection of relapse and microchimerism. Despite the additional cost and time allocation, incorporating cell subsets and developing PT can increase the reliability of lineage-specific cell subset MC monitoring. The utilization of lineage-specific cell subset NGS-based MC testing in a medium-to-high volume allo-HSCT center is justified by the shorter turn-around time. Lastly, the pre-analytical advantages of utilizing low DNA input and the freedom to conduct multiple runs on both recipient and donor genomic samples make NGS-based MC monitoring assays reign supreme.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Approval was obtained from the Saskatchewan Cancer Agency (SCA) Privacy Office to utilize de-identified residual samples for method validation and scientific research.

Author contributions

PK: data curation, formal analysis, investigation, writing–original draft, and writing–review and editing. YZ: data curation and writing–review and editing. HP: formal analysis and writing–review and editing. FW: writing–review and editing. TP: methodology and writing–review and editing. DW: methodology and writing–review and editing. ME: data curation and writing–review and editing. WS: data curation and writing–review and editing. LK: resources, and writing–review and editing. LP: project administration and writing–review and editing. MB: data curation and writing–review

and editing. KS: writing–review and editing. JL: writing–review and editing. JT: writing–review and editing. RL: writing–review and editing. PK: writing–review and editing. AM: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.1282947/full#supplementary-material

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Population genetics and external proficiency testing for HLA disease associations

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Numerous associations of HLA variants with susceptibility to diseases, namely, those with an immunopathological component, have been described to date. The strongest HLA associations were incorporated into the standard algorithms for the diagnostics. Disease-associated HLA variants are routinely detected by various techniques including DNA-based assays. For the identification of HLA markers or their combinations with the highest diagnostic value and those with frequent clinical indications (e.g., HLA-B*27, -B*57:01, -DQ2/-DQ8, -DQB1*06:02), diagnostic tests that focus on a single or limited number of specific HLA antigens/alleles, have already been developed; the use of complete typing for particular HLA loci is a relevant alternative. Importantly, external proficiency testing (EPT) became an integral part of good laboratory practice for HLA disease associations in accredited laboratories and not only supports correct "technical" identification of the associated HLA variants, but also adequate interpretation of the results to the clinicians. In the present article selected aspects of EPT for HLA disease associations related to population genetics are reviewed and discussed with the emphasis on the optimal level of HLA typing resolution, population-based differences in disease associated HLA alleles within the allelic group, distribution and linkage disequilibrium of HLA alleles in particular populations and interpretation of the presence of less common HLA variants/haplotypes. In conclusion, the laboratories that perform and interpret the tests to the clinicians, producers of the certified diagnostics and EPT providers should consider, among others, the genetic characteristics of the populations in order to optimise the diagnostic value of the tests for disease-associated HLA variants.

KEYWORDS

external proficiency testing, HLA, disease association, polymorphism, genetic susceptibility

1 Introduction

Early after the discovery of the polymorphic human major histocompatibility complex (MHC) encoding for the human leukocyte antigens (HLA), the first reports on the association of particular HLA variants with diseases were published (Brewerton et al., 1973; Woodrow, 1973; Thorsby, 2009). These studies were based on comparisons of the presence of HLA variants between the patients and the age/sex/ethnically matched control subjects. Such pioneer empirical observations were not usually accompanied by any data explaining the causal mechanism of the associations. Nevertheless, it is important to remember, that exact mechanisms of many associations between the HLA system and diseases are not currently clear, despite several plausible hypotheses being available. An enormous number of HLA associations have thus far been described based on both a

candidate gene and genome-wide approach. Particularly in autoimmune diseases, the variability of the MHC genes at the short arm of chromosome 6 substantially contributes to the genetic susceptibility of these conditions, e.g., in type 1 diabetes or rheumatoid arthritis (Wellcome Trust Case Control Consortium, 2007). Of the confirmed HLA disease associations, those with the strongest relationship with the diseases were integrated as part of the complex diagnostics of the associated diseases, namely, due to their high negative predictive value (Altman and Bland, 1994; Tye-Din et al., 2015).

Great effort has been dedicated to development of rapid and reliable laboratory procedures to identify variants with the highest value for the assessment of disease susceptibility under reasonable costs (Rouvroye et al., 2019). HLA variants with the most frequent clinical indications are represented, e.g., by HLA-B27 in rheumatology (Dequeker et al., 1978), HLA-DQ2/-DQ8 for celiac disease (Tye-Din et al., 2015) or HLA-DQB1*06:02 in narcolepsy (Tafti et al., 2014). HLA tests that support (contra) indications of the specific pharmacotherapy, such as HLA-B*57:01 for abacavir application in HIV treatment (Mallal et al., 2002) or HLA-A*02:01 for indication of targeted therapy of metastatic uveal melanoma by tebentafusp (Nathan et al., 2021) have recently been established. It is worth remembering, that there are further enormously important applications of the HLA disease association studies beyond direct detection of HLA variants for diagnostics reasons, namely, for understanding the molecular pathogenesis of complex diseases with immunopathological component (e.g., Ciacchi et al., 2022), but this area is out of scope of this rather practically oriented minireview.

Clinical application of the HLA tests for the diagnosis of associated diseases must always be based on the strong scientific evidence of a relationship between the HLA variant (or combination of HLA variants) and the disease. Detailed and independently confirmed data of the associated HLA variant should optimally be available for particular ethnics/populations, including the frequency of the HLA variant in the general population, relative risk of the condition conferred by the variant, the specificity, sensitivity, positive/negative predictive values, population attributable risk and further epidemiological parameters of the association. Because of the great complexity of the polymorphic HLA system, in the present article we focus on the selected aspects of population genetics in the HLA disease associations that may be relevant for design, organisation, interpretation and outcome of the EPT schemes in order to not only support correct "technical" identification of the associated HLA variants, but also adequate interpretation of the results by laboratories and their application in the diagnostic process.

2 HLA polymorphism, population genetics and disease associations

Currently known global HLA polymorphism is enormous (Barker et al., 2023) and may be demonstrated, e.g., by the fact that for the substantial percentage of patients seeking a donor for haematopoietic stem cell transplantation, no fully HLA matched unrelated donor can actually be found among the more than 41 million currently available donors in World Marrow Donor Association database (WMDA, 2023). The vast amount of data shows that global distribution of HLA alleles strongly differs in various ethics and populations (Gonzalez-Galarza et al., 2020; Arrieta-Bolaños et al., 2023). This fact has important consequences for identification, evaluation, clinical application and interpretation of associations of HLA variants with diseases. There is clear evidence that the distribution of HLA alleles in the concrete population may directly affect the prevalence and clinical manifestation of HLA associated diseases (Yazici et al., 2018). In cases of tight disease association with the common HLA variant in the population, the frequency of the variant correlates with the occurrence of the disease and confers high values of population attributable risk for the condition.

The impact of population genetics to the HLA disease association studies is, however, complex and may not be reduced to the relationship between the prevalence of the disease and frequency of the predisposing HLA variant in the population. It has been shown that different alleles within the same HLA allelic groups or even those from different allelic groups and HLA loci, may confer susceptibility to the same disease, e.g., to Vogt-Koyanagi-Harada disease, in various populations (Huang and Brown, 2022). Furthermore, linkage disequilibrium (LD, see Section 3) observed in the MHC region is reflected in different haplotypes characteristic for the particular populations, including those possessing predisposing HLA or other linked causal variants. Importantly, the relationship between the HLA risk variants and diseases may be modified by the polymorphism outside the HLA genes, and even outside the MHC genetic region. The role of such gene-gene interactions between the variants from different genes (epistatic effect) depends, among others, on the overall occurrence of these variants in particular populations. There is, for example, a well-known epistatic effect of the *ERAP1* (endoplasmic reticulum aminopeptidase 1) gene to the HLA-B51 association with Behçet's disease (Kirino et al., 2013). In this case the specific *ERAP1* alleles strengthen the effect of HLA-B51 on disease susceptibility. This fact supports the implication of the HLA-B51 associated peptidome in the pathogenesis of Behçet's disease. Finally, both qualitative and quantitative patterns of the associations between HLA variants and diseases or conditions are modified by, often unknown, environmental factors and disease triggers that may substantially vary between the regions and populations (Sparks and Costenbader, 2014).

3 Linkage disequilibrium in MHC region

There is no doubt that linkage disequilibrium (LD), defined as nonrandom association between particular alleles at linked loci (Slatkin, 2008), is very common within the MHC genetic region at chromosome 6p and is responsible for the occurrence of numerous HLA haplotypes often characteristic for particular populations (Creary et al., 2021). From the viewpoint of the search for HLA disease associations, it was the strong LD in the HLA region that enabled substantial proportion of initial observations of the relationship between the HLA polymorphism and diseases, despite these studies only identifying the HLA marker in LD and not the causal HLA variant. On the other hand, tight LD in the HLA region complicates identification of the HLA or non-

TABLE 1 Linkage disequilibrium (LD) in HLA disease association studies: historical examples of evolving knowledge on HLA variants associated with particular diseases based on LD.

HLA associated disease	Associated HLA variants	References
Celiac disease	HLA-B8	Evans (1973)
	HLA-DR3	Ek et al. (1978)
	HLA-DQ2	Corazza et al. (1985)
	HLA-DQ2.5 heterodimer	Vartdal et al. (1996)
Type 1 diabetes	HLA-B8	Cudworth and Woodrow (1975)
	HLA-DR4	Farid et al. (1979)
	HLA-DQ8	Owerbach et al. (1989), Baisch et al. (1990)
Psoriasis (psoriatic arthritis)	HLA-B13	Karvonen et al. (1976)
	HLA-B17	Gunn et al. (1979)
	HLA-Cw6	Tsuji et al. (1979)
Narcolepsy (cataplexy)	HLA-B7	Seignalet and Billiard (1984)
	HLA-DR2	Juji et al. (1984)
	HLA-DQB1*06:02	Mignot et al. (1994)

HLA variants really causal for the disease (Moutsianas and Gutierrez-Achury, 2018). Together with the enormous development of methods for HLA typing, the conduction of large candidate gene and genome-wide association studies from various populations followed by fine mapping of local polymorphisms within the HLA and nearby loci, supported by accompanying functional data, enabled the specification of causal HLA variants and haplotypes for numerous diseases (Gutierrez-Achury et al., 2015; Sciurti et al., 2018). Due to the LD in the HLA system, one can observe a long-term history of the evolving knowledge on many HLA associations with particular diseases. For example, currently well-documented association of celiac disease with the presence of HLA-DQ2.5 heterodimer (encoded in "cis" configuration by DQB1*02:01 and DQA1*05:01 alleles at the same HLA haplotype) (Tye-Din et al., 2015) was originally identified as an association with HLA-B8 (Evans, 1973), followed by HLA-DR3 (Ek et al., 1978); both these HLA antigens are encoded on the same and very common HLA haplotype together with DQB1*02:01 and DQA1*05:01 alleles. Further historical examples of HLA associations originally identified based on LD in the MHC region are listed in Table 1. From the practical point of view, HLA laboratories are sometimes faced with requirements to conduct typing of the HLA variants already obsolete for particular disease associations based on older literary reports. The example is provided in the guidance to the current Standards for Accredited Laboratories of the American Society for Histocompatibility and Immunogenetics (ASHI, 2023) where laboratories testing for narcolepsy risk would be expected to type for DQB1*06:02 and not for DRB1*15. In such cases, and if the laboratory registers a requirement for an unusual HLA test for particular disease, it is recommended to individually consult those indications with the clinicians in order to ensure the optimal diagnostic value of the test, and its proper interpretation, and use that as an opportunity for physician education.

4 Methodical aspects of HLA disease association studies

Historically, numerous tests based on various methodical principles were developed to identify HLA variants associated with diseases. Among the techniques that use antibodies specifically targeting the HLA molecules expressed on the cell surface, the lymphocytotoxicity test (complement-dependent cytotoxicity, CDC) or flow cytometry (Albrecht and Müller, 1987) are widely used to date. Nevertheless, these approaches are limited by the level of resolution of the HLA variants, because they are usually able to provide information at "low resolution" level (e.g., the presence of HLA-B27). Such disadvantage is eliminated by DNA-based assays that recognise HLA polymorphism directly on the HLA genes and, therefore, any level of HLA resolution reliable for particular disease association may be obtained. In order to detect HLA variants associated with disease susceptibility, one approach aims at identification of the single associated HLA variant in the subject; such tests are particularly used for their lower costs. The second approach is based on the evaluation of the disease-associated HLA variants from the complete HLA type of the individual at particular HLA locus (loci). The knowledge of the complete HLA type at relevant locus may be preferred in situations when the information on the heterozygous/homozygous status of the HLA allele provides important additional information for the disease susceptibility (i.e., risk stratification of the subjects based on the gene-dose effect) (Megiorni and Pizzutti, 2012). In general, any laboratory technique intended for identification/typing of disease-associated HLA variants, both commercially available or "in house" (when allowed for particular purpose) should undergo a validation/verification process in order to demonstrate that it is able to provide reliable and reproducible results with adequate diagnostic value.

TABLE 2 Distribution of HLA variants associated with diseases/conditions in various populations.

HLA association	HLA allelic group	Associated HLA allele(s)	Population*	Sample size	HLA allelic group (%)	Associated HLA alleles in the allelic group (%)	Associated HLA alleles carriers (%)
Ankylosing spondylitis	HLA-B*27	B*27:02 B*27:04 B*27:05 B*27:07	Germany DKMS - German donors	3,456,066	4.5	98.2	8.6
			USA NMDP Vietnamese	43,540	2.5	55.6	2.8
			USA NMDP Filipino	50,614	3.4	29.4	2.0
Tebentafusp indication	HLA-A*02	A*02:01	Germany DKMS - German donors	3,456,066	29.6	95.9	48.7
			USA NMDP African American pop2	416,581	18.1	68.2	23.2
			Israel Morocco Jews	36,718	14.5	17.1	4.9
Narcolepsy (cataplexy)	HLA-DQB1*06	DQB1*06:02	Germany DKMS - German donors	3,456,066	25.7	50.4	24.2
			USA NMDP Hispanic South or Central American	5,764	17.5	42.7	14.4
			USA NMDP Vietnamese	1,032	10.8	10.8	2.3

The data (including designation of example populations*) was adopted from the publicly available Allele frequency net database (http://www.allelefrequencies.net/, Gonzalez-Galarza et al., 2020). For each population, frequency of the allelic group (e.g., HLA-A*02), percentage of susceptible allele(s) (e.g., HLA-A*02:01) out of all alleles in the allelic group, and calculated estimation of the carriers of the allele(s) associated with the condition are given.

5 HLA typing resolution

In order to characterise HLA type of the individual for different clinical/research purposes, several levels of HLA typing resolution by DNA techniques have been established (Nunes et al., 2011). "Low resolution" typing identifies HLA allelic groups (e.g., HLA-B*27) and, in the majority of cases, reflects the level of typing obtained by antibody-based techniques such as CDC (e.g., HLA-B27). By contrast, "high resolution" (e.g., HLA-B*27:05P) and "allelic resolution" (e.g., HLA-B*27:05:02:01) provide more detailed information on HLA variants within allelic groups. Before the arrival of modern DNA-based assays, a limited number of elemental HLA "specificities" could be recognised by serological or antibody/cell–based techniques such as CDC. Nevertheless, these techniques enabled discovery of numerous currently known HLA disease associations.

Based on the results from genetic-association studies, the required level of HLA typing resolution was defined for appropriate assessment of the genetic susceptibility conferred by particular HLA variants. For example, information about the presence of HLA-DQB1*02/-DQA1*05 at low resolution level is usually accepted for the interpretation of the test for predisposition to celiac disease (heterodimer DQ2.5). In contrast, a low resolution level (HLA-DQB1*06) is not informative for the evaluation of the genetic risk to narcolepsy, where the HLA-DQB1*06:02 allele is strongly susceptible but another common DQB1*06:03 variant confers protection from the disease (Tafti et al., 2014). Similarly, only patients carrying HLA-A*02:01 and not those with other HLA-A*02 variants are eligible, from the genetic point of view, for the therapy of metastatic uveal melanoma by tebentafusp (Table 2; Nathan et al., 2021).

For a very long time there was a consensus that the presence of HLA-B27 is strongly associated with the rheumatic diseases, such as ankylosing spondylitis (Brewerton et al., 1973) or Reiter's syndrome (Woodrow, 1973). Antibody-based HLA typing techniques, such as CDC or flow cytometry, are therefore still in use for selected HLA disease associations. Accordingly, several EPT programs offer identification, e.g., of HLA-B27, by serological or antibody-based techniques (INSTAND, 2023). However, there is evidence that in addition to the HLA-B*27 alleles conferring the risk of the diseases (e.g., HLA-B*27:05, -B*27:02), other HLA-B*27 variants are protective (HLA-B*27:06, B*27:09) (Costantino et al., 2018). In recent years substantial differences in the distribution of susceptible/neutral/protective HLA-B*27 variants in various populations have been debated, including the fact that such variability affects the diagnostic value of the HLA-B27 test. Based on the publicly available population data on HLA polymorphism, the occurrence of HLA-B*27 protective alleles in European Caucasoid populations is rare (Table 2; Gonzalez-Galarza et al., 2020). In other words, almost each Caucasoid patient with the presence of HLA-B27 is a carrier of the HLA-B*27 risk allele. In contrast, in some Asian populations (e.g., Filipino, Vietnamese), susceptible HLA-B*27 alleles are much less frequent among all HLA-B*27 alleles (only approx. 30% in the Filipino population, Table 2). Therefore, the majority of patients of Filipino ancestry possessing the HLA-B27 antigen are not at increased genetic risk of the rheumatologic diseases mentioned above. From this point of view diagnostic value of the HLA-B27 defined at low resolution level may significantly differ in various populations. Accordingly, there is a possibility to consider the distribution of HLA-B*27 alleles in the population for which the laboratory provides the tests and, if appropriate, to apply identification of HLA-B*27 alleles at a high

resolution level for all samples or those from subpopulations with a lower proportion of susceptible HLA-B*27 variants. From the same reason, EPT programs that will provide both levels of resolution in EPT for single HLA-B27 testing, i.e., for the presence of HLA-B27 and for the presence of particular susceptible HLA-B*27 alleles, may improve the overall value of HLA-B27 testing in clinical applications.

6 EPT in HLA disease association studies

EPT is considered to be an integral part of the HLA testing for disease associations. For example, according to the current standards for accreditation of laboratories by the European Federation for Immunogenetics (European Federation for Immunogenetics, 2020), the laboratory must document all tests performed for HLA disease association (diseases, spectrum of tested HLA variants) and participate in EPT for each performed investigation including the detection of single HLA variants for disease associations. The minimum of 10 samples per year should be tested and reported by the laboratory to the organiser of the EPT. If an EPT scheme or EPT workshop/trial for the identification of a specific HLA variant is not available, the laboratory must at least participate in an inter-laboratory exchange of samples. Very similar rules for EPT and testing for HLA disease associations are also valid for laboratories accredited by the American Society for Histocompatibility and Immunogenetics (ASHI, 2023). Furthermore, both the ASHI and Asia-Pacific Histocompatibility and Immunogenetics Association (APHIA) conduct their own proficiency testing programs in order to support improvements of overall quality of HLA testing (APHIA, 2023).

Complete typing for particular HLA loci, which is a relevant possibility for HLA disease association diagnostics, is covered by numerous EPT providers (see, e.g., the list of EPT Providers registered at EFI at https://efi-web.org/committees/ept-committee). On the other hand, the spectrum of commercially available certified tests that were designed for the detection of a single or limited number of associated HLA antigens or alleles continually grows. Unfortunately, relatively few EPT providers offer proficiency testing for the tests that target particular HLA variants and do not provide complete HLA typing. This problem could be partially resolved by the providers of EPT for standard (complete) HLA typing who will allow participants to only report the presence/absence of the HLA variant of interest, and such reports being analysed in a specific manner. This approach has already been established, e.g., by the ASHI proficiency testing program. This EPT scheme distributes the same EPT samples for both complete HLA typing and/or HLA-B27 detection based on the requirements of participating laboratories. In a similar model situation, EPT for any HLA-A*02:01 single allele test used for indication of the therapy by tebentafusp could be involved in the analysis of standard EPT for HLA typing, because the carriers of this variant represent up to 50% of the Caucasoid population. However, this approach will have a limitation for the less common HLA variants that may be absent in the EPT samples, except in case that the samples possessing those HLA variants will be specifically selected for the EPT.

To our knowledge established providers of EPT for HLA disease associations offer the schemes that follow the current knowledge and recommendations in HLA disease association studies and undergo regular evaluation by the EPT program steering committees. In order to properly reflect the developments in the field, EPT providers (e.g., the Institute of Hematology and Blood Transfusion in Prague) organise regular workshops with attendance of the experts in immunogenetics, opinion leading clinicians representing medical societies in particular fields of HLA disease associations (rheumatology, gastroenterology, neurology, etc.), manufacturers of the reagents and EPT participants. Such workshops also serve as a forum for interlaboratory harmonisation and updating the recommendations for the interpretation of the tests for HLA disease associations.

7 Conclusion

Identification of selected HLA variants or their combinations that predispose to the diseases or conditions were involved in the standard algorithms for the diagnostics of associated diseases, namely, for their negative predictive value. External proficiency testing (EPT) is considered to be an integral part of HLA testing for disease associations and not only supports the technical point of the tests, but also interlaboratory harmonisation in terms of their reporting, interpretation and outcome for the clinical application. In this article we focused on selected aspects of HLA disease association studies that are related to population genetics and may be relevant for the providers and participants of EPT for HLA disease associations. In general, all parties involved in the application of HLA tests for diagnostic purposes (clinicians indicating the tests, HLA laboratories, producers of the specific reagents and providers of EPT schemes) should be familiar with the current knowledge in the field including the standards and guidelines of the relevant medical societies. Regarding population genetics, both EPT providers and participants should consider HLA disease association at the level of resolution that reflects distribution of risk HLA variants in the relevant populations and ethnics, select HLA markers with the strongest informative value for the diagnostics (avoid obsolete HLA markers in LD), apply adequate methods and reagents for their identification, and, if beneficial for patients, interpret the results taking specific characteristics of the particular populations and ethnics (e.g., LD, allele frequency, distribution of genotypes, gene-gene and gene-environment interaction, epidemiological parameters) into account. In addition, the laboratories and EPT providers should have the mechanisms for how to interpret the presence of less common HLA variants/haplotypes without sufficient supportive data on their implication in disease susceptibility.

Author contributions

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Seventy-five years of service: an overview of the College of American Pathologists' proficiency testing program in histocompatibility and identity testing

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The Histocompatibility and Identity Testing Committee offers an overview of the College of American Pathologists' (CAP) Proficiency Testing (PT) program, commemorating its significant 75th anniversary in 2024. The CAP PT program has undergone significant growth and evolution over the years, ultimately achieving Centers for Medicare and Medicaid Services approval. In 1979, CAP's partnership with the American Association for Clinical Histocompatibility Testing marked a pivotal moment, leading to the creation of the first proficiency testing survey in 1980. This laid the foundation for various PT programs managed by the CAP Histocompatibility and Identity Testing Committee, including HLA antibody testing, HLA molecular typing, engraftment monitoring, parentage/relationship testing, HLA disease associations and drug risk, and HLA-B27 typing. Each program's distinctive considerations, grading methodologies, and future prospects are detailed here, highlighting the continual evolution of histocompatibility and identity testing PT to support emerging technologies and evolving laboratory practices in the field.

KEYWORDS

College of American Pathologists (CAP), proficiency testing, histocompatibility and identity testing committee, HLA antibody testing, HLA molecular typing, monitoring engraftment, disease association, HLA-B27

1 Introduction

The CAP's proficiency testing (PT) program will celebrate its 75th anniversary in 2024. In 1946, founding CAP Board member F. William Sunderman, MD, partnered with a group of Pennsylvania-based pathologists to conduct a statewide survey to evaluate the accuracy of some common chemical measurements. Following this initial survey, the CAP Standards Committee submitted a proposal to the Board of Governors in November 1947 to distribute a national survey of up to approximately 200 laboratories to assess the accuracy of laboratory determinations in anticipation of later distributing standards for calibration and equipment methods. By 1949, the program had expanded to 650 participants per mailing, with some 500 participants returning their results. Samples provided for analysis included water-based solutions of glucose, urea, chloride, and calcium, and a chloroform-based solution of cholesterol, each supplied at two levels.

In 1961, the CAP Board of Governors committed to expand the PT program "to develop and maintain the highest possible technical standards in the field of clinical pathology." Surveys were no longer restricted to CAP members but were open to all laboratories. The CAP's PT program was available as a Centers for Medicare and Medicaid Services (CMS) approved PT program when the Clinical Laboratory Improvement Amendments (CLIA) PT regulations were implemented in 1994 and has maintained approval every year since this implementation. The CAP continues to be a deemed organization by CMS and is in compliance with associated regulations (e.g., Title 42 of the Code of Federal Regulations Part 493). Additionally, the PT program is accredited by the American National Standards Institute (ANSI) National Accreditation Board (ANAB) and continues to be a distinguished laboratory quality improvement program designed and evaluated by panels of diverse experts from around the country, including the expertise from members of the CAP's 28 scientific committees.

Just over 10 years later, the CAP partnered with the American Association for Clinical Histocompatibility Testing (AACHT; name changed to American Society for Histocompatibility and Immunogenetics in 1984) to form the AACHT/CAP Joint Committee for Histocompatibility Testing in 1979. The joint program produced its first Histocompatibility Survey in 1980, the same year CAP began accrediting Clinical Histocompatibility Laboratories. As of 2003, all CAP Histocompatibility surveys have been produced independently by the CAP. The CAP Histocompatibility and Identity Testing Committee (HITC), formed of members with expertise in histocompatibility and immunogenetics, designs the scope and focus of PT challenges and reviews all PT results prior to reporting to costumers. This insures the complexities of histocompatibility testing are accounted for when analyzing the results. The following sections detail the different PT programs that fall under the purview of the HITC. Each section is subdivided into four subsections, including an introduction to the PT, unique considerations, grading, and future prospects specifically related to each section's content.

2 MX: HLA crossmatching, antibody screen, and antibody identification proficiency testing

2.1 Introduction to proficiency testing for anti-HLA antibodies

Antibodies against allogeneic human leukocyte antigen (HLA) molecules can cause rejection of solid organ allografts, platelet transfusion refractoriness, and delayed/non-engraftment in hematopoietic stem cell transplantation (Anasetti et al., 1989; Trial to Reduce Alloimmunization to Platelets Study Group, 1997; Girnita et al., 2005; Smith et al., 2011; Wiebe et al., 2012). Thus, accurate detection and characterization of anti-HLA antibodies is essential to support clinical transplantation. While a thorough description of the testing methods used and their strengths and limitations is beyond the scope of this current discussion, understanding the multiple assays used for clinical detection and characterization of anti-HLA antibodies is essential to identifying and meeting PT needs for histocompatibility laboratories. Crossmatch testing, both complement-dependent cytotoxic crossmatch (CDC) and the flow cytometry crossmatch (FCXM), directly test patient serum reactivity against cells from a given donor (de Moraes et al., 2019). This is highly informative for evaluating reactivity for a potential transplant pair but does not enable prospective assessment of anti-HLA antibody status before donor specimens become available. Antibody screening tests, currently formatted as flow cytometry-based immunoassays, directly determine the absence or presence of anti-HLA antibodies and estimate the breadth of reactivity against allogeneic HLA molecules, but may not identify the antigenic specificities of these antibodies (Bray et al., 2004). Information about specific reactivities of anti-HLA antibodies can be obtained by single-antigen bead (SAB) flow cytometry-based immunoassays, which enable precise identification of the antigenic specificities of anti-HLA antibodies present. More recently, SABs have been modified to use complement proteins such as C1q or C3d as detection reagents with a goal of differentiating "high risk" anti-HLA antibodies (though a physiologic basis for distinction based on this testing remains debated) (Lee et al., 2018). The CAP MX PT program groups these assays according to shared performance and function (Table 1).

The CAP MX survey provides comprehensive PT for anti-HLA antibody testing. MX survey challenges ship three times per year, with each shipment containing 4 samples (0.4 mL recalcified plasma) to be tested. Anti-HLA antibody testing can be performed using 6 screening and/or SAB assays and/or complement-binding-SAB assays (Table 1). Each shipment also contains 2 donor cell populations (>6 × 10^6 peripheral blood lymphocytes) for cellular crossmatch testing. All samples are shipped at ambient temperature. Donor cell HLA genotyping information is also provided to enable laboratories to interpret crossmatch results as per routine clinical practice.

TABLE 1 Methodologies tested by MX PT.

MX PT category	Testing methodologies supported	Test results reported
Antibody reactivity	Mixed antigen beads, PRA screening beads, SAB, PRA/SAB combination, SAB-C1q	Present/Absent
Antibody specificity	SAB, SAB-C1q	HLA specificities detected, in descending order of assay output
Crossmatch	CDC, anti-human globulin-augmented CDC, FCXM	Positive/Negative

[a]PRA, panel-reactive antibody; SAB, single-antigen bead assay; SAB-C1q, C1q-binding SAB; CDC, complement-dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch.

2.2 Unique considerations for proficiency testing for anti-HLA antibody testing

The genetic and phenotypic diversity of HLA is the most obvious challenge for developing and maintaining a robust PT program for anti-HLA antibody testing. While it is theoretically possible to cover each of the approximately 165 well-characterized HLA serologic reactivities (Holdsworth et al., 2009; Marsh et al., 2010) in the yearly PT samples, it is highly impractical and may not reflect the most clinically-relevant reactivities assessed in laboratories. Thus, care is taken to ensure that a representative range of potential antibody reactivities against HLA are assessed across PT samples within a year.

Another significant consideration for anti-HLA antibody testing is that most laboratories utilize multiple methods, either in isolation or in aggregate depending on the clinical context. Supporting multiple testing methodologies requires not only sufficient sample for different tests but also distinguishing how various methodologies can produce varying results on the same sample. For example, an antibody may be detectable by a screening assay and SAB, but not necessarily detectable by less analytically sensitive methods such as SAB-complement, CDC, or FCXM (Tait, 2016). However, this seeming discrepancy does not necessarily represent a failure of any of the methods to produce a "correct" result, but rather reflects the different clinical information provided by each assay. Thus, it is essential to grade PT results within similar methodology, or peer group, independently.

Finally, a significant challenge for grading PT results, even within peer groups, for anti-HLA antibody testing exists due to the diverse approaches that laboratories use to test and interpret anti-HLA antibody data. Anti-HLA antibody testing platforms, particularly SAB, have several known limitations to their performance including complement interference, non-specific reactivity, saturation, and non-linearity (Sullivan et al., 2017; Abraha et al., 2022). Although all laboratories are expected to have procedures in place to address these issues (Tait et al., 2013), there is no consensus or adoption of universal approaches which can affect the output of these assays. This may initially not seem to be a critical limitation for qualitative assays measuring anti-HLA antibodies, however, the raw output of these assays is semi-quantitative and laboratories establish their own policies for interpretation of these results (Liu et al., 2012; Sullivan et al., 2017). These interpretive approaches can be more or less stringent depending on the laboratory's methods and the clinical needs (i.e., laboratories performing testing for hematopoietic stem cell transplantation (HSCT)) programs may use higher cutoff values for identifying potentially pathologic anti-HLA antibodies as it has been demonstrated that low-level antibodies may not present significant immunologic risk (Gladstone and Bettinotti, 2017). While this variability in the detection and characterization of anti-HLA antibodies may be clinically appropriate, it can create significant challenges when attempting to determine consensus results for PT samples.

2.3 Grading of proficiency testing for anti-HLA antibody testing

MX results are graded by consensus within each peer group, defined as the laboratories using a specific methodology to test the PT category. Antibody screening and crossmatch test results are reported as present/absent and positive/negative respectively. Antibody specificity results are reported as the specificities identified, listed in decreasing order of assay output. MX result reporting has 20 fields for listing antibody specificities for a given sample by a given method, with the ability to list additional specificities via free text entry. This approach of ranked listing is designed to minimize the effects of variation in interpretive approaches (more conservative interpretations are not necessarily penalized by more liberal interpretations of results, both of which may be clinically appropriate). This enables a reasonable approach to ensuring laboratories are detecting and characterizing the most clinically significant anti-HLA antibodies while preserving autonomy in medical practice. Results with 90% agreement within the peer group are determined as reaching consensus.

2.4 Future of proficiency testing for anti-HLA antibody testing

A practical challenge for anti-HLA antibody testing PT is the need to grade results in a way that ensures accuracy of the results obtained by individual laboratories, but enables flexibility in clinical practice. The degree of technical variation between methods, combined with the diversity in clinical practice, precludes approaches to PT that would be proscriptive. CAP HITC has focused on ensuring the reporting of meaningful qualitative data by participating laboratories (such as inclusive listing of identified anti-HLA antibody specificities) while avoiding quantitative data that may be imprecise and prone to misinterpretation (such as mean fluorescence intensity (MFI) data for SAB, which is known to vary widely between laboratories). However, the scale of potential diversity of PT challenge responses presents a challenge for data entry by PT participants as well as data collection and analysis by CAP. Efforts are continuing to find technical approaches to PT result reporting that are both intuitive and useful. Additionally, as new technologies are developed that may enable truly quantitative immunoassay output, HITC will need to evaluate the potential for quantitative as well as qualitative assessment of PT results.

Related to the challenge of providing PT that is clinically relevant across the spectrum of technical and clinical practice in transplantation, the HITC committee will need to address the increasing use of virtual crossmatch analysis. The practice of virtual crossmatch analysis, (Jackson, 2014), or determination of immunologic compatibility using donor HLA genotyping and recipient anti-HLA antibody testing data, is ever more common, driven primarily by changes in deceased donor organ allocation practices that expand access to donors outside of the recipient's locality. While virtual crossmatch analysis is conceptually straightforward, intricacies related to anti-HLA antibody interpretation and differing clinical practices between transplant programs preclude universal approaches to PT grading of anti-HLA antibody interpretation and virtual crossmatch analysis. To date, CAP MX has assessed clinical interpretation of anti-HLA antibody testing results via informational (ungraded) written challenges, with the goal of providing information to MX participants regarding their clinical practice as compared to their peer group. The increasing prevalence and importance of virtual crossmatch analysis will continue to push the HITC committee to find new and improved approaches for assessing this in the MX survey.

3 DML: HLA molecular typing proficiency testing

3.1 Introduction to proficiency testing for HLA molecular typing

Clinical HLA typing can be performed at different levels of resolution, depending on the clinical application. Solid organ transplantation and transfusion support typically require identification of the serologic reactivity of the HLA molecules present (focused on a relatively limited set of polymorphisms on the surface-exposed elements of the HLA molecules) while HSCT requires determination of the specific HLA allele genotypes based on at least the regions encoding the antigen recognition domains (Exons 2 and 3 of HLA class I genes and Exon 2 of HLA class II genes), with the capability of differentiating common null alleles. Both "low-resolution" serologic split-level and "high-resolution" allele-level HLA typing are performed by DNA analysis methods. To support molecular typing of HLA, the CAP provides the DML PT survey which challenges the laboratory on their ability to provide accurate HLA genotyping results to the same level resolution at which the lab provides results to physicians for clinical decision making and patient care. The DML survey is shipped to participants twice per year (surveys DML-A and DML-B) and each sample containing 2 mL of whole blood in Citrate Phosphate Dextrose (CPD) or CPD-adenine (CPD-A) anticoagulant for 5 specimens in each shipment. Participants are expected to isolate DNA from each specimen using the lab's routine DNA isolation procedure and perform molecular typing for each including all HLA genes tested in the lab. Laboratories participating in any given PT program are graded for their success in achieving results which are identical to or concordant with the majority of participants (based on 90% consensus).

The DML survey includes PT assessment for HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1 genes. Each participating lab submits results for each specimen in the shipment and for each level of HLA typing performed. Reporting levels include generic typing (low-resolution 1st-field typing), Bw4 and Bw6 for each B-locus typing result, serologic equivalents associated with each generic typing result (if different from the first field molecular type and in concordance with United Network for Organ Sharing [UNOS] solid organ transplant patient requirements), and high-resolution 2nd-field typing, which meets HLA community standards as well as National Marrow Donor Program (NMDP) requirements for HSCT patients and donors.

Along with each molecular typing result submission, participants submit the specific typing methodologies and techniques used. The Participant Summary (PS) is published with this information included. Current categories of typing methodologies include real time qPCR, reverse sequence-specific oligonucleotide probe (SSOP), forward SSOP, sequence-specific primer (SSP), next-generation sequencing (NGS), and Sanger sequencing (Dunn, 2011). For each HLA locus, all typing results reported by participants, the frequencies, and the associated PT grade are also summarized in the PS to aid participants in understanding how their results compare to all other survey participants.

3.2 Unique considerations for proficiency testing for HLA molecular typing

PT of HLA molecular typing presents obstacles stemming from the intricate biological complexity and extensive genetic diversity of HLA genes, along with the technical intricacies of molecular testing methodologies. Biologically, HLA genes exhibit remarkably high polymorphism, boasting over 30,000 protein-coding variant alleles (https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/) (Holdsworth et al., 2009; Marsh et al., 2010), hindering the creation of comprehensive PT samples covering all variations. Additionally, the presence of sequence similarities among many HLA alleles results in typing ambiguities that require further testing and expertise to resolve. Analyzing the vast array of alleles at each locus and managing ambiguities becomes challenging when striving to reach a consensus.

Compounding the biological intricacies are the technical aspects of HLA typing, which encompass a multitude of methodologies and technologies employed in various laboratories, such as SSP, SSOP, Sanger sequencing, and NGS (Dunn, 2011; De Santis et al., 2013). Each method bears its own advantages and limitations, producing distinct levels of resolution, ranging from low-, intermediate-, and high-resolution typings. For example, intermediate-resolution may result in a long list ("string") of possible alleles, whereas high-resolution typing can generally resolve the allele to two fields (e.g., HLA-A*02:01). These variations in methodologies, reagents, and reports across different laboratories make it challenging to compare and harmonize results. Furthermore, the analysis of complex molecular data and interpretation of HLA typing outcomes necessitate bioinformatics expertise, but PT software limitations mandate manual reviews, prolonging the consensus attainment and reporting processes.

To address the challenges inherent in molecular HLA typing and reporting, the CAP HITC continuously seeks improvement and implements measures to standardize data collection and grading. As of 2023, the committee has eliminated free text entry for reporting HLA alleles, requiring participants to input results into designated fields: one-field for generic typing and two- or three-fields for higher resolution typing. To circumvent the reporting of extensive strings of less common alleles that remain indeterminate but have uncertain clinical relevance, the data entry result form permits the selection of G (gene) and P (protein) groups. This grouping system diminishes the number of individual alleles or allele combinations reported while still preserving salient genetic diversity. Moreover, comprehensive DML kit instructions are included with examples defining criteria for high-resolution typing reporting, facilitating comprehension, and ensuring standardization. For instance, participants are reminded to report according to WHO-defined nomenclature (Marsh et al., 2010), exemplified by the following instruction: "If the consensus result is A*02:01:01G, then reporting A02:01G (incorrect nomenclature and not a WHO defined G group) or A*02:01:02G (also not a WHO defined G group) will be graded Unacceptable."

By using these measures, HITC aims to enhance standardization, improve accuracy, streamline reporting, and expedite data analysis for HLA molecular typing PT. Collectively, these efforts contribute to the overarching goal of fostering consistency and precision in HLA molecular typing across laboratories.

3.3 Grading proficiency testing for HLA molecular typing

Grading of participant submissions for PT assessment is often more complicated than it may seem. Although HITC strives to provide a simple and straightforward interface for submission of results, differences in laboratory practice with respect to the level of typing resolution and even misinterpretation on how to appropriately enter PT results for the assessment create challenges. All results submitted in the result entry form for the "1st Type" and "2nd Type" (corresponding to each of the 2 alleles expected in each sample for each locus) are most readily summarized and graded based on consensus. However, many laboratories additionally enter free-text comments along with each locus submission including information on additional alleles, which they have not ruled out using their typing methodology or reporting procedure. These comments need to be individually reviewed by the committee to assess whether or not their inclusion still leads to an appropriate high-resolution HLA typing result with respect to G groups and P groups as required and defined for high-resolution typing requirements per the most-recent catalog version of Common, Intermediate and Well-Defined (CIWD) HLA alleles and the resolution of common null alleles which may be present in G groups being reported (Hurley et al., 2020).

As a PT participant, some of the additional grading challenges routinely encountered by the committee, which the laboratory should be keenly aware of, include the submission of more than two antigenic groups for any locus, or the submission of results utilizing incorrect nomenclature which does not align with current WHO standards. An example of a generic typing submission which would receive a grade of Unacceptable is any entry in which more than two antigens are submitted for a single locus (when considering the comment field in addition to the DML 1st Type and 2nd Type entry fields). Examples of high resolution submissions which would receive a grade of Unacceptable include HLA-A*01:01G, as the correct nomenclature is HLA-A*01:01:01G (Acceptable), HLA-B*39:11P (Unacceptable) when no such P group exists, and HLA-DQB1*02:02G (Unacceptable) where the correct nomenclature is HLA-DQB1*02:01:01G (Acceptable) and the allele identified by consensus is HLA-DQB1*02:02 (Acceptable).

Based on the above assessments of participant result entries, nomenclature, and anything additional entered into the comments field on the data submission form, results that achieve 90% participant consensus (based on a combination of Good and Acceptable grade categories) are formally graded for each participant. Results that do not achieve such consensus are reported as Ungraded. Table 2 provides examples of the grading categories while Table 3 provides the consequences of receiving unacceptable PT results.

3.4 Future of proficiency testing for HLA molecular typing

The continuous advancement of HLA molecular typing poses future challenges for PT. The ongoing discovery of novel and rare HLA alleles leads to an expanding pool of variants, necessitating continual updates to PT samples and data entry forms for comprehensive coverage. The emergence of new NGS technologies facilitates interrogation of HLA gene sequences including introns and non-coding regulatory sequences, and the clinical relevance of this increased level of genotyping is actively under investigation (Mayor et al., 2019; Mayor et al., 2021). As some clinical laboratories will undoubtedly begin to report this additional genotype information for clinical use, this will require PT that supports this level of testing. Additionally, evolving regulations and accreditation requirements may impact PT grading procedures.

To address these challenges proactively, the HITC must remain dynamic and responsive to HLA molecular typing advancements. Regular updates to the data entry form will ensure alignment with the evolving HLA landscape, accommodating new alleles and high-throughput sequencing technologies. Along with the discovery of new alleles comes the assignment of appropriate serological equivalents. As the HLA community reaches a consensus on serological equivalents for these new alleles, the committee will need to update PT reporting accordingly. Furthermore, the committee anticipates a transition to universal high-resolution typing, making generic typing PT potentially obsolete.

4 ME: monitoring engraftment proficiency testing

4.1 Introduction to proficiency testing for monitoring engraftment

Chimerism testing quantifies the relative amounts of donor and recipient-derived hematopoietic cells based on measurement of

TABLE 2 HLA high resolution genotyping grading categories.

Molecular typing result	Grading category
Unambiguous results consistent with consensus	Good
Ambiguous results consistent with the consensus and including alternate alleles (provided in the Comments) which are in the same G/P group	Acceptable
Ambiguous results consistent with the consensus and including well-documented or rare alternate alleles outside of the same G/P group	Acceptable
Ambiguous results consistent with the consensus and including common or intermediate alternate alleles outside of the same G/P group	Unacceptable
Results inconsistent with the consensus (incorrect alleles identified and/or submitted using incorrect nomenclature)	Unacceptable

TABLE 3 Consequences of receiving unacceptable PT results.

PT performance	Definition	Impact on testing
Unsatisfactory	Receiving an unacceptable result for a given analyte or test during a PT event	Can continue testing until the next PT event
Unsuccessful	Receiving unsatisfactory PT performance on 2 consecutive or 2 of 3 PT events	Can continue testing until the next PT event
Critical	Receiving unsatisfactory PT performance on 3 consecutive or 3 of 4 PT events	Can continue testing until the next PT event. Laboratory will be warned they are at risk of a cease testing directive
Repeat Critical	Receiving unsatisfactory PT performance on 4 consecutive or 4 of 5 PT events	May be directed to cease testing on given analyte or test until reinstatement requirements have been met

[a]These consequences are applicable to all CAP PT programs.

distinguishable genetic polymorphisms. Chimerism testing is most commonly used to evaluate donor engraftment after HSCT. Underlying malignant or non-malignant disease, patient conditioning regimen, graft cellular content, graft manipulation, and posttransplant treatment for Graft-versus-host disease (GVHD) and infections, affect the chimerism kinetics and should be considered in interpretation. Engraftment can be associated with relapse and GVHD and must be performed before donor lymphocyte infusion (DLI) or consecutive transplants. A panel of experts has agreed on the definitions of cellular recovery, graft failure, poor graft function, secondary graft failure, and which chimerism test should be used to diagnose these complications and interpret them accordingly (Kharfan-Dabaja et al., 2021). Chimerism testing can be performed on peripheral blood, bone marrow, and specific cellular subsets, including myeloid or lymphoid compartments. For purposes of the CAP Monitoring Engraftment (ME) survey, "full" chimera is present if donor DNA is >95% for both myeloid and lymphoid lineages, "mixed or partial" if the result is 5%–95%, and "absent" if donor DNA is less than 5%. The panel recommended chimerism testing time points and type of subsets to analyze according to disease type and conditioning regimen. Besides the HSCT setting, chimerism testing is critical to detect rare but high-risk occurrences of GVHD associated with transfusion and liver transplantation.

The ME survey is conducted twice per year. For each survey, samples are mixed to form 5 admixtures from unique pairs, referred to as 'A' donor and 'B' recipient. Thus, over the course of a year, the survey assesses a total of 10 admixtures and 4 individual blood samples (2 pairs). These samples, each containing 0.5 mL and preserved in either CPD or CPT-A anticoagulant, are kept at room temperature.

4.2 Unique consideration for proficiency testing for monitoring engraftment

Multiple methods for chimerism analysis exist, with the analytical sensitivity and specificity determined by 2 main factors: 1) selection of informative markers to distinguish the recipient from the donor; and 2) detection method. Historically, blood groups and gender-specific markers were tested using agglutination, flow cytometry, or conventional cytogenetics methodology, but today most of the clinical laboratories are using molecular assays. Molecular markers vary from SNP (single nucleotide polymorphism), indels (insertion/deletion), VNTR (variable number of tandem repeats), STR (short tandem repeats), or a combination of them. Multiple quantification methods include FISH (fluorescence *in situ* hybridization), RFLP (restriction fragment length polymorphism), DNA fragment analysis sequencing, Real-time PCR, digital PCR, and NGS. Though many methods have been developed, most participants (115 and 104 from 135 participants from ME-A, 2023) are using commercial kits and capillary electrophoresis STR analysis (respectively) as the detection method.

4.3 Grading proficiency testing for monitoring engraftment

The participants receive acceptable grades if the final donor ("A") proportion (percentage) reported is within the range of consensus, which is defined as mean ±3 standard deviations (SDs). The PS includes lower and upper limits and SDI (Standard deviation index) as additional information for participants to evaluate their performance. The result is the

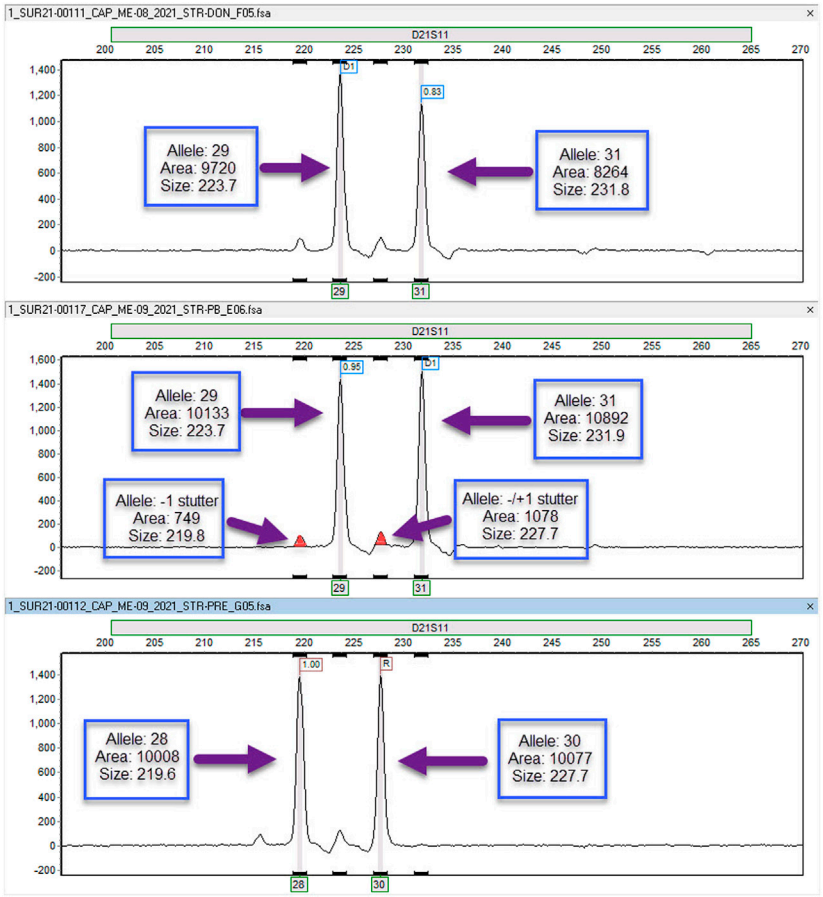

FIGURE 1
Electropherograms demonstrating stutter Electropherograms for the D21S11 short tandem repeat (STR) marker. The top panel is the electropherogram for the donor while the bottom panel is the electropherogram for the recipient pre-transplantation. The middle panel is the electropherogram for the recipient post-transplantation (allele, area = peak area, size = allele size). The post-transplant sample demonstrates that the recipient alleles (allele 28 and allele 30) are in the stutter of the donor alleles (allele 29 and allele 31). The presence of stutter peaks (represented by the smaller solid peaks) introduces complexity when attempting to interpret the percentage of donor and recipient contributions. This challenge arises due to the overlapping nature of recipient and donor markers. Typically, markers exhibiting stutter peaks are not considered informative for determining percent chimerism. Nevertheless, it is possible to utilize these markers if specific calculations are employed to accommodate for the average percent stutter associated with the particular marker in question.

average percentage of all informative markers and can be inaccurate if only a few informative markers are used. Unfortunately, the selection of too few markers continues to be an issue, 28/120 laboratories used only 1-2 informative markers for calculation (e.g., ME-A, 2023).

ME evaluation is also challenging when the sample only contains component "A" or "B", due to limitations in analytic sensitivity [STR sensitivity is around 1%–5% (Picard et al., 2023)]. Of note, a small number of laboratories use methods like NGS, real-time PCR or digital PCR, which can reach a limit of detection down to 0.01%–0.1% (Picard et al., 2023). Discrepant results are usually due to wrong interpretation of "stutter" bands, which are PCR errors due to strand slippage during primer extension (Levinson and Gutman, 1987), resulting in 1 less STR. Most of the time, markers with "stutter" bands should not be used as informative markers (Hancock et al., 2003). In a 2021 survey, seven markers were identified as having "stutter" bands (Sample ME-14, ME-B, 2021) and 3 electropherogram examples were provided (see Figure 1 for 1marker: D21S11) in the discussion to address multiple participants' concerns for discrepant grading. Nonetheless, participants are expected to distinguish monotypic samples from admixtures.

4.4 Future of proficiency testing for monitoring engraftment

The current survey faces a persistent challenge with the limited availability of human blood samples and transplant scenarios. As we look ahead to future surveys, it will be imperative to explore potential solutions for this issue. For example, the detection of informative markers typically relies on pre-transplant blood samples. However, an alternative option lies in using DNA isolated from buccal brushes or hair, which is sometimes employed by clinical laboratories. It is worth noting that the quantity and quality of buccal DNA can be compromised,

significantly impacting the amplification reaction. Therefore, it may be advisable to include buccal samples in future survey evaluations.

The source of PT samples should be a focal point in future surveys as well. Currently, the PT relies on samples obtained from healthy donors, which contrasts with the reality of clinical scenarios where samples are sourced from patients with hematologic malignancies. This discrepancy between PT samples and clinical samples deserves consideration. However, obtaining DNA from immunosuppressed transplant patients presents formidable challenges in terms of both quantity and quality, rendering it nearly impractical to incorporate such scenarios into the survey.

Chimerism testing also offers a valuable tool in cases involving multiple transplants. In these situations, chimerism testing can effectively distinguish the admixture of DNA from more than two distinct individuals. To accommodate this aspect, expanding the survey's scope by including PT samples from two or more individual donors could prove beneficial. Additionally, chimerism testing finds relevance in identifying monozygotic twins, a potential factor in HSCT donor selection. In such cases, the patient and their identical twin lack informative markers, precluding engraftment chimerism assessment.

In terms of assay sensitivity, several emerging methodologies like NGS, real-time or digital PCR, with varying degrees of sensitivity, have been adopted by some laboratories, which can create evaluation challenges. In connection with the growing adoption of high-throughput technologies, the utilization of expanded sets of genetic markers is evident in distinguishing admixture genotypes. The integration of large panels of SNPs or markers, different from the predominantly employed STRs, should not necessarily impact the final determination of the proportion of admixture. However, it poses a challenge in furnishing participant data related to the incorporation and performance of individual markers in the PS. As the use of these high-throughput assays increases, HITC will discontinue the inclusion of this data in the PS in 2024.

The sensitivity of capillary electrophoresis STR analysis can be improved using cell subsets (Antin et al., 2001); 68% of respondents of a survey perform T cell chimerism where T cells are separated using CD3 magnetic beads and others also test the myeloid/granulocyte population (Clark et al., 2015; Blouin et al., 2021). Purity assessment by flow cytometry is recommended before DNA isolation. Cell purity is yet another parameter that could be evaluated by future surveys.

In summary, the ME survey is poised to evolve and adapt to the rapidly changing methodologies that have emerged in recent years. It will encompass not only various sample types but also diverse transplant scenarios to ensure its continued relevance and effectiveness.

5 PARF: parentage/relationship test—filter paper proficiency testing

5.1 Introduction to proficiency testing for parentage/relationship

The PARF (Parentage/Relationship Test—Filter Paper Proficiency Testing) survey was first offered jointly by the CAP and the American Association of Blood Banks (AABB, now the Association for the Advancement of Blood and Biotherapies) in 1993 as the PI survey, or Parentage Identity (Polesky et al., 1996). When the Parentage survey was introduced, the laboratory results reported by participants included red cell antigens, red cell enzymes, serum proteins, HLA, and DNA results. The early surveys' DNA results were RFLP and later autosomal STRs. With the reported phenotypes, the calculated paternity index/likelihood ratio (LR) values were also submitted. As technology advanced, fewer participants reported non-DNA results, and RFLP results gradually dwindled until no laboratories currently report RFLP results. Presently, participants exclusively report standardized STR testing, which includes autosomal STR, Y-STR, and emerging X-STRs.

Laboratories accredited by the AABB are mandated to partake in PT, which assesses their capability to deliver accurate phenotyping results and analyses crucial for constructing relationship testing reports. Participants must report results for all loci and assays used in casework, along with the corresponding LR. The CAP is a PT provider accredited by the ANSI National Accreditation Board (ANAB) to the international standard ISO/IEC 17043:2010, as recommended by the Paternity Testing Commission of the International Society of Forensic Genetics (Morling et al., 2002). PARF meets this standard.

Triannually, four different biological specimens obtained from volunteers are distributed to participating relationship testing laboratories through the CAP PARF survey consisting of three mailings: PARF-A, PARF-B, and PARF-C. The biological specimens previously consisted of whole blood and/or buccal swabs. In recent years, the biological specimens mimic the majority of specimens received by relationship testing laboratories for paternity testing, which consist of mock buccal swabs and bloodstains on filter paper. All shipments consist of a mother, child, tested man #1 (alleged father #1), and tested man #2 (alleged father #2). For one shipment, the four samples include blood-stained filter cards for each biological specimen. For two shipments each year, they contain blood-stained filter paper for the mother and child specimens and mock buccal swabs for the two alleged fathers. The survey testing of these biological specimens aims to maintain standard paternity trios but includes two alleged fathers in each submitted case—one alleged father is included while the other is excluded. Notably, closely related alleged fathers, such as biological siblings or a father and son, have never been used as the two alleged fathers.

Also included in each PARF survey is a calculation challenge (also known as a paper challenge or a dry challenge). Most often, the paper challenge consists of a case scenario, phenotyping results, and frequencies for each allele at each locus, and a few questions for each participating laboratory to answer. For the paper challenge, the participants are asked to report the LR result for each locus as well as the combined LR. No biological specimens are distributed for the paper challenge. By providing the phenotyping results and the allele frequencies, the responses address reporting and not the laboratory work conducted, which removes any differences in reported phenotypes and any differences due to the frequencies in the database used.

5.2 Unique consideration for proficiency testing for parentage/relationship

The methods used and the nomenclature of reporting laboratory results have a high degree of standardization today. As the

technology has advanced, so has the consistency in reporting. Nonetheless, typing discrepancies are encountered and must be taken into consideration:

1) The 9.3 allele of the HUMTH01 locus is often observed in reporting this locus due to its high frequencies in the commonly used populations. In the surveys offered through the 1990s, the resolution from the technology in use at that time for the 9.3 and the 10 alleles was not always precise enough for the reporting laboratories; the participants would pool these results into reporting a 9.3/10 result instead of a distinct 9.3 allele and a 10 allele. Today, with the one base pair resolution that is easily achieved with the different capillary electrophoretic instruments, reporting of the 9.3 at this locus is possible.
2) Rarely, consensus is not achieved due to the use of different commercially available kits; the kit manufacturers are known to have different primer sequences and different amplification efficiencies. Typically, there are two to three kit manufacturers that are used for the majority of the reporting laboratories. However, more often, differences are observed when a participating laboratory designs its own kit internally.
3) The most common reporting difference amongst the participants is the reporting of genotypes *versus* phenotypes. Reporting the phenotype is scientifically accurate. When a participant reports a homozygous genotype, *e.g.,* 13, 13, it is flagged with a footnote that the "participant is incorrectly reporting genotype rather than reporting phenotype when a single allele is visualized." For a homozygous result, a grade of "good" is reported when the participant reports the correct one allele at the locus and a grade of 'acceptable' is reported when the participant reports the correct allele but has two alleles at the locus. The participants should report the phenotype rather than the genotype when only one allele is visualized using STR methods.

5.3 Grading proficiency testing for parentage/relationship

A grading scheme for the 3 levels of performance (good, acceptable, and unacceptable) was introduced in 1997 for responses reported by the participants (Allen et al., 2003). With a minimum of 10 participants reporting a result, that result would be graded if 9 participants reported the same result (Polesky et al., 1998). This grading evolved and for qualitative results, consensus is now established when at least 10 participants report results and at least 80% of those results are in agreement. When consensus is achieved, grading is provided at each locus. Grading is reported on both interpretation results (e.g., alleles) and numerical values (parentage index/likelihood ratios). STR results that do not reach the minimum number of participants or do not reach consensus are not graded. Further, the calculation paper challenge is not graded.

Over the many years of distributing this and versions of this survey, reporting differences have been observed and documented. In 2003, Allen et al. reported the percent of incorrect quantitative results for the LRs may be due to the limited number of allele frequency databases in use by participants (Allen et al., 2003). Further, Allen et al. proposed increasing the magnitude of the standard deviations used for grading to increase the range of acceptable responses and reducing the unacceptable response rate. Through 2023, quantitative results of the LRs have been graded after the outliers were removed with a mean based on the submitted responses and 3 standard deviations.

However, the committee recognizes that pseudoreplication (Hurlbert, 1984; Lazic, 2010) is possibly the cause of some of the unacceptable responses graded by the CAP Subcommittee and that most of these participants are likely accurately applying the correct LR calculations but possibly using a database very different than some of the commonly used databases. Hence, one to three databases used make up the majority of the responses and therefore, are not independent. In short, pseudoreplication can be an effect of replicates that are not statistically independent. Therefore, when the mean and standard deviation were calculated for the LRs, all of the responses were not independent since many participants used one of three databases. This leads to multiple observations from the same database, which in turn leads to dependencies that are skewed. Therefore, in 2023, the subcommittee decided not to grade some of the results by committee decision for PARF-B 2023 and PARF-C 2023; starting in 2024, the LRs will not be graded.

5.4 Future of proficiency testing for parentage/relationship

With the increasing use of the sex chromosomal testing of Y-STRs and X-STRs, it is anticipated that more participants will be reporting these results and thus, consensus may be achieved in some of these systems that have not been previously achieved. Further, with the increasing use of new technologies, such as NGS, there will be reporting strategies that must be considered and reporting of these results compared to the standard STR results today and those obtained from the different technologies. To address these challenges proactively, the CAP HITC must remain dynamic and responsive to molecular typing advancements. Regular updates to the data entry form will ensure alignment with the evolving landscape, accommodating new alleles and high-throughput sequencing technologies.

6 DADR: disease association and drug risk proficiency testing

6.1 Introduction to proficiency testing for HLA disease association and drug risk

In addition to utility for transplantation, HLA genotypes have significant associations with several disease states, most notably autoimmunity and adverse hypersensitivity drug reactions (Gough and Simmonds, 2007; Jeiziner et al., 2021). PT to support focused HLA typing for HLA disease association and drug risk (DADR) is provided by the CAP. Three 0.1 mL specimens, each containing 200 µg/mL of extracted human DNA are shipped in kits with specific instructions twice a year for each analyte. The specimens are intentionally sent at ambiently. Upon opening, the specimens are stable for 48 h when tightly capped and stored at room temperature. Unopened specimens are stable at room temperature for a duration

of 30 days. All laboratories are asked to measure and adjust nucleic acid concentrations according to standard laboratory protocols prior to performing nucleic acid amplification procedures. Both surveys also contain paper (dry) challenges regarding the clinical relevance of HLA genotypes in non-transplant-related diseases for educational purposes.

Drug risk (DADR1) assesses the detection of HLA-A*31:01, HLA-B*13:01, HLA-B*15:02, HLA-B*57:01, and HLA-B*58:01. This survey challenges the laboratory to accurately identify the presence or absence of alleles associated with the adverse reactions to specific drugs such as carbamazepine-induced Stevens-Johnson syndrome, allopurinol-induced Stevens-Johnson syndrome, abacavir hypersensitivity, and dapsone hypersensitivity. Disease association (DADR2) tests the detection of the following alleles: HLA-A*29:01, HLA-A*29:02, HLA-DQA1*04:01, HLA-DQA1*05:01, HLA-DQB1*03:02, HLA-DQB1*06:02, HLA-DRB1*03:01, HLA-DRB1*03:02, HLA-DRB1*04:02, HLA-DRB1*04:03, HLA-DRB1*04:06, HLA-DRB1*08:02, HLA-DRB1*08:04, HLA-DRB1*14:04, HLA-DRB1*14:05, HLA-DRB1*14:08, HLA-DRB1*15:01, HLA-DRB1*15:02, DQA1*02, DQA1*03, DQA1*05, DQB1*02:01, and DQB1*02:02. This survey tests the laboratory ability to accurately recognize the presence or absence of alleles associated with disease states such as celiac disease (CD), narcolepsy (N), pemphigus vulgaris (PV), psoriasis (P), anti-glomerular basement membrane disease (ABM), birdshot retino-choroidopathy (BR), and idiopathic myopathy (IM). Table 4 presents examples of well-established HLA drug and disease associations for specific alleles.

6.2 Unique considerations for proficiency testing for HLA disease association and drug risk

This is a rather straightforward survey with good agreement among participants. The survey currently has DQB1*02:01 and DQB1*02:02 as separate analytes. They both belong to the same DQB1*02:01P and DQB1*02:01:01G group and are associated with celiac disease (Sciurti et al., 2018). Participants may not be able to differentiate between these alleles, depending upon the test methodology used, which may occasionally cause disprepant results.

6.3 Grading proficiency testing for HLA disease association and drug risk

The HLA typing methodologies used by participants include real-time PCR, NGS, Sanger sequencing, forward SSOP, reverse SSOP, and SSP in various combinations. Methods that do not fall under the listed methods can be entered under the "other test methodology category." Results are simply recorded as present or absent. Performance grading for the DADR1 and DADR2 Surveys is based on 80% participant consensus and/or the intended response as established by the referee laboratory's result. Survey results are not stratified by test methodology. There must be at least 10 laboratories in the peer group to report a result. The CAP uses exception reason codes for ungraded results. The laboratory must identify all analytes with an exception reason code, review, and document the acceptability of performance as outlined in the instructions and retain documentation of review for at least 2 years. The survey occasionally has educational dry challenge with multiple choice questions, which help keep participants' knowledge up-to-date regarding HLA disease association and drug risk.

6.4 Future of proficiency testing for HLA disease association and drug risk

The field of pharmacogenomics and research in HLA disease associations is rapidly growing and new disease associations and HLA-drug risks are being identified. For example, the survey currently does not include an HLA-A*02:01 analyte, which is increasingly utilized as a requisite for cancer immunotherapies. As an example, the frontline treatment for unresectable or metastatic uveal melanomas is Tebentafusp, an immune-mobilizing monoclonal T cell receptor that has a high binding affinity for the melanoma-associated antigen gp100 presented by HLA-A*02:01 (Chen and Carvajal, 2022). Incorporating this analyte, along with others as they become integrated into routine clinical practice, is a significant undertaking essential for maintaining the relevance of this survey.

7 B27: HLA-B27 typing proficiency testing

7.1 Introduction to proficiency testing for HLA-B27 typing

Patient HLA-B27 screening for ankylosis spondylitis and associated spondyloarthropathies is performed by many US and international CAP accredited laboratories. Following CLIA guidelines, CAP provides PT material: 5 whole blood specimens twice a year. PT specimens are shipped at room temperature and stay stable for 3 days once opened and 7 days unopened. Participating laboratories are expected to test these specimens as if they were patient specimens and submit their results as HLA-B27 "present" or "absent" online by the deadline. Results submitted after the deadline are not graded. Participating laboratories are required to indicate the test methodology on the result form: antibody-based flow cytometry, micro-cytotoxicity, molecular methods: PCR-SSO, PCR-SSP, and real-time PCR. An "other test methodology category" is provided for methods that do not fall under the listed methods. Exception codes can be used if a laboratory cannot perform the PT. The survey occasionally includes some dry challenge questions, which are educational and ungraded.

7.2 Unique considerations for proficiency testing for HLA-B27 typing

It is well known that HLA-B27 shows remarkable polymorphism, and its disease association varies in populations and with different alleles (Khan, 2017). HLA-B*27:05 is the most common disease-associated subtype in the world (Reveille, 2006a), whereas HLA-B*27:02 is more commonly seen in Mediterranean populations and HLA-B*27:04 in Asian populations. Other subtypes, namely, HLA-B*27:06 (a common subtype in Southeast Asia) and HLA-B*27:09 (a rare subtype found primarily on the Italian island of Sardinia), seem to lack the disease

TABLE 4 DADR analytes for disease association and drug risk.

DADR1		
Analyte	Associated drug or disease risk	References
HLA-A*31:01	carbamazepine hypersensitivity	NEJM 2011; 364:1134–1143 (McCormack et al., 2011)
HLA-B*13:01	dapsone-induced cutaneous adverse reactions	NEJM 2013; 369:1620–1628 (Zhang et al., 2013)
HLA-B*15:02	carbamazepine hypersensitivity	NEJM 2011; 364:1126–1133 (Chen et al., 2011)
HLA-B*57:01	abacavir hypersensitivity	NEJM 2008; 358:568–79 (Mallal et al., 2008)
HLA-B*58:01	allopurinol hypersensitivity	BMJ 2015; 351:h4848 (Ko et al., 2015)
HLA-A*29:01	birdshot retino-choroidopathy	Ocul Immunol Inflamm 2011; 19: 397–400 (Brezin et al., 2011)
HLA-A*29:02	birdshot retino-choroidopathy	Ocul Immunol Inflamm 2011; 19: 397–400 (Brezin et al., 2011)
DADR2		
Analyte	**Associated drug or disease risk**	**References**
HLA-DQB1*06:02	Narcolepsy	Immunol Res 2014; 58: 315–339 (Mignot, 2014)
HLA-DRB1*03:01	Sjögren's syndrome	Autoimmun Rev 2012; 11: 281–287 (Cruz-Tapias et al., 2012)
HLA-DRB1*04:02	pemphigus vulgaris	Br J Dermatol 2012; 167: 768–777 (Yan et al., 2012)
HLA-DRB1*04:03	pemphigus vulgaris	Br J Dermatol 2012; 167: 768–777 (Yan et al., 2012)
HLA-DRB1*04:06	pemphigus vulgaris	Br J Dermatol 2012; 167: 768–777 (Yan et al., 2012)
HLA-DRB1*14:05	pemphigus vulgaris	Br J Dermatol 2012; 167: 768–777 (Yan et al., 2012)
HLA-DRB1*14:08	pemphigus vulgaris	Br J Dermatol 2012; 167: 768–777 (Yan et al., 2012)
HLA-DQA1*02	celiac disease	Hum Immunol 2020; 81: 59–64 (Choung et al., 2020)
HLA-DQA1*05	celiac disease	Hum Immunol 2020; 81: 59–64 (Choung et al., 2020)
HLA-DQB1*02:01	celiac disease	Hum Immunol 2020; 81: 59–64 (Choung et al., 2020)
HLA-DQB1*02:02	celiac disease	Hum Immunol 2020; 81: 59–64 (Choung et al., 2020)
HLA-DQB1*03:02	celiac disease	Hum Immunol 2020; 81: 59–64 (Choung et al., 2020)

association (Reveille, 2006b). Thus, HLA-B27 allelic typing provides a better understanding of disease association. This is possible by high resolution typing methodologies like NGS. This PT survey is a screen for HLA-B27 and does not differentiate between the alleles. A review of responses to dry challenge questions demonstrates that most participants understand that allele-level HLA-B27 typing results can inform clinical interpretation and how they impact the risk association (Pena et al., 2023).

7.3 Grading proficiency testing for HLA-B27 typing

Results are provided by test methodology for laboratories to evaluate their performance in their "methodology" peer group. However, grading is performed by consensus of >90% and not by methodology. The CAP uses exception reason codes for ungraded results. The laboratory must identify all analytes with an exception reason code, review, and document the acceptability of performance as outlined in the instructions and retain documentation of review for at least 2 years. Laboratories performing B27 testing by flow cytometry may need additional confirmation by an alternate method and may send a patient specimen to another center for additional testing. Since this is not permitted for PT specimens, these laboratories must report their flow cytometry result and indicate that they would normally send such a patient sample for additional testing. Laboratories with the indeterminate result indicating that they would send this patient specimen out for additional testing are graded acceptable.

7.4 Future of proficiency testing for HLA-B27 typing

Flow cytometry is the most used methodology due to its simplicity, cost-effectiveness, and fast turn-around time. However, it is also associated with higher error rates compared to all other methods due to cross-reacting antibodies and altered or masked antigenic epitopes (Kirveskari et al., 1997; Levering et al., 2003). There has been a gradual decrease in the number of laboratories performing flow cytometry methodology and an increase in molecular methods (Pena et al., 2023). Molecular typing would allow for a more accurate risk association and obviate the need for reflex confirmatory testing when "indeterminate results" are obtained by flow cytometry. The HITC may

consider incorporating high-resolution typing results along with racial/ethnic data in future PT to aid in clinical interpretation and diagnostic classification.

8 Conclusion

Inaugurated seven and a half decades ago, the CAP PT program has not only withstood the test of time but has also evolved, mirroring an unwavering dedication to enhancing laboratory practices. This evolution is enabled by HITC providing subject expertise in the design and review of PT challenges that support the rapidly-evolving landscape of histocompatibility and identity testing.

The future trajectory of PT in these domains is aptly characterized as a dynamic and responsive continuum, inextricably aligned with the ever-evolving terrain of clinical diagnostics and patient welfare. The HITC, in recognition of its pivotal role, acknowledges the imperativeness of harmonizing with the needs of the HLA community. This entails catering to the specific lexicons and benchmarks delineated by organizations such as the National Marrow Donor Program (NMDP) and the United Network for Organ Sharing (UNOS). Furthermore, the committee anticipates an expansion of its PT purview to encompass burgeoning diagnostic modalities as well as the needs of international laboratories. For instance, on the horizon, donor-derived cell-free DNA is an emerging evaluative tool in the clinical assessment of solid organ transplantation rejection (Kant and Brennan, 2022). As laboratories incorporate this technology into their test menus, innovative PT will be needed to ensure quality and patient safety. In the domain of customer service, the committee recognizes the importance of expeditious and precise responses to participant queries, which will be aided by integration of enhanced bioinformatics and data analytics software. Through the implementation of continuous improvement measures, the committee aims to remain proactive and responsive to the challenges arising from the ongoing advancement of HLA antibody testing and molecular typing.

In summation, the HITC hopes the contents of this review serve as a comprehensive resource, shedding light on the rich history and promising future of the CAP PT program, a cornerstone of laboratory quality assurance and proficiency assessment in the field of histocompatibility and identity testing. It amplifies the CAP's enduring pledge to excellence and commitment to continuous improvement, ensuring that laboratories across the country and beyond maintain the highest standards in patient care and clinical testing.

Author contributions

HS: Conceptualization, Project administration, Writing–original draft, Writing–review and editing. MG: Supervision, Writing–review and editing. SG: Writing–original draft, Writing–review and editing. RN: Writing–original draft. KG: Writing–original draft. SP: Writing–original draft, Writing–review and editing. RR: Writing–original draft, Writing–review and editing. GcM: Writing–original draft. HK: Writing–original draft. JS: Writing–original draft, Writing–review and editing. JM: Writing–original draft. MS: Writing–original draft. CL: Supervision, Writing–review and editing. GpM: Conceptualization, Project administration, Writing–original draft, Writing–review and editing.

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Conflict of interest

Author GcM was employed by the Laboratory Corporation of America Holdings. Author JS was employed by the Versiti Wisconsin Inc.

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Current status and perspectives of the quality system in histocompatibility laboratories in Poland

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Allogeneic transplantation is a multi-step process involving many clinicians and laboratory personnel working together to achieve a common goal—to maximize the recipients' chance of survival and to improve their quality of life. One of the key elements of the process is to ensure high quality, accuracy, and reliability of histocompatibility testing. This manuscript presents: the development and organizational principles of the national system of supervision and control of histocompatibility laboratories in Poland, problems faced by these laboratories, availabe proficiency testing schemes, as well as suggestions and prospects for the future raised by members of the Polish histocompatibility community.

KEYWORDS

HLA, histocompatibility, laboratories, Poland, transplantation, quality

Introduction

Many factors determine the success or failure of allogeneic transplantation of vital cells, tissues or organs. This type of transplantation involves a significant burden on the recipient's immune system. An optimal immunological match between the recipient and a potential donor increases the chance of obtaining well-functioning allografts over a long period of time.

The principles of immune matching depend on the type of cells, tissues or organs to be transplanted, with different algorithms of matching for hematopoietic stem cell transplantation, vascularized organs, and multi-tissue transplants. Biostatic transplants and non-vascularized grafts usually do not require matching between the recipient and the donor.

Regardless of the selected algorithm, it is usually necessary to determine major histocompatibility complex (HLA) antigens of the patient from the waiting list and of the potential donor, with the scope of loci depending on the type of the allograft and requirements of the respective national transplantation program. In certain situations, monitoring of the immunological status of both the potential and actual recipient is also carried out.

Here we describe the development and organizational principles of the national system of supervision and control over histocompatibility laboratories in Poland, proficiency testing schemes, problems faced by these laboratories, as well as suggestions and prospects for the future raised by members of the Polish histocompatibility community. This description is based on publicly available data, data from control reports at Histocompatibility Laboratories (hereafter referred to as HcL), and data obtained from surveys returned by HcL.

Histocompatibility laboratories (HcL) in Poland

In Poland, laboratory procedures in the field of transplantation immunology are performed in medical diagnostic laboratories, traditionally referred as Histocompatibility Laboratories (HcL). The nineteen laboratories involved in transplant immunology in Poland are part of various parent entities with a different legal status: regional blood centers, hospitals established by regional municipalities, university hospitals, and research institutes. HcL can be stand-alone units within the parent entity or be part of medical laboratories with a broader scope of activities.

The scope of tests performed by HcL depends on the involvement of an individual HcL in specific transplantation programs and/or programs for recruiting potential donors, especially potential unrelated hematopoietic stem cell donors, the type of parent entity, as well as contracts with healthcare providers.

Polish HcL use a wide range of techniques: HLA typing by molecular techniques at a low, high, and allelic resolution, anti-HLA antibody screening and identification, determination of PRA (panel reactive antibodies), anti-C1q antibody presence, and cross-matching, the latter also with the use of flow cytometry. Serological HLA typing is currently used only by two laboratories in parallel with molecular techniques: one laboratory uses it for HLA-B27 testing and one performs class I tests as an auxiliary method for evaluation of deceased donor cells' reactivity in complement dependent tests.

Organizational principles of the national system of HcL supervision and control

In Poland, transplantation immunology laboratories are supervised by dedicated institutions appointed by the Minister of Health (hereafter referred to as MoH) and, unlike other laboratories, are also directly responsible to MoH. For the past 17 years, Polish HcL must have complied with requirements of legal acts applicable to all medical laboratories (hereafter referred to as the general Laboratory Law) and additional requirements defined in the so-called Transplantation Law dated 2005 (Tinyurl, 2023a) implementing the provisions of European Union 2004/23/EC (Data Europa, 2004) and 2010/45/EU (Data Europa, 2010) directives, which came into force in January 2006. Moreover, every Polish medical laboratory must comply with common legal requirements relating to the medical profession, documentation, healthcare, patient rights, *etc.*

The general Laboratory Law details the requirements relating to personnel, premises, laboratory equipment, obligatory participation in external proficiency schemes, as well as pre-laboratory and laboratory activities that every medical laboratory must meet. None of these require *expressis verbis* the need to develop, implement, maintain, and continuously improve the quality management system (hereafter referred to as QMS) in laboratories or penalize non-adherence. Obtaining confirmation that the laboratory and/or the parent entity meet the requirements of ISO 9001:2015, 15189:2022 and/or 17025:2017, is voluntary in Poland. Certification regarding compliance with standards developed by professional societies such as European Federation for Immunogenetics (EFI) or American Society for Histocompatibility and Immunogenetics (ASHI) is also voluntary. The requirement for HcL to have a QMS is, on the other hand, included in the Transplantation Law.

Polish medical laboratories are currently in the transitional period with regard to the requirements of the general Laboratory Law. In December 2022, the Laboratory Medicine Act (Tinyurl, 2022a) came into force, replacing the Laboratory Diagnostics Act (Tinyurl, 2022b). Until December 2023, a number of implementing acts issued under the Laboratory Diagnostics Act are still in effect. Supervision over personnel in all laboratories, including HcL, is carried out by a dedicated body of self-government, the National Board of Laboratory Diagnosticians (Polish: KRDL). KRDL issues the laboratory diagnostician's license, maintains a register of laboratory diagnosticians, and is responsible for keeping records of all health care-related laboratories, including HcL.

Additional supervision over HcL activities is also exercised directly by MoH who, under the Transplantation Law (Tinyurl, 2023a), grants permission to operate valid for 5 years from the moment it is granted. The procedure for obtaining such a permission is a multi-step process, currently free of charge to the applicant. Submitting an application describing the laboratory and its quality system is the first step in this process. Since 2009, the application is submitted to a dedicated entity, the so-called National Centre for Tissue and Cell Banking (Polish: KCBTiK). KCBTiK is a budgetary unit responsible to the Polish MoH, obliged to supervise tissue and cell banks, as well as to organize training in the field of procurement, testing, processing, sterilization, storage, and distribution of cells and tissues.

Following verification of the submitted application by KCBTiK, MoH conducts a control at HcL or orders it to be conducted. Such controls are carried out by MoH-authorized KCBTiK staff and sometimes by external experts designated by MoH in cooperation with the National Clinical Immunology Consultant. The ministerial control includes verification that the HcL in question meets both the requirements of the general Laboratory Law and the Transplant Law (**1**) and covers all areas of a given laboratory's operations (Supplementary Table S1). In principle, controls are carried out at an HcL's premises by two persons authorized by MoH.

During the COVID-19 pandemic, controls were carried out remotely. Regardless of the form of control, all activities are documented using a form developed by KCBTiK, based on the deliverables of the EU-funded project EUSTITE ("European Union Standards and Training in the Inspection of Tissue Establishments").

The report prepared after the control may include certain post-control recommendations. Following verification that the HcL meets all legal requirements, KCBTiK submits an application to MoH for permission to perform specific tests, and with specific research techniques. MoH consults the application with the National

Transplant Board and decides whether or not to grant permission, which is an administrative decision within the meaning of the Polish Code of Administrative Procedure (Tinyurl, 2023b). Critical non-conformities defined as imposing significant risk to patient health or life result in suspension of the permission to operate until effective corrective measures are implemented.

Legal requirements for HcL to obtain MoH permission to conduct its activities are presented in Supplementary Table S2.

All allotransplantation procedures in Poland are coordinated by a government agency POLTRANSPLANT, a budgetary unit subordinate to the MoH. POLTRANSPLANT maintains a number of transplant registries: the Central Registry of Objections, the National Transplant Waiting List, the Registry of Unrelated Potential Donors of Hematopoietic Stem Cells and Cord Blood, and the Transplant Recipient Registry. POLTRANSPLANT is also, along with the National Health Fund and the Ministry of Health, one of the payers for a specific catalog of such procedures.

Proficiency testing schemes

All medical laboratories in Poland, including HcL, are required to participate in internal and external quality control schemes (hereafter referred to as EPT) to assess the qualifications of the laboratory personnel and quality of histocompatibility testing. The HLA Proficiency Testing for Central and East Europe (formerly the Proficiency Testing of HLA class I Typing for Central and East Europe) was the first attempt at EPT initiated in the early 1990s by Prof. Andrzej Lange (Bogunia-Kubik et al., 2000). Currently, this EPT is organized by the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw under the auspices of the Polish Society for Immunogenetics. It is supervised and directed by Prof. Katarzyna Bogunia-Kubik, who also serves as regional coordinator for EFI Region 5. This EPT scheme is the only Polish EPT provider, and has been serving Polish standardization system for over 20 years.

Since 1999 when the first round of EPT has been organized, it been has extended to the wider Central-Eastern European area (Bogunia-Kubik and Lange, 2004; Bogunia-Kubik et al., 2006; Bogunia-Kubik and Lange, 2008; Bogunia-Kubik and Lange, 2009; Bogunia-Kubik, 2019). In total, 67 HcL from 16 countries, also from outside of Central and Eastern Europe, participated in Wroclaw EPT (Bogunia-Kubik, 2019). Over the years, the scheme has significantly evolved with respect to both the clinical material provided and number of HLA loci to be tested (Bogunia-Kubik, 2019), covering serological typing of HLA class I loci, as well as DNA typing of 11 HLA loci (A, B, C, DRB1, DRB3/4/5, DQB1, DQA1, DPB1, and DPA1).

The participants are provided with blood and/or DNA samples to test HLA class I antigens by serology and/or to perform genomic assessment of HLA class I and class II alleles at low or high resolution (two fields allele assignment) level. This year, the XXX round is being organized. Polish EPT fulfils EFI rules for EPT providers and participants (Tinyurl, 2021).

All laboratories in Poland that serve transplantation purposes use commercially available kits for PCR-SSP and/or PCR-SSO HLA genotyping. NGS technology has been introduced in nine HcL and a few more are currently implementing this technology. SBT technology is employed in three HcL. The real-time PCR technique has been recently implemented in HcL involved in deceased organ donor matching.

Analyses of the past 10 years of Wroclaw's EPT activity allow to compare results in the following categories: HLA class I serological typing, HLA class I and class II molecular typing at low and high resolution level (for details see Supplementary Figure S1). Starting from the XIX trial, only 2 Polish participants did not comply with the required consensus (none/only one divergent result) due to discrepancies in serological typing of HLA class I antigens (in 2016) and in 2017, due to few discrepancies in results of HLA typing at DNA level (Supplementary Figure S2), including mistyping of a given allele. No discrepant results have been detected in HLA-DRB1 locus genotyping. The improvement in relation to previous rounds of the EPT (Bogunia-Kubik et al., 2006; Bogunia-Kubik and Lange, 2008) confirms the usefulness of participation in EPT. Direct benefits for the laboratory include elimination of incorrect typing of HLA alleles/antigens, reduction in the number of methodological errors, and overall improvement of quality, credibility, and repetitiveness of histocompatibility testing.

Polish HcL may apply for EFI accreditation. Currently, 3 laboratories hold EFI accreditation (from Wroclaw, Poznan and Warsaw) and two others (from Wroclaw and Warsaw) previously accredited by EFI, plan to regain this privilege. Some HcL (their parent entities) also hold various ISO certificates, i.e. 9001:2015, 27001:2017, 45001:2018) or AQAP (2110:2016).

The current EPT system in Poland does not cover procedures other than HLA typing. The interested laboratories participate in EPT schemes for cross-matching, PRA and anti-HLA testing provided by Eurotransplant or INSTAND e.V. and disease association studies (HLA-B27, HLA-DQ2/DQ8) provided by the Institute of Hematology and Blood Transfusion in Prague or INSTAND e.V. (Efi Web, 2023).

One laboratory tests samples provided by ASHI. Six HcL currently participate in the Eurotransplant scheme and 3 in INSTAND EPT. Besides PRA, anti-HLA, anti-C1q, and crossmatching, laboratories participating in the EPT organized by the Eurotransplant Reference Laboratory use the provided samples also for HLA typing. Four laboratories use samples provided by CET or INSTAND in parallel to Wroclaw EPT (Efi Web, 2023).

Discussion

Problems faced by Polish HcL

Recent survey and observations from ministerial controls reveal that major problems faced by HcL result from financial constraints. Insufficient number of personnel, inadequate salaries in relation to qualifications and legal responsibility, a whole range of additional office tasks increase the risk of potential laboratory errors and result in a high staff turnover, especially among young diagnosticians at the beginning of their careers. Although appropriate working conditions with adequate separation of processes have been granted as required, suboptimal environmental conditions in office spaces or archives have been reported in some cases. Access to modern equipment is partially financed by MoH, but high costs of its maintenance, regular inspections and servicing covered by parental entities largely affect laboratory's budget. Maintenance and calibration procedures are gradually improved and better documented, and non-compliance with regular periodical technical inspections of equipment, or the scope of inspections by service providers is less frequently reported. Remote monitoring systems supervise temperature-controlled equipment in most laboratories.

Lack of local EPT providers for procedures other than HLA testing, especially those required for organ transplantation, high costs of participation in international EPTs (registration sample, shipment fees), costs of reagents needed to perform EPT testing, and inconsistency in legal requirements may lead to insufficient control over several procedures. On the other hand, laboratories participating in external EPTs for these procedures face several problems associated with sample quality, which even though remaining beyond control of an EPT organizer (force majeure) may affect overall results. This issue, however, must be urgently formally resolved in the near future. Currently, new contracts are signed only with laboratories participating in EPT.

In general, laboratory methods used in transplantation immunology are well described in medical literature. Dedicated *in vitro* attested commercial kits and reagents are used whenever available. However, several reagents were or are currently expected to be unavailable due to the new *in vitro* Diagnostic Reagent Law - Regulation (EU) 2017/746 (IVDR) (Data Europa, 2017). Lack of access or high costs of IVDR attested reference cells, rabbit complement, and serum controls will probably significantly limit the ability to evaluate patients' immunization status. Solid phase-based methods face similar problems with IVDR licensing. At this point, however, due to difficulties caused e.g., by the COVID-19 pandemic, the transition periods for the aforementioned regulation have been extended by several years (Eur Lex Europa, 2022).

According to the Transplant Law, all QMS documentation and relevant records must be kept for 30 years from the date of test results delivery. Insufficient or inadequate storage areas and problems with access to electronic documentation for the required period pose another challenge. Since July 2021, all medical data should be stored in dedicated repositories in the form of Electronic Medical Records. High costs associated with the change of laboratory software or its adaptation result in a delay in its implementation. A dedicated platform called e-Transplant, expected to be available in 2024, is planned to cover all elements of the national transplant system and to replace the existing POLTRANSPLANT registries and associated paper documentation.

Suggestions and perspectives for the future raised by members of the Polish histocompatibility and immunogenetics community

Since the first successful kidney transplant performed in Poland in 1966, surgical techniques have improved, and new methods of organ preservation and better suppressive regimens have been developed. Tremendous developments in immunological diagnostics allowed for the complete replacement of serological HLA typing by molecular methods, monitoring of a recipient's immunization status with solid phase methods, and crossmatching with higher sensitivity. Shortly, it will be necessary to extend local EPT and/or grant support for participation in external EPT schemes.

None of the above developments or routine operations of HcL will be possible without ensuring further stable funding. Significant funds allocated by MoH support the purchase of modern equipment and introduction of new diagnostic methods in HcL, but salaries in publicly funded entities will have to be gradually increased to prevent high turnover of personnel and an exodus of young diagnosticians. Members of the Polish histocompatibility community hope that the new Act on Laboratory Medicine will both make it easier for young diagnosticians to become specialists in laboratory medical immunology and guarantee them adequate salaries. After all, HcL's most important resource is well-educated specialists.

In summary, various aspects of obtaining and maintaining quality at Polish HcL, including legal requirements and their fulfillment, supervised by a number of public organizations, have been described in detail. The actual ability to fulfill all requirements is influenced by a variety of factors, both at a general and local level. The common denominator is that promoting the implementation of pro-quality solutions in HcL requires the provision of stable funding.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

IU-T: Funding acquisition, Methodology, Writing–original draft, Writing–review and editing. KB-K: Visualization, Writing–original draft, Writing–review and editing, Funding acquisition, Data curation, Formal analysis. MB-K: Conceptualization, Writing–review and editing, Funding acquisition. BP: Methodology, Supervision, Writing–original draft, Writing–review and editing, Funding and data acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1322414/full#supplementary-material

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Evaluation of 19 years of international external proficiency testing for high-resolution HLA typing

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The international high-resolution external proficiency testing (EPT) started in 2004 with high-resolution typing of human leucocyte antigen (HLA) class I (HLA-A,B,C) and HLA class II (HLA-DRB1, DRB345, DQB1, and DPB1) alleles, since possibilities for such an EPT within Europe were limited and all existing EPTs at that time made use of the comparison of HLA typing results without a reference. This EPT was set up as a collaboration between the HLA laboratory of Leiden, providing DNA samples to the participants, and the laboratory of Maastricht, performing the high-resolution typing as the reference result and evaluating the results of all participants according to the prevailing European Federation for Immunogenetics (EFI) standards. Once a year, 12 samples were sent to the participating laboratories, and evaluation and certificates were provided at the end of that same year. During the years, the EPT was extended to low-resolution HLA class I and II typing, high-resolution typing including DQA1 and DPA1, and allelic resolution typing for HLA class I, the latter one being unique in this field. Evaluation of the high-resolution typing results of the last 19 years showed a clear increase in the number of loci tested by the participating laboratories and a clear change of method from Sanger sequencing with additional other techniques (SSO/SSP) to the nowadays widely used next-generation sequencing method. By strictly using the EFI rules for high-resolution HLA typing, the participants were made aware of the ambiguities within exons 2 and 3 for class I and exon 2 for class II and the presence of null alleles even in a two-field HLA typing. There was an impressive learning curve, resulting in >98% correctly typed samples since 2017 and a 100% fulfillment of EFI rules for all laboratories for all loci submitted in the last 2 years. Overall, this EPT meets the need of an EPT for high-resolution typing for EFI accreditation.

KEYWORDS

histocompatibility, immunogenetic tests, quality control, HLA high-resolution typing, sequencing, NGS, quality assessment, accreditation

Introduction

Matching donor and recipient for human leucocyte antigens (HLAs) has been and still is important for patient and graft survival in solid organ and stem cell transplantation. For both kinds of transplantation, national and international exchange programs exist, and therefore, it is necessary to have a reliable HLA typing using identical nomenclature all over the world,

irrespective of the HLA typing laboratory. For the use of identical HLA nomenclature worldwide, the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System was set up in 1968 having the responsibility for naming of new HLA genes, allele sequences, and their quality control (WHO Nomenclature Committee, 1968). Furthermore, in 1998, a unique specialist database was set up for sequences of the human major histocompatibility complex (MHC), nowadays known as the IPD-IMGT/HLA database, which is an important and highly appreciated resource for the HLA community (Barker et al., 2023).

The American Society of Histocompatibility and Immunogenetics (ASHI) and the European Federation for Immunogenetics (EFI) have both established an accreditation program for Histocompatibility and Immunogenetics (H&I) laboratories, and one of the aims is to ascertain accurate and correct HLA typing (Harmer et al., 2018; ASHI accreditation available at: https://www.ashi-hla.org/page/Accreditation. Accessed August 23, 2023). One of the requirements for both EFI and ASHI accreditation is adequate performance of (external) proficiency testing [(E)PT] for all techniques in use for accredited activities. Already from the start of the EFI accreditation program, performing high-resolution typing of at least DRB1 was one of the prerequisites to become accredited for the clinical accreditation category of unrelated stem cell transplantation (EFI standards 5.5; I2.210 available at: https://efi-web.org/committees/standards-committee. Accessed August 23, 2023). Nowadays, high-resolution typing of HLA-A, -B, -C, and -DRB1 is the minimum requirement for this clinical service (EFI standards 8.0, E5.3.4.3.1.2.1). Furthermore, for solid organ transplantation, high-resolution typing of both the recipient and the donor is now the preferred choice because this will ultimately facilitate the virtual cross matching that has recently been implemented by Eurotransplant.

Although several external proficiency testing (EPT) schemes on HLA typing were available in 2004, in many of them, not all HLA loci or no high-resolution typing was provided. Moreover, at that time, all of them made use of a comparison of HLA typing results of all participants and definition of the consensus based on the most frequently reported assignment. Therefore, we set up a high-resolution EPT for the HLA loci HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB1, and -DPB1 as a collaboration between two laboratories: the laboratory of Leiden provided the samples, whereas the laboratory of Maastricht performed the high-resolution typing by hemizygous, group-specific Sanger sequence-based typing, providing the reference consensus typing (Voorter et al., 2014; Voorter et al., 2016). This EPT scheme is now running for the 20th year in a row. During these years, the EPT was extended to low-resolution HLA class I and II typing, high-resolution typing including HLA-DQA1 and -DPA1 and allelic resolution typing for HLA class I, the latter one being unique in this field. In this report, we evaluated the high-resolution typing results from the past 19 years.

Materials and methods

For this EPT exercise, each year, 12 DNA samples were shipped to the participants by the Department of Immunology (formerly Immunohematology and Blood Transfusion) of the Leiden University Medical Center (LUMC). The amount of DNA was approximately 20 μg in a concentration of 100 ng/μL. The shipment was scheduled at the end of May, whereas the results had to be submitted before the 1st of October, giving the participants at least 4 months to collect their results. An introduction letter stating the rules of evaluation of results was sent together with the samples and predetermined forms to fill in the results obtained. Both letter and forms were also sent by e-mail, to fill in digitally and send back by mail. In this introduction letter, it was indicated that the typing analysis must be performed using an IPD-IMGT/HLA database that has been released not more than 1 year prior to the shipment of the samples (in accordance with the EFI standards) and that the database used (release number) must be reported.

The reference high-resolution typing was performed by the Department of Transplantation Immunology of the Maastricht University Medical Center (MUMC+) from 2004 till 2019 with the in-house method of hemizygous, group-specific Sanger sequencing (Voorter et al., 2014; Voorter et al., 2016) and from 2019 on by next-generation sequencing (NGS) using the AllType FASTplex kit (One Lambda, Life Technologies, Carlsbad, California, United States) in combination with sequencing on Illumina MiSeq. In case of phasing or other problems with the latter method, the previous hemizygous Sanger sequencing method was used in addition to resolve any ambiguities.

In 2004, the EPT started with high-resolution typing of the HLA loci A, B, C, DRB1, DRB3/4/5, DQB1, and DPB1. In 2007, the locus DQA1 and in 2021, the locus DPA1 were added to this EPT exercise. On request of several participants also, low-resolution typing was supported and evaluated since 2016 and allelic resolution typing for the HLA class I loci A, B, and C. For this allelic resolution typing, restrictions were set to the part of the gene that had to be analyzed as a minimum to comply with this EPT.

Evaluation of the results was carried out at the Maastricht laboratory, comparing the submitted typing of the participants with the reference HLA typing. The rule for high-resolution typing as described in the EFI standards was taken into account. From the beginning, it was strictly followed that all genotype ambiguities (i.e., ambiguities within exons 2 and 3 for class I and within exon 2 for class II) were counted as an error (error 1). In addition, not excluding the possible null alleles present within the indicated high-resolution typing result was counted as a mistake from 2010 for class II and from 2011 for class I (error 2). In the evaluation letters of 2009 and 2010, this was clearly stated with, as an example, DRB4*01:03 and A*03:01 that will both be counted as an error if the potential null alleles (DRB4*01:03:01:02N, A*03:01:01:02N) were not excluded. Other results that were counted as an error were (error 3) reporting a typing that is different from the consensus; this could be reporting an extra allele not present in the consensus, missing an allele that is present in the consensus, or reporting an allele different from the consensus and reporting an allele twice, whereas the allele was detected only once (no family results are present for these samples), and (error 4) reporting only a one-field result instead of two and the usage of incorrect nomenclature by the laboratory.

After the evaluation, an overview of the results of all participating laboratories including the reference result was provided to the participants, together with a certificate for each laboratory, clearly stating the number of samples performed for each HLA locus, the number of correct and incorrect samples, and the percentage of concordance with the reference result.

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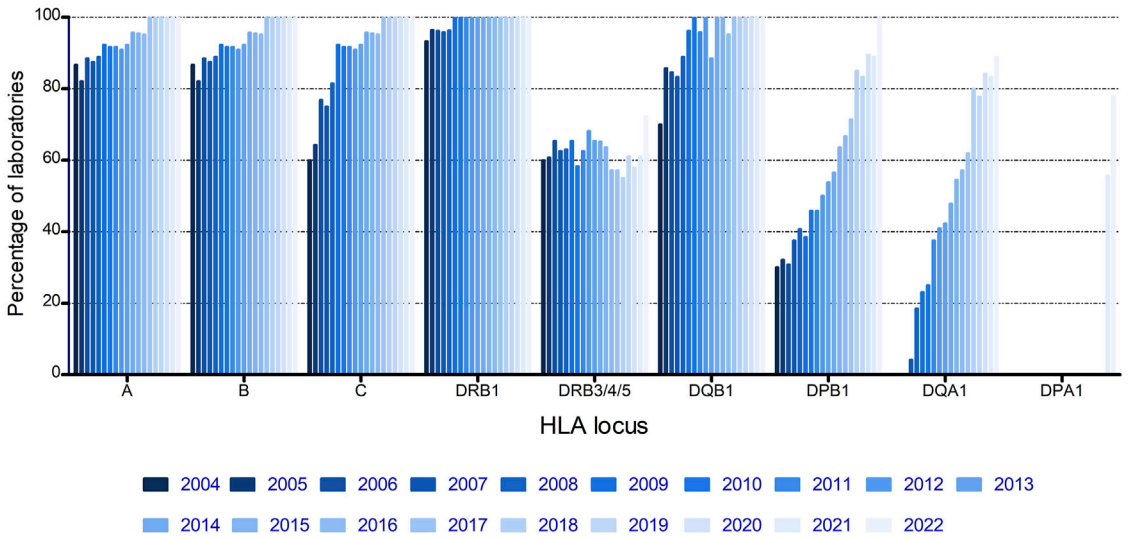

FIGURE 1
Percentage of laboratories submitting results for the HLA loci during the years.

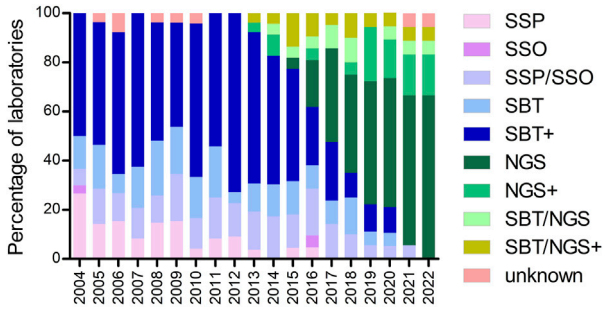

FIGURE 2
Percentage of laboratories using the methods indicated for high-resolution HLA typing during the years. Footnote: SBT+ = SBT/SSP; SBT/SSO; SBT/SSP/SSO; SBT/RLB; SBT/SSP/RLB. NGS+ = NGS/SSP; NGS/SSO; NGS/qPCR. SBT/NGS+ = SBT/NGS/SSP; SBT/NGS/SSO; SBT/NGS/SSP/SSO.

Results

During the past 19 years, the number of participating laboratories to this international high-resolution EPT has been fluctuating between 18 and 30, always outnumbering the minimum required number of 10 as demanded by the EFI standards for EPT providers (vs. 7.3, standard 7.1; EFI standards for EPT providers available at: https://efi-web.org/fileadmin/Efi_web/Committees/EPT/EFI_EPT_Standards_for_Providers_v7-3_approved_April_2021.pdf. Accessed August 23, 2023). The participating laboratories were located in 12 different countries: Austria (two), Belgium (five), Denmark (two), France (two), Germany (twelve), Greece (one), Ireland (one), the Netherlands (five), Romania (one), Slovenia (one), Sweden (three), and Turkey (one).

From the start of the EPT, most laboratories submitted results for HLA-A, -B, and -DRB1, the loci that were thought to be most important at that time, and with DRB1 being mandatory for unrelated stem cell transplantation (Figure 1). Both HLA-C and -DQB1 showed a fast increase with >90% of laboratories submitting results for these loci from 2009 onwards. DPB1 and DQA1 showed a more gradual increase, whereas DRB3/4/5 showed no increase at all, with a steady 55%–70% of laboratories submitting results for these loci throughout the complete period of evaluation (Figure 1).

Evaluation of the methods used for high-resolution HLA typing by different laboratories demonstrates that SBT in combination with other techniques has been the prevalent method in the period from 2004 to 2016 (Figure 2). After 2016, NGS either alone or in combination with other techniques was the method of choice for the majority of the laboratories. Moreover, SSP and the combination of SSP/SSO gradually disappeared throughout the evaluation period (Figure 2).

The percentage of correctly typed samples per HLA locus and per year was calculated and is displayed in Table 1. Although the percentage of incorrectly typed samples was >20% for several loci in the initial stage of the EPT scheme, for all loci, a clear learning curve was present, resulting in >98% correctly typed samples since 2017. Notable decreases in correctly typed samples were observed in 2010 for HLA-DRB3/4/5 and in 2011 for HLA-A and -B. In those years, we started to count not excluding the null alleles as an error. In 2010, all typing results of DRB4*01:03, without mentioning that the null allele (DRB4*01:03:01:02N) was excluded, were counted as a mistake, and in 2011, the same was made for the class I results. The presence of A*01:01, A*03:01, and B*15:01, for which the null alleles (A*01:01:01:02N, A*03:01:01:02N, and B*15:01:01:02N, respectively) had to be excluded, but were not, resulted in a clear decrease in correctly typed samples in 2011, although overall 73% of the laboratories correctly excluded these null alleles.

To investigate the type of errors that occurred for different loci, we analyzed the errors per year and per locus (Figures 3A–H). From these figures, it is clear that the increase in the incorrect results in 2011 for HLA-A and -B and in 2010 for HLA-DRB3/4/5 was due to

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TABLE 1 Percentage of samples correctly typed by all laboratories together.

Year	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DRB3/4/5	HLA-DQB1	HLA-DPB1	HLA-DQA1
2004	87.7	90.4	77.2	94.4	78.7	94.8	79.6	
2005	95.3	96.4	91.5	98.8	99	97.9	91.7	
2006	96.4	95.6	92.9	97.7	94.1	97	86	
2007	99.1	99.6	97	99.2	98.8	96.8	90.9	100
2008	97.5	97.8	98.4	97.7	98.4	99.6	97.5	96.9
2009	99.6	99.3	96.7	99	96.5	98.3	98.3	96.9
2010	93.8	94.6	94.6	99.3	72.2	98.9	92.9	98.6
2011	84.9	91.9	96.1	98.2	89.1	98.9	95.2	98
2012	99.6	99.1	98.3	99.6	94.8	99.6	98.4	100
2013	94.7	94.3	99.6	99.7	92.9	98.5	96.3	92.1
2014	98.5	100	99.6	100	98.9	99.6	99.4	94.6
2015	96.8	100	99.2	96.9	100	98.9	97.6	100
2016	100	99.1	99.1	99.6	94.7	100	98.1	100
2017	98.4	98.8	99.2	99.6	99.2	98.7	98.3	99.3
2018	98.7	99.6	100	99.6	98.5	99.6	99	99
2019	99.1	99.1	98.1	100	98.5	99.1	99.4	100
2020	99.1	98.2	98.7	99.6	97.7	99.1	99.5	99.5
2021	99.5	99.5	100	99.5	99.2	99.5	99.5	100
2022	99.5	100	98.9	99.5	98.6	100	99	100

not excluding the null alleles (error 2). An error 4 mistake at the beginning of the EPT was due to reporting of low-resolution results instead of high resolution by the participants. In later years, error 4 was primarily due to usage of incorrect nomenclature, the majority concerned incorrect reporting of allele ambiguities (i.e., ambiguities outside exons 2 and 3 for class I and outside exon 2 for class II) (see Figure 3 legends and footnote for details). A clear learning curve for resolving genotype ambiguities (error 1) could be observed for all loci. The percentage of error 3, typing an allele incorrect, is fluctuating for all the loci throughout the years, in general varying between 0% and 4% but with some outliers. For the B locus, the outlier in 2013 was due to the allele B*07:161N that was present in one of the samples, which was mistyped as B*07:02 by 55% of the laboratories. For DRB3/4/5, not only there was a 20% increase in not excluding the null alleles in 2010 but also incorrect allele typing was exceeding 4% because in one sample, the DRB4*01:03:01:02N allele was present, and this was mistyped as DRB4*01:03 by 46% of the participants. The main problem with DPB1 typing in the earlier years, up to 2010, was the incorrect reporting of an allele, without taking alleles into account with different exon 1 or exon 3 sequences that were not analyzed in the laboratory. For example, laboratories were reporting DPB1*03:01, whereas the correct allele typing was DPB1*104:01, which has an exon 2 sequence identical to DPB1*03:01, but a difference in exon 3. Reporting DPB1*03:01/104:01 would have been correct, but reporting only DPB1*03:01 is incorrect. The allele DPB1*104:01 (previously known as DPB1*0502) was included for the first time in the IPD-IMGT/HLA database of January 2005 and, therefore, had to be reported in the EPT since 2006. The outlier for DQA1 in 2014 was due to mistyping DQA1*01:01 as 01:05 and DQA1*03:03 as 03:02.

Another interesting feature to check was how many laboratories had no mistakes in any of the submitted loci and whether the EFI criteria for EPT were fulfilled (i.e., >90% of results are correct). Figure 4 shows three different lines: line A shows the percentage of laboratories without any mistake in all submitted loci, line B shows the percentage of laboratories that fulfilled the EFI EPT criteria for all submitted loci, and line C shows the percentage of all submitted loci that fulfilled the EFI criteria. The steep drop in 2013 is again due to the mistyping of B*07:161N; although 45% of laboratories typed this B allele correct, some of these laboratories had another incorrect typing for a different locus. From 2014, >95% of all submitted loci fulfilled the EFI EPT criteria. In the last 2 years, all laboratories fulfilled the EFI EPT criteria for all submitted loci. Since the percentage of laboratories with 100% correct is varying here between 60% and 70%, this indicates that 30%–40% of laboratories have incorrect typing results, but maximum 1 per locus.

Discussion

Due to the global use of HLA test results obtained by Histocompatibility and Immunogenetics (H&I) laboratories all over the world and the high clinical importance for transplantation outcome, there are strict rules for quality and accreditation

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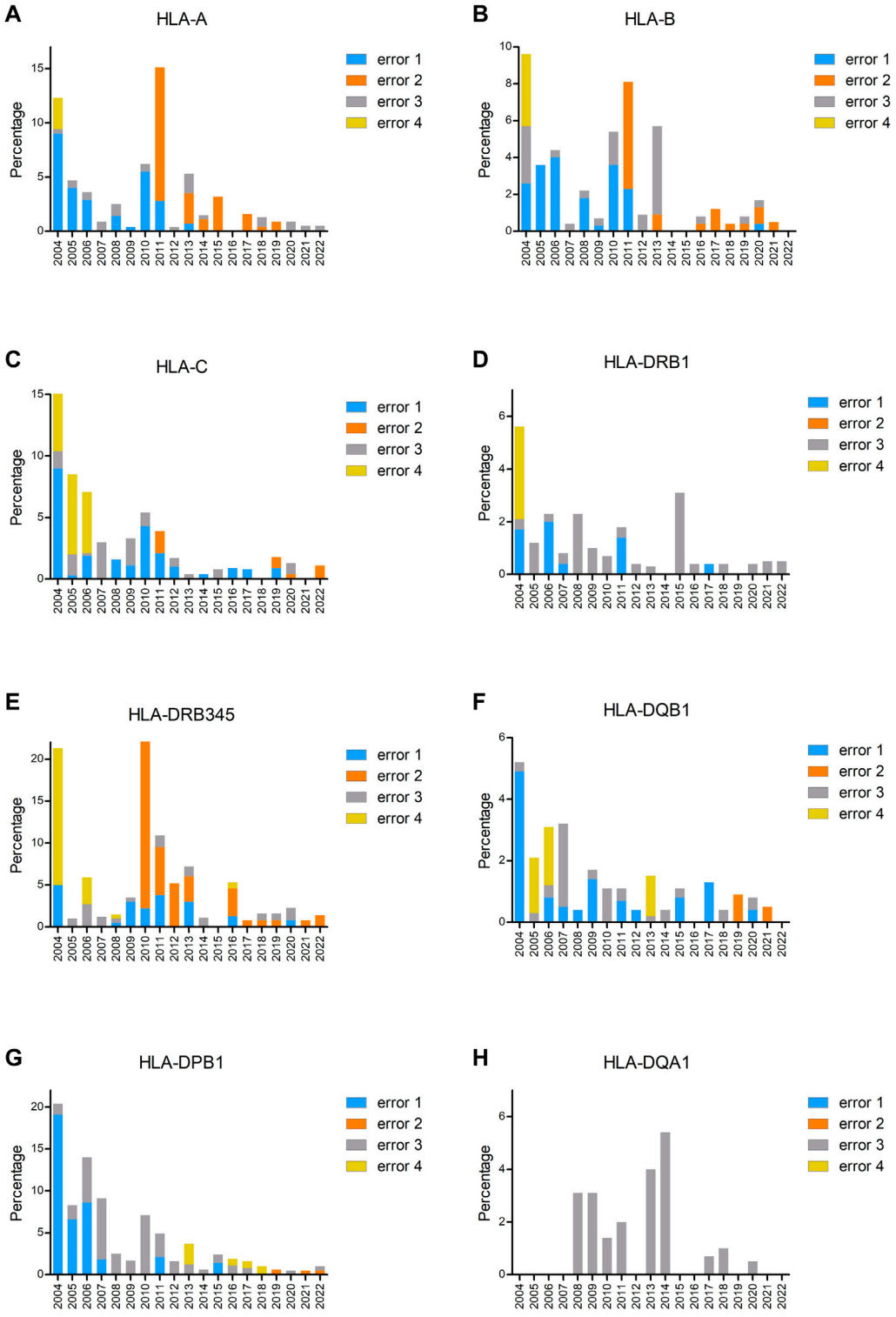

FIGURE 3
Percentage of incorrectly typed samples split up into the different types of errors for HLA-A **(A)**, HLA-B **(B)**, HLA-C **(C)**, HLA-DRB1 **(D)**, HLA-DRB3/4/5 **(E)**, HLA-DQB1 **(F)**, HLA-DPB1 **(G)**, and HLA-DQA1 **(H)**. Error 1 is genotype ambiguity (i.e., ambiguities resulting from polymorphisms located within exons 2 and 3 for HLA class I loci and exon 2 for HLA class II loci), error 2 is null alleles not excluded[A], error 3 is incorrect allele type (i.e., allele different from consensus and missing allele or extra allele, the latter one also in case of a homozygous result), and error 4 is others (i.e., one-field typing till 2006, incorrect nomenclature from 2008)[B]. Footnote: [A]Error 2 not excluding the null alleles concerns the following null alleles: HLA-A: *01:01:01:02N, *03:01:01:02N, *26:01:01:03N, *31:01:02:03N. HLA-B: *15:01:01:02N. HLA-C: *03:03:01:50N, *03:03:01:52N, *07:02:01:17N, *15:02:01:08N. HLA-DRB4: *01:03:01:02N, *01:03:01:13N. HLA-DPB1: *04:01:01:24N. HLA-DQB1: *03:276N. [B]Error 4 incorrect nomenclature (from 2008 on) concerns the following: DRB3/4/5: *03:01 instead of 3*03:01; 4*01:03N instead of 4*01:03:01:02N. DQB1: *06:03/39, *06:04/41 instead of *06:03/41, *06:04/39. DPB1: *04:01/105:01, *04:02/126:01 instead of *04:01/126:01, *04:02/105:01; *02:01/105:01, *04:02/416:01 instead of *02:01/416:01, *04:02/105:01.

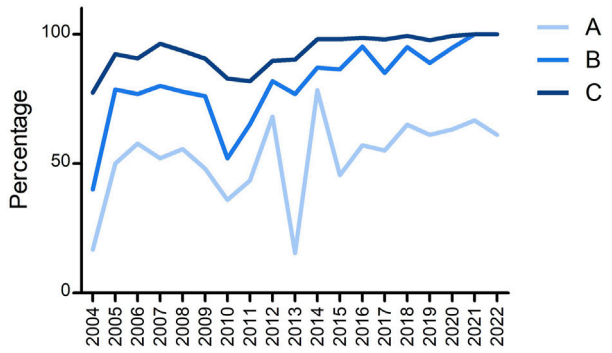

FIGURE 4
Overview of the percentage of (A) laboratories that were 100% correct for all submitted loci, (B) laboratories that fulfilled the EFI criteria for all submitted loci, and (C) submitted loci that fulfilled the EFI criteria.

requirements for these laboratories. One way of testing whether a laboratory meets these high-quality standards is by performing an external quality control for all techniques in use by the laboratory. Within Europe, there are different EPT schemes for the different EFI accreditation categories for H&I laboratories, listed on the EFI website (EFI overview of EPT providers available at: https://efi-web.org/fileadmin/Efi_web/Committees/EPT/Overview_EPT_provider_registration_January2021.pdf. Accessed August 23, 2023).

This report concerns one of the high-resolution HLA typing EPT schemes that is available within Europe. For EFI accreditation, a minimum of 10 samples is required for each molecular method and each locus. For this EPT, we decided to send 12 samples each year to anticipate any problems that might occur with a specific sample, like contamination, loss of material, or otherwise. Furthermore, in contrast to many other EPTs for HLA typing that use a consensus rule, this EPT makes use of a reference HLA typing performed by the laboratory of Maastricht. There are two major advantages of this approach, one being that the samples can always be graded, which might not be the case when the consensus rule is used and the consensus threshold is not reached. The second advantage is that if the majority of laboratories use a certain method/kit that results in an incorrect typing for a certain sample, the consensus will be the incorrect result, applying unnecessary pressure on the participants who do have the correct typing result. This was, for example, the case shortly after the discovery of the allele DPB1*104:01, which had an exon 2 sequence identical to DPB1*03:01, but a difference in exon 3. One year after this discovery, the majority of laboratories still typed DPB1*03:01, whereas the reference typing was DPB1*104:01, determined by sequencing exon 3. A potential disadvantage could be if the reference laboratory has an incorrect result, but one assumes that the reference laboratory will start evaluating their sequencing results, when all participants have a discordant result.

The different aspects that are tested with this EPT are correct sample tracking, correct HLA typing with the method used, and correct reporting of the HLA typing results. With this EPT, we are not testing the complete specimen handling, since we are providing DNA samples, whereas in most laboratories, blood or buccal swab samples will be obtained. This made the exclusion of null alleles extra challenging for the participating laboratories, since all typing information had to be obtained using a molecular method and could not be performed using serological methods, showing the presence of the molecule on the cell surface. Furthermore, from 2011, it was counted as incorrect if the null alleles were not excluded, creating awareness among the participants that even a two-field HLA result might not be a truly high-resolution typing, since there can be a null allele amongst the two-field typing results (e.g., A*03:01 can be A*03:01:01:02N). There is one exception to this rule, in case a sample is typed by full length sequencing as DQB1*03:01 homozygous, the presence of DQB1*03:276N as the second allele cannot be excluded. DQB1*03:01 and 03:276N have identical sequences from exon 2, the null allele is missing exon 1 and part of intron 1. However, for patient care, this is not a problem, since there is a DQB1*03:01 present as a molecule on the cell surface, whether the second allele is expressed or not.

The main goal of HLA typing EPTs in general is to assess the reproducibility, accuracy, and reliability of the HLA typing performed by each participating center, and as such, it contributes to high-quality level in the participating laboratories. The evaluation of all results including an error analysis is very useful for the participants to improve their diagnostic work. Therefore, we always provided an overview of all results with the errors highlighted and an explanation of the errors in detail in the accompanying letter with the intention to raise specific awareness about the presence of null alleles, the presence of genotype ambiguities, and the correct reporting of allele ambiguities.

Lin et al. (2022) described the EPT results with a national proficiency scheme from China performing HLA typing by NGS by 24 laboratories in 2021. Comparing their results with ours revealed an overall concordance rate for all HLA alleles typed of 99.2% in the China EPT and 99.5% in ours for both 2021 and 2022. The percentage of Chinese laboratories that were 100% correct for all alleles reported was 54.1%, whereas it was 66.7% in 2021, and 61.1% in 2022 in ours (see Figure 4). Apparently, although the overall performance is rather high in both EPTs, there are still a substantial number of laboratories with one or more errors in the EPT results.

Since EFI standards (vs. 6.3, effective Okt 2015; EFI standards available at: https://efi-web.org/committees/standards-committee. Accessed August 23, 2023) implemented the definition of allelic resolution, we started to assess allelic resolution typing for HLA class I in 2016, but with a very limited number of participants. According to the EFI EPT standards for providers (vs. 7.3, standard 7.2; EFI standards for EPT providers available at: https://efi-web.org/fileadmin/Efi_web/Committees/EPT/EFI_EPT_Standards_for_Providers_v7-3_approved_April_2021.pdf. Accessed August 23, 2023), it should be regarded as an EPT workshop, since the number of participants is below 10. Nevertheless, as far as the authors are aware, this is the only EPT workshop on HLA typing at the allelic resolution level. In the EFI overview of EPT providers (EFI overview of EPT providers available at: https://efi-web.org/fileadmin/Efi_web/Committees/EPT/Overview_EPT_provider_registration_January2021.pdf. Accessed August 23, 2023.) from January 2021, UK NEQAS has also indicated to provide EPT for HLA allelic resolution typing, but according to their website (UK NEQAS for H&I schemes available at: https://ukneqashandi.org.uk/schemes/. Accessed August 23, 2023), they provide HLA typing to the second and third field resolution, whereas allelic resolution is defined as a four-field typing result. The difficulty with this four-field typing is that with each update of the database, the sequence at the 5′ and/or 3’ UTR sites might have been extended, with differences between alleles located in these newly submitted sequences. No exact boundaries have

been set for the HLA genes, and therefore, it is not known to what extend the gene must be sequenced to enable allelic resolution typing. For our EPT workshop on allelic resolution, we kept the boundaries of −50 and +500, implying that all ambiguities located within 50 nucleotides ahead of the start codon and 500 nucleotides after the stop codon must be resolved. These boundaries enable ongoing allelic resolution without continuous adaptation of primers, kits, and/or procedures. The participants fulfilled EFI rules for all class I loci for this allelic resolution EPT in the last 5 years.

Further improvement of this EPT will be a web-based submission in the near future in collaboration with the Eurotransplant Reference Laboratory, with upcoming possibilities to send results automatically from the laboratory information system after authorization of the results to minimize any clerical errors and to resemble the normal working flow of the laboratories.

In summary, the results of our high-resolution HLA typing EPT showed that the quality of high-resolution typing of the participants has been improved over the years, enabling EFI accreditation for all submitted loci. To keep this high quality standard, the continuing participation in external proficiency testing is of utmost importance and mandatory to be granted accreditation by the specific H&I accreditation programs of EFI and ASHI.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials, further inquiries can be directed to the corresponding author.

Author contributions

CV: writing–original draft. LG: writing–original draft. SH: writing–original draft. LW: writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Advancing precision in histocompatibility and immunogenetics: a comprehensive review of the UCLA exchange program

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Precise typing of human leukocyte antigens (HLA) is crucial for clinical hematopoietic stem cell and solid organ transplantations, transfusion medicine, HLA-related disease association, and drug hypersensitivity analysis. The UCLA Cell Exchange program has played a vital role in providing educational and proficiency testing surveys to HLA laboratories worldwide for the past 5 decades. This article highlights the significant contribution of the UCLA Cell and DNA Exchange Programs in advancing HLA antibody testing, genotyping, crossmatches, and, more recently, virtual crossmatches. Additionally, we discuss future directions of the UCLA Cell Exchange program to support histocompatibility testing to adapt to the fast-evolving field of immunotherapy, tolerance and xenotransplantation.

KEYWORDS

HLA, KIR, MICA, virtual crossmatch, physical crossmatch, HLA antibody

Introduction

The UCLA HLA Clinical Laboratory Exchange Program has played a pivotal role in advancing the field of Human Leukocyte Antigen (HLA) testing and transplantation diagnostics for nearly 50 years. Established in 1974 with a vision to foster international collaboration, to exchange knowledge, and to advance transplant immunology research, the program has evolved over the years from one that initially provided challenges just for serological-based tests to one that now sends analytes appropriate for molecular-based assays. Many of the cell lines cultured and sent out by the program have been used as reference cells in the International Histocompatibility Workshops. In 1993, the original aim of the program of internal laboratory quality control and standardization of HLA antigen-level typing reagents was modified to incorporate proficiency testing (PT) for accreditation of HLA allele-level typing through the HLA DNA exchange. Subsequently, we now provide PT for several of our surveys including HLA serum antibody identification, cytotoxicity and flow cytometry crossmatch tests, and Killer-cell Immunoglobulin-like Receptors (KIR) genes. Online reporting was added in 2011, in which labs submit results though a website: https://cell-exch.ctrl.ucla.edu/register/. Exchange results are sent to all participating centers, summarized at ASHI annual meetings, and periodically published as milestone reports

(Loon et al., 1987; Lau et al., 1989; Lau et al., 1990; Park et al., 1994; Locke et al., 2023). Participants of UCLA Exchange Program include laboratories from 30 countries worldwide. In this article, we present a fresh account of the impact of the UCLA HLA Clinical Laboratory Exchange Program in the field of Histocompatibility and Immunogenetics.

HLA typing

Since the first discovery of the HLA-A2 antigen in 1958, the field of Immunogenetics and Histocompatibility has seen tremendous advancement (Dausset, 1958). Today, the field plays important roles in disease association, transfusion support, solid organ transplantation (Bosanquet et al., 2015; Wehmeier et al., 2017; Zhang et al., 2018; Frischknecht et al., 2022) and hematopoietic stem cell transplantation (HCT) (Morishima et al., 2015). Advancements were slow at first due the extreme polymorphic and multi-locus nature of the HLA system, and the lack of standardization among serological typing reagents. Establishing reproducible relationships between antigens and functional polymorphisms by a single laboratory was problematic. Consequently, International Histocompatibility Workshops were organized by WHO bringing together a handful of established laboratories (designated as "reference" labs) who exchanged their reagents, methodologies and results with all participating workshop laboratories with the goal of standardizing results. The UCLA International Cell Exchange was launched in 1974 to continue this collaboration with 85 participating laboratories, which then expanded to more than 290 participates worldwide by 1997 (Figure 1). During its early years, the UCLA exchange program focused on building relationships with international HLA laboratories. These exchanges led to significant breakthroughs, enhancing the accuracy and efficiency of HLA typing (Lau et al., 1989; Lau et al., 1990). For example, the percent agreement in detection of the antigen HLA-A23 among laboratories went from 30% to 97% over the 23-year period (1974–1997).

One of the major challenges of serological HLA typing was the need of viable cells. The UCLA Cell Exchange was made possible with the breakthrough of shipping viable lymphocytes at room temperature worldwide and later on provide a reliable shipping method (Park and Terasaki, 1974). As efforts were made to standardize specificities for international consensus, the exchanges identified instances of duplicate names for the same specificity and identical names assigned to different specificities. Examples of variants which were extensively studied in previous cell exchanges and received formal designations by the WHO Nomenclature Committee are: A9.3 (A*24:03), BN21 (B*40:05), B5.35 (B*51:02), 5Y/8w58/BSNA (B*78:01, B*78:02), numerous B15 variants (B*15:08, B*15:11, B*15:12, B*15:15), and DT (B*81:01). The Cell Exchange data has provided vital correlation between alleles and serologic names in many cases, such as establishing B*15:18 as B71 and Cw*17:01 as a short Cw7.

In 1987, HLA class II typing was initiated with first shipment of two lymphoblastoid cell lines. By 1990, DNA typing, including restriction fragment length polymorphism (RFLP), PCR-sequence-specific primer (PCR-SSP) and PCR-sequence-specific oligonucleotide probe (PCR-SSOP) was implemented in the HLA DNA exchange survey, which, as was mentioned above, became the first graded proficiency test service offered by the UCLA Exchange program in 1993. Subsequently, PCR-sequencing-based typing (PCR-SBT) was added 1996. In 2016, the exchange program

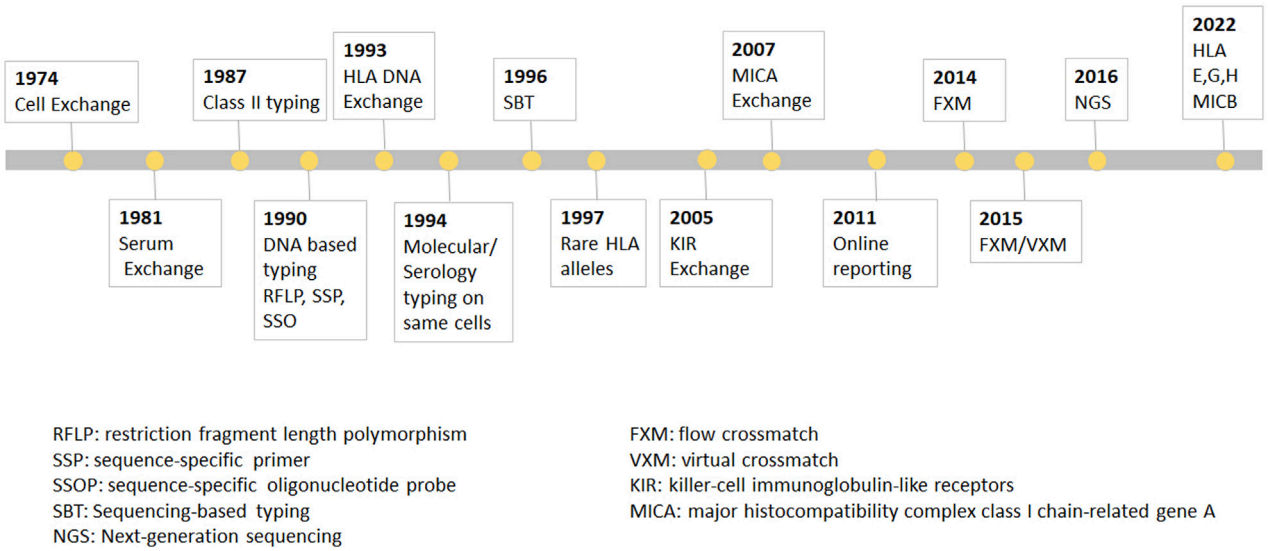

FIGURE 1
The UCLA cell Exchange Program. The UCLA cell Exchange Program was first established in 1974 followed by serum exchange in 1981. In 1987, HLA class II typing was initiated with first shipment of two lymphoblastoid cell lines. By 1990, DNA typing, including restriction fragment length polymorphism (RFLP), PCR-sequence-specific primer (PCR-SSP) and PCR-sequence-specific oligonucleotide probe (PCR-SSOP) was implemented in the HLA DNA exchange survey, which became the first graded proficiency test service offered by the UCLA Exchange program in 1993. Subsequently, PCR-sequencing-based typing (PCR-SBT) was added 1996. In 2016, the exchange program embraced cutting-edge methodologies for the next-generation sequencing (NGS) HLA tying.

embraced cutting-edge methodologies for the next-generation sequencing (NGS) HLA typing. The Cell Exchange has also been graded since 2018 and many laboratories have used the exchange to satisfy their proficiency test requirement for clinical laboratory accreditation. In 1994, the Cell Exchange initiated offering the same cells for molecular typing as for serologic typing for Class I. The data from the parallel typing was instrumental in identifying serologic equivalents for Class I and Class II alleles that previously had little or no serologic information.

In contradistinction to other histocompatibility PT programs (e.g., ASHI and CAP), the UCLA International Cell Exchange has often focused on uncommon HLA alleles. This provides laboratories and companies to validate and improve their HLA typing techniques and reagents (Supplementary Table S1). Since 1994, a total of 120 alleles (21 HLA-A locus, 49 -B locus, 7 -C locus, 1 DQB1, 38 DRB1 and 4 DRB3/4/5) typed in exchange cells were considered uncommon, as listed in Supplementary Table S1. Often, these challenging HLA types were found to include "variants," which represented new alleles that were not defined by serology. In addition, certain HLA alleles do not possess a serologically defined antigenic counterpart. As a result, it is not consistently feasible to associate a serological equivalent with each HLA allele. This information was routinely added to the HLA Dictionary. The data from the UCLA Cell Exchange has been invaluable in establishing correlations between alleles and serologic names. From 1974 to 2021, a total of 1704 cells were sent out worldwide. Among them, 14 cells were initially typed in the Cell Exchange and now serve as reference cells. The UCLA cell exchange has greatly contributed to the HLA Dictionaries (Schreuder et al., 1999; Schreuder et al., 2001; Holdsworth et al., 2009), as well the publication of Common and Well documented alleles (Cano et al., 2007).

HLA antibody detection and identification

Donor-specific alloantibody (DSA) either present at the time of transplantation or arising *de novo* posttransplant is a risk factor for antibody mediated rejection (AMR) and potentially allograft loss in solid organ transplants (Lefaucheur et al., 2023). The development of HLA antibody detection has been significantly advanced in clinical transplantation over the past decades. The initial approach for identifying anti-HLA antibodies involved the use of the complement-dependent cytotoxicity (CDC) assay, a method pioneered by Terasaki and McClelland in 1964 (Terasaki and McClelland, 1964). Over the past 50 years, prospective CDC crossmatches and flow crossmatches (FXM) have been standard practices for solid organ transplantation to detect donor-specific reactivity. For CDC crossmatch, donor lymphocytes and recipient serum are mixed with complement. The membrane attack complex forms when DSA bind to donor HLA antigens on the cell surface leading to donor cell lysis. The CDC assay is a functional test, but with a low sensitivity that only detects high titered complement fixing antibodies. The FXM introduced in early 80s significantly increased the sensitivity of the lymphocyte crossmatch test (Garovoy et al., 1983). In the mid-90s, the introduction of HLA antibody detection by flow cytometry and Luminex technology using purified HLA class I and II antigens on solid phase platforms have revolutionized the ability to detect HLA antibodies with high sensitivity and specificity. However, solid phase assays are also subject to issues with prozone (Schnaidt et al., 2011), interfering substances (Goldsmith et al., 2020) and false positive reactivities to cryptic epitopes (El-Awar et al., 2009). UCLA Serum Exchange was initiated in 1981 for HLA antibody identification. Since then, more than 1,300 well characterized reference sera have been sent out to participating laboratories for HLA antibody evaluation. The goal is to facilitate HLA laboratory to accurately detect the presence of HLA antibodies, HLA antibody specificity identification and crossmatching. For single HLA class I and class II antigen bead (SAB) testing, data collected include serum pre-treatment, median fluorescence intensity (MFI) cut-off, vendor, and reagent lot numbers. The UCLA Serum Exchange provides the concordance and discordance in HLA antibody detection across multiple laboratories as well as intra- and inter laboratory variability to participating laboratories. The exchange results recently showed that laboratories using sera pretreated with DTT or EDTA have 10%–15% less variability compared to laboratories not using serum pre-treatment (Locke et al., 2023).

The advancement of solid phase assays, particularly the SAB assay, allows laboratories to predict physical crossmatch (PXM) results with high accuracy. The American Society for Histocompatibility and Immunogenetics defines virtual crossmatch (VXM) as an assessment of immunologic compatibility based on patient's alloantibody profile compared with donor's histocompatibility antigens. However, the VXM is performed based on the agreement between the transplant centers with their supporting HLA laboratories and it is highly variable from center to center. To address this gap, in 2015, UCLA Virtual Crossmatch Exchange was launched. It is a two-phase challenge that assesses laboratory consensus in HLA antibody detection, VXM and FXM reporting. The UCLA VXM Exchange is the first program designed to provide laboratories with the opportunity to compare VXM with an actual FXM. In Phase I, participating laboratories are sent two sera for HLA Class I and Class II antibody testing by SAB and VXM with the complete HLA typing of 3 virtual donors (HLA A, B, C, DRB1/3/4/5, DQA1, DQB1, DPA1, and DPB1) for a total of six VXM challenges. Each VXM challenge is given a fictional clinical vignette including if the patient is a primary or regraft recipient based on prior transplantation. In Phase II, laboratories that are part of the program receive four recipient sera samples and lymphocytes from two donors. These samples are used for SAB testing and FXM. An interesting aspect of the UCLA VXM Exchange Survey is that, in Phase II, one recipient-donor pair is sent as a blinded sample to the participating laboratory. This pair was originally included in the VXM survey during Phase I and is now used for FXM testing. This setup enables a comparison between FXM and VXM (Figure 2). By October 2023, 58 donor blood samples and 116 well defined HLA reference sera were sent to participating laboratories to peform HLA antibody testing, flow crossmatch (FXM) and VXM since consisting 232 T/B cell FXM pairs and 18 T/B cell VXM pairs. Despite the fact that participating laboratories used different standard operating procedures (SOP) and reagents from different manufacturers, approximately 80% concordance between the VXM predictions and the physical FXM was achieved in the presence of HLA DSA. Significant variability was observed in sera with 1) very high titer antibodies

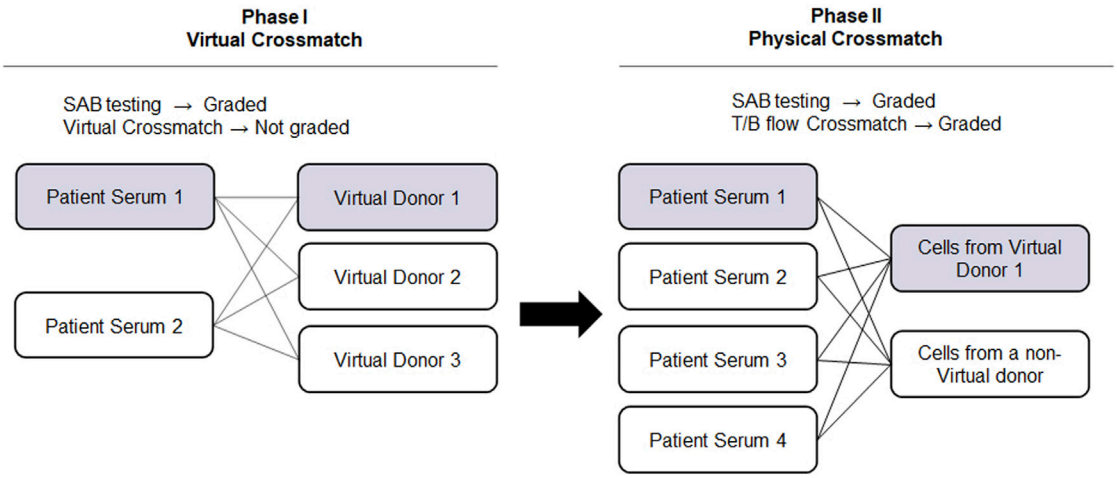

FIGURE 2
UCLA Virtual Crossmatch Exchange Survey. UCLA VXM Exchange consist of two Phases. In Phase I, two serum samples and three virtual donors are provided to the laboratories to perform SAB tests and 6 VXM. In Phase II, four recipient serum samples and lymphocytes from two donors are provided to perform SAB tests and 8 FXM. In Phase II, one recipient-donor pair will be selected from the VXM of Phase I, therefore a direct comparison between FXM and VXM can be achieved. VXM, virtual crossmatch; SAB, single antigen bead test; FXM, flow cytometry crossmatch; MFI, median fluorescence intensity.

that exit prozone effect; 2) weak-to-moderate DSA, particularly in the presence of multiple weak DSAs; and 3) DSA against lowly expressed antigens. The results were recently summarized and reported in Transplantation (Locke et al., 2023). With the increasing use the VXM, standarization and continuous learning via exchange surveys will provide better understanding and quality controls for VXM to improve accuracy across all centers.

KIR gene typing

The effects of natural killer (NK) cell alloreactivity on disease relapse and transplant-related mortality following allogeneic stem cell transplantation have been recognized over the past decades (Jennifer Zhang, 2022). Killer immunoglobin-like receptors (KIRs), which recognize HLA class I molecules, are the key receptors in regulating NK cell functions. In 2005, the International KIR Exchange was integrated within the framework of the International Cell Exchange. This was the first program to offer reference DNA samples for KIR genotyping. As background, KIR genes are organized in a complex of loci (~150–200 kb) in chromosome 19q13.4 (Wilson et al., 2000). The human KIR family consists of 15 KIR genes (*KIR2DL1-4*, *KIR2DL5A*, *KIR2DL5B*, *KIR3DL1-3*, *KIR2DS1-5*, and *KIR3DS1*) and the two pseudogenes (*KIR2DP1*, and *KIR3DP1*). KIR genes are inherited in haplotypes: A haplotype, which consists of nine genes (*3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2*) and B haplotypes, which has variable gene content (*2DS1*, *2DS2*, *2DS3*, *2DS5*, *2DL2*, *2DL5*, and *3DS1*) and more activating KIRs. In addition, four framework genes divide the *KIR* haplotypes into centromeric (*KIR3DL3* to *KIR3DP1*) and telomeric (*KIR2DL4* to *KIR3DL2*) regions. Since established in 2004, 248 DNA samples have been shipped with 56 unique haplotypes (Supplementary Table S2). The KIR gene complex is extremely diverse due to allelic polymorphism and gene copy-number variation. Moreover, KIR genes display substantial sequence homology, with a high degree of similarity ranging from 85% to 98% between alleles from any two genes (Roe et al., 2020). This similarity can lead to recombinations and deletions within these genes. These unique features make KIR typing a highly complicated process. Comprehensive analysis of all results using SSP, SSO, real-time PCR and NGS greatly improves the accuracy of the results and the understanding of the KIR haplotypes. For example, *KIR2DS2* has a strong linkage disequilibrium with *KIR2DL2* (Moesta and Parham, 2012). However, exchange sample KIR#231 carries *KIR2DS2* in the CenB haplotype but misses *KIR2DL2*, *KIR2DL5B* and *KIR2DS3* genes as a result of recombination. This recombination also resulted in–*KIR2DS2*005* —a hybrid allele sharing the first six exons with *KIR2DS2* and the exons 7 to 9 (cytoplasmic regions) of *KIR2DS3*. *KIR2DS2*005* has been reported to present in 1.2% of Caucasoids (Ordonez et al., 2011). On the contrary, in sample #150, *KIR2DL2* is present in the absence of *KIR2DS2*. This haplotype (*KIR2DL1- 2DL2-2DL4-2DL5B-3DL1-3DL2-3DL3-2DS4FULL-2DS5-2DP1-3DP1*$_{DEL}$) is exclusively reported in African American population at a frequency of 3.5% (Middleton and Gonzelez, 2010). Among all the samples have been sent, the highest error rate (5 out of 242 samples) was found in *KIR2DS3* with controversial results on the presence or absent of the gene. There are 71 KIR2DS3alleles been documented with 2 null alleles (*2DS3*003:01:01N* and *2DS3*003:01:02N*). *KIR2DS3* can be present on either a Cen-B or Tel-B haplotype. *KIR2DS3*003N* is identical to *KIR2DS3*002* except for a nucleotide change in exon 5 that results in a premature termination. If the primers or probes do not cover exon 5, the *KIR2DS3*003N* will be mistyped as *KIR2DS3*002*. The prevalence of *KIR2DS3*003N* in the Caucasian population has been documented at 0.8% (Luo et al., 2007). 2/246 of samples (#0019 and #0045) did not reach consensus for KIR2DS4 typing due to the inability to distinguish the presence of null alleles. Currently, 41 KIR2DS4 alleles exist and 22 of them are

defined based on null alleles. It is reported that KIR2DS4 exists in two versions: one with the full-length sequence (**001*, **016*, **017*, **018*, **020*) and the other with a deletion of 22 bp in exon 5 (**003*, **004*, **006*, **007*, **008*, **009*, **010*, **012*, **013*, **014*, **015*). This deletion leads to a frame shift causing a stop codon in exon 7 which truncates the soluble KIR2DS4 protein. However, no typing errors of *KIR2DS4* have been reported since 2007. Next, *KIR3DL1/S1* gene locus contains reciprocal genes either encoding the inhibitory receptor *KIR3DL1* or the activating receptor *KIR3DS1*. *KIR3DS1* shares >95% homology with *KIR3DL1* in their extracellular domains, yet they have different ligand binding profiles. Unlike *KIR3DL1*, which displays a wide range of polymorphism with a total of 189 alleles, *KIR3DS1* shows a relatively restricted diversity, with 91 alleles having been identified. In the sample KIR#229 *KIR3DL1* typing did not reach consensus with 68% laboratories reported presence while 32% laboratories reported absence of the gene. Since *KIR3DS1* and *KIR3DL1* have identical sequences in exon 3, typing methods purely focusing on exon 3 typing will cause false positive results on the presence of the *KIR3DL1*. Currently, the majority of laboratories use SSP and SSO KIR typing methods. Employing NGS for full-length characterization of KIR genes would undoubtedly enhance typing resolution, but it would also reveal previously undiscovered alleles and haplotypes, advancing the field.

MICA gene typing

MHC class I chain-related gene A (MICA) is a non-conventional MHC-encoded class I molecule located in the HLA complex. Over 500 *MICA* alleles have been reported to date (https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/). Since its discovery (Bahram et al., 1994; Zhang et al., 2011; Carapito et al., 2022), multiple reports have shown the involvement of MICA in solid organ transplantation (Zou et al., 2007). February 2007, a pilot study for *MICA* genotyping was initiated by sending samples to a select number of laboratories and was expanded to all laboratories a year later. It currently serves as the only proficiency testing program for MICA genotyping, providing an opportunity for laboratories around the world to compare results from different typing methods and to identify new MICA alleles. Since established in 2007, 204 DNA samples have been shipped, including two novel MICA alleles: MICA*018new and MICA*041new. MICA nucleotide variations are mainly located in exons 2, 3, and 4, relating to 3 extracellular domains. MICA exon 5 encodes the transmembrane region (TM) of the MICA protein. It contains the trinucleotide repeat microsatellite polymorphism (GCT)n with eight alleles encoding a variable number of alanine residues: A4, A5, A6, A7, A8, A9, A10, and A5.1. The A5.1 allele contains an extra guanine (G) insertion after 5 GCT repeats, which causes a frameshift leading to a premature stop codon, resulting in a shorter and more easily cleaved protein from the cell surface. 7/204 samples manifested ambiguous results, either because of polymorphism in exons the number of the GCT repeats. For example, the ambiguous result between *MICA*007/MICA*026* is due to the number of the GCT repeats, which *MICA*007* has 4 GCT repeats compared to 6 GCT repeats in *MICA*026*. Similarly, ambiguity among *MICA*002/*020/*055* is due the number of GCT repeats. *MICA*002* has 9 GCT repeats, *MICA*020* has 10 GCT repeats, while *MICA*055* has 8 GCT repeats. Another common ambiguous involves *MICA*009:01* and *MICA*049:01*. *MICA*049:01* differs from *MICA*009* only at codon 333 in exon 6 of the cytoplasmic domain by a single nucleotide substitution (ACG- > ATG), which results in an amino acid substitution from threonine to methionine. Continuing exchange programs play a crucial role in collecting essential data that enables the comparison of typing methods and their outcomes across various laboratories, thereby enhancing typing accuracy.

Future directions

The International Cell Exchange has a long standing history of service and contributions to the field of Histocompatibility and Immunogenetics. With the rapid and continued advancement of technical innovations over the past 50 years, the UCLA International Cell and DNA Exchange Programs endeavor to design, develop and provide up-to-date surveys reviewed by experts in the field to achieve technical and diagnostic relevance.

HLA is the most polymorphic gene complex in the human genome, Wehmeier et al. demonstrated that current SAB panels encompass approximately 98.5% of HLA eplets, yet there is still a lack of representation for HLA alleles within minority populations (Wehmeier et al., 2020). The use of extended SAB antibody detection panels will be important for increasing the precision of HLA antibody detection and improve the accuracy of the VXM, particularly in highly sensitized patients (Zavyalova et al., 2021). Serum samples displaying prozone phenomenon, as well as, sera from patients treated with immune suppressive/immunomodulatory drugs (e.g., rituximab), could be selected as challenges for use in educational surveys. Despite HLA DSA, patients still may lose their grafts due to antibodies directed against non-HLA antigens expressed on the donor endothelium (Zhang and Reed, 2016; Butler et al., 2020)There is a growing need for assays that can identify non-HLA antibodies and their impact on graft injury in context of solid organ transplantation. A future direction for the field will be focusing on the concordance and proficiency in the detection of non-HLA antibodies.

Allorecognition is mediated by T and B lymphocytes, which are responsible for the cellular and humoral mediated immunity, respectively. T and B lymphocytes are activated via the recognition of non-self epitopes by their antigen receptor complexes at their cell surfaces, known as the B cell receptor (BCR) and the T cell receptor (TCR). Recent advances in HLA sequencing technology allows the study of the donor-recipient incompatibility at the molecular level. Still in its infancy, the Predicted Indirectly Recognizable HLA Epitopes (PIRCHE) predicts the T cell epitopes that can be presented by HLA class II molecules to the recipient CD4 T cells (Otten et al., 2013; Lemieux et al., 2022). However, the prediction is currently limited to HLA-DR, and does not include HLA-DQ and DP antigens. Eplets are defined as clusters of polymorphic amino acids situated on the surface of HLA molecules. They serve as functional B cell epitopes, encompassing specific amino acids recognizable by anti-HLA antibodies within the larger amino acid structure comprising an HLA epitope. There are a number of tools that can be used to predict B cell epitopes, including HLAMatchmaker (Duquesnoy, 2001),

HLA epitope mismatch algorithm (HLA EMMA) (Kramer et al., 2020) and the three-dimensional electrostatic mismatch score (EMS3D) (Kim et al., 2023). Recent publications suggest that these analyses may provide improved precision in HLA matching and optimal donor selection, and lead to substantial improvements in transplant outcomes and increased graft and patient survival rates (Wiebe et al., 2019; Senev et al., 2020; Mohammadhassanzadeh et al., 2021).

A new direction in the field is Histocompatibility Testing for Swine Leukocyte Antigen (SLA). Histocompatibility testing for xenotransplantation is in its infancy. Methods to detect and define xenoantibodies to swine HLA include flow cytometry crossmatching, complement dependent lymphocytotoxicity and red blood cell agglutination (Ladowski et al., 2021). However, only a few reagents exist to characterize the specificity of the human anti-swine HLA antibodies. Hence, this is a much needed area for clinical research and translation to the clinic to achieve significant advances that will benefit the future of Xenotransplantation.

The future directions of the International UCLA Cell and DNA Exchange Programs will likely involve various aspects of these new ideas.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies involving humans because this is a clinical proficient test result summary. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from a by-product of routine care or industry. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

QZ: Data curation, Formal Analysis, Investigation, Methodology, Supervision, Writing–original draft, Writing–review and editing. AL: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing–review and editing. AA: Data curation, Methodology, Writing–review and editing. MC: Data curation, Writing–review and editing. LL: Data curation, Methodology, Writing–review and editing. BA: Data curation, Methodology, Writing–review and editing. DG: Conceptualization, Investigation, Supervision, Writing–review and editing. ER: Conceptualization, Investigation, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1352764/full#supplementary-material

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External proficiency testing exercises: challenges and opportunities

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Proficiency Testing (PT) provides the participant a certificate proving the competence and reliability of the laboratory. PT was very disputed before accreditation and certification of the laboratories performing diagnostic workout for patients. Historically, the report of the first two surveys of the Committee on Laboratories of the Medical society of the State of Pennsylvania of the year 1946 (Belk and Sunderman, 1947), revealed a for today's standards unacceptable situation. For the determination of hemoglobin (37 satisfactory results vs. 35 unsatisfactory results) and for glucose (60 satisfactory results vs. 43 unsatisfactory results respectively). Honestly, these results were not only not adequate, but they also reflected the situation at that time. Similar findings were observed for almost all disciplines. These results promoted the use of PT on a primarily voluntarily basis. For Histocompatibility and Immunogenetics (H&I) PT was introduced during the International Histocompatibility Workshops to assure that the submitted data were reliable. Both the schemes of the International Cell Exchange (Lau et al., 1992) and the Eurotransplant Scheme (Doxiadis et al., 2000; Doxiadis and Claas, 2003) were introduced to improve the reliability of the participating laboratories. Especially in the field of organ transplantation, in which organs are offered and transported from center to center or from country to country according to the organ exchange organizations, the reliability of the laboratories is an imperative (Doxiadis et al., 2000). Furthermore, in stem cell transplantation the life of the patient relies on the accurate information from the H&I laboratory. PT is an integral part of the package a laboratory performing diagnostics must fulfill. At the beginning, the samples for PT were send out *ad hoc*. The laboratories received a small piece of the spleen from an organ donor, which was used for typing. The report of Schreuder et al. (1986) show that the results were far from adequate. They showed the efficacy and reliability of the participating centers during duty hours at that time. An increase of reliability from 40% in 1977 to 91% in 1981 was reported. Furthermore, Opelz et al. (1991) in 1992 showed that 25% of the reported HLA-DR serological results were incorrect when compared to molecular typing. A significant increase of reliability and efficacy of the laboratory in H&I was needed. Molecular typing was introduced in Eurotransplant and then worldwide. Interestingly, Sunderman (1992) the pioneer of PT mentioned that even "proficiency testing had its probable beginnings in the Paleolithic age ... Neanderthal man tested his lethal stone axes for strength, weight, and serviceability before using them for the onslaught of his enemies".

In the beginning of the nineties of the past century, the schemes changed from an *ad hoc* manner to fixed dates. Exercises including the number of samples to be tested, their analyses, the way of calculation of discrepancies, the certificates, etc. were documented. This is mainly due to the PT Committee of the European Federation of Immunogenetics (EFI) and the respective Committees of the sister societies, like ASHI. The number of samples to

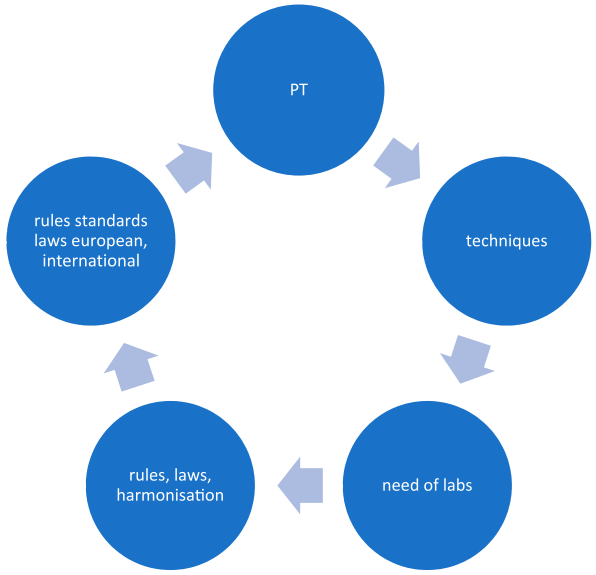

FIGURE 1
The evolution of PT.

be tested, and discrepancy calculation were institutionalized. A view how the evolution of PT occurs is presented in Figure 1. Here the influence of external factors is given. The report of the PT results changed with time. At the beginning the reports included the name of the laboratories, in an open way of reporting data. This had to be changed because of the privacy rules within the community. If this was a step in a good direction remains open. Nowadays laboratories and their services can be valued in social media. The main opportunity of the organizers is to cope with the daily work of the participants and promote flexibility. New methods as well as new ways of sample workouts ask for new schemes, but outdated methods should be stopped.

Among the evolution of PT is the digital input of the retrieved information. The digital input of the PT data and the information retrieved from PT is from the point of view of the organizers a major (evolutionary) step forward, because it helps the analysis and reduces as good as possible any clerical error from their part. However, this is not the case if the view of the participant is considered, who must enter the data mainly manually. Even if they follow the four eyes principle, meaning that two persons check the results prior to submit them, clerical errors can and will occur, following the saying: good lab but bad supervisor. This step must be automated in the future since it is one very important step for reliable PT results. In some countries the electronic patient file is or will be introduced soon, while PT organizers keep the manual entry of the results. To our opinion the entry of the data must follow the way results are reported to clinicians.

Besides Accreditation and Certification from the national bodies internal and external proficiency testing exercises are needed. Comparison of a laboratory to all the others is done in the modern times via professional European Institutions like NEQAS, (*www.ukneqas.org.uk*), Instand, (*www.instand.ev.de*), or the Eurotransplant Reference Laboratory (*www.etrl.eurotransplant.org*) and others. World-wide there are many institutions and laboratories organizing PT in a professional way on a scientific or diagnostic basis nationally or internationally, e.g., CAP, UCLA. The flow is similar for all organizers and participants. The participants apply for participations, the organizers inform the participants about the dates when the samples will be sent. The report of the results meets a deadline, and the results are sent to the organizer. A certificate of either participation or fulfil of the criteria set by the respective international or national society, e.g., APHIA, ASHI, EFI or others is issued. The certificate can be issued for every send out or annually.

Only when it was decided that accreditation/certification makes use of the results of PT to grant accreditation/certification, PT became mandatory. Furthermore, it received a status of "a must" for diagnostic purposes in H&I. All steps reported above led the laboratories to a diagnostic path with reliable results. PT must mimic the workflow laboratories perform their work. Furthermore, PT must be established for all diagnostic related testing performed in a laboratory. The possibility to receive accreditation without a PT is possible since the International Standard Organization (ISO) DIN ISO 15189, required for medical laboratories in diagnostics, offers the possibility to make use of intra laboratory control for the case no established PT is available. In the meantime, the different Societies follow this possibility. Within the Immunogenetical Societies (as ASHI, APHIA and EFI), in the past, the mixed lymphocyte culture assay and the T cell precursor assays could not be accredited because no PT was present or could be established. Laboratory comparisons are not specified in more detail, they are intended to test or compare what is required for practical application and are therefore a substitute or surrogate for an external PT. Among those assays, the monoclonal antibody immobilization of platelet antigens (MAIPA) assay is not offered by any provider, but an intra laboratory testing allows the possibility to receive an accreditation via ISO, as currently done in Germany. Other new techniques such as the Oxford Nanopore Technology (Liu, 2021), the modern absorption/elution method (Liwski et al., 2022) can be accredited without waiting long time until official PT are established. Similarly, the complement cytotoxicity assay (CDC) can be allowed in the future, since in some regions this assay is used for the final decision before transplantation, while in other regions this assay is not anymore performed.

The allocation of organs is influenced by the accuracy of the definition of HLA specific antibodies. Here, several methods can be used, with a high spectrum of sensitivity and reliability. Besides the complement dependent cytotoxicity (CDC), Luminex based assays can be used. Two different providers are available making the comparison of the results difficult (Israeli et al., 2015), furthermore, a new reader has been introduced, with an increased sensitivity, jeopardizing the comparison of the results. It is for no saying that the results of the screening for HLA specific antibodies influences allocation, transplantation, and post-transplant treatment of the patient. Currently, PTs have been established for these methods and are analyzed separately. Unacceptable HLA antigens defined as mismatches to be avoided in transplantation, are defined according to the results. To our opinion, PT organizers should

concentrate on establishing PTs for the definition of such unacceptable HLA antigens, which in their turn are used in the virtual crossmatching procedure. A method-based analysis might offer valuable data, but it reflects more the ability of the participant to perform in comparison with others (Israeli et al., 2015), than the use of the results which directly influence a medical procedure. To our opinion there is here a strong discussion need. To our opinion specific calibration of the Luminex based assays and the readers might help in the comparison of the results. PT exercises are required for the medical procedures according to ISO 15189 such as definition of unacceptable antigens. To our observation the expertise within laboratories regarding the CDC assay is steadily decreasing, especially since the introduction of molecular typing techniques. In addition, CE-labelled commercial trays with frozen cells will be not available from 2025 on so that they cannot be used for diagnostic purposes in Europe. Reestablishing local test is quite cumbersome and difficult because of the *In-Vitro* Diagnostic Regulation (https://euivdr.com).

State-of-the-art laboratories in the meantime have introduced electronic storage of data, analyses of them, and reports to the clinicians. The main reasons are not only quickness, or storage of the results but especially prevention of man-made errors. This is one of the points needed for the state-of-the-art PT in the future. The report of the results of a PT to the organizer must be done electronically using reports in an up-to-date manner, as mentioned above, mainly using open but reliable protocols. This should be discussed between the PT organizer and the participants.

PT organizers must adhere to the changing requirements. Regarding the position of PT organizers in the complex situation within the different organizations and the accreditation/certification bodies we propose a direct contact and discussion which will lead to a reduced workload of the participants while reliability will increase. Till now the results of the PT exercises are regarded as an additive information for the performance of a PT participant. To our opinion the PT exercises must be used for future policies of the transplantation procedures within the National Bodies and Societies. One important aspect are the costs which should be optimal to reduce inconsistencies and allow access to PT world-wide.

In addition, PT exercises should be used nationwide for reimbursement from Medical Insurance Bodies. Here, Organizers covering laboratories Europe- or worldwide should be accepted by the National Medical Insurance Bodies, if the respective program is certified by the Societies or Organizations, EFI, ASHI, APHIA, etc.

In summary.

- PT is an integral step in an accreditation/certification procedure and is directly bound to the reliability of laboratory results.
- Entering the results electronically via reliable protocols and report the results to the accreditation bodies is an imperative.
- Use of the PT reports for accreditation/certification purposes must be done electronically to avoid unnecessary nature resources and costs
- Besides the "usual" PT, experimental PT could, and should be organized for new methodologies. In this case the Societies are asked to provide procedures
- All methods with no established PT should not be used for certification/accreditation unless interlaboratory prove the opposite
- The PT should keep the costs as low as possible to allow access for all laboratories. It is imperative to avoid unnecessary use of nature resources.
- PT providers need to evolve and develop their PT offering with the evolution of current/new laboratory techniques.
- The opportunities offered for PT organizers and participants are mainly in the flexible use of the programs. The modern view of PT allows short term schemes in which a few participants are taking part. Instead of waiting until a method is well established, experimental PT should be offered meeting the laboratory requirements for a good laboratory practice.

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External proficiency testing for histocompatibility and immunogenetics in today and future

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The Histocompatibility and Immunogenetics laboratories provide disease association and pharmacogenetic analyses as well as the tests required for transplantation immunology and transfusion medicine. They perform Human Leukocyte Antigen (HLA) genotyping in patients/recipients and potential donor candidates for solid organ and stem cell transplants using various molecular methods, and determine mismatches. In addition, they also perform HLA antibody tests to detect anti-HLA antibodies in patients and flow cross-matches to evaluate donor-recipient compatibility. Evidence-based clinical guidelines have emphasized the importance of laboratory tests in clinical practices for a long time. Understanding the principles of Quality Control and External Quality Assurance is a fundamental requirement for the effective management of Tissue Typing laboratories. When these processes are effectively implemented, errors in routine assays for transplantation are reduced and quality is improved. In this review, the importance of Quality Assurance, Quality control and proficiency testing in Histocompatibility and Immunogenetic testing, the necessity of external proficiency testing (EPT) for accreditation, and existing and potential EPT programmes will be reviewed and evaluated in the light of the literature.

KEYWORDS

external proficiency testing, EPT, HLA, quality control, QA

Introduction

Histocompatibility and Immunogenetics Laboratories (Tissue Typing Labs) play an active role in both solid organ and hematopoetic stem cell transplants. Histocompatibility testing is essential for donor identification and risk assessment in solid organ and hematopoietic stem cell transplant. Additionally, it is useful for identifying donor specific alleles for monitoring donor specific antibodies in post-transplant patients.

Post-transplant chimerism test to evaluate engraftment especially in hematopoietic stem cell transplantation (HSCT) transplant patients and donor specific antibody monitoring in renal transplants are routine tasks. In addition, Human Leukocyte Antigen (HLA) Laboratories provide disease association and pharmacogenetic analyses as well as the tests for transfusion medicine.

In recent surveys performed with expert clinicians in Germany and United States, it was reported that 60%–70% of clinical decisions were influenced by the results of laboratory tests performed both in hospitals and in external centers (Rohr et al., 2016). Evidence based

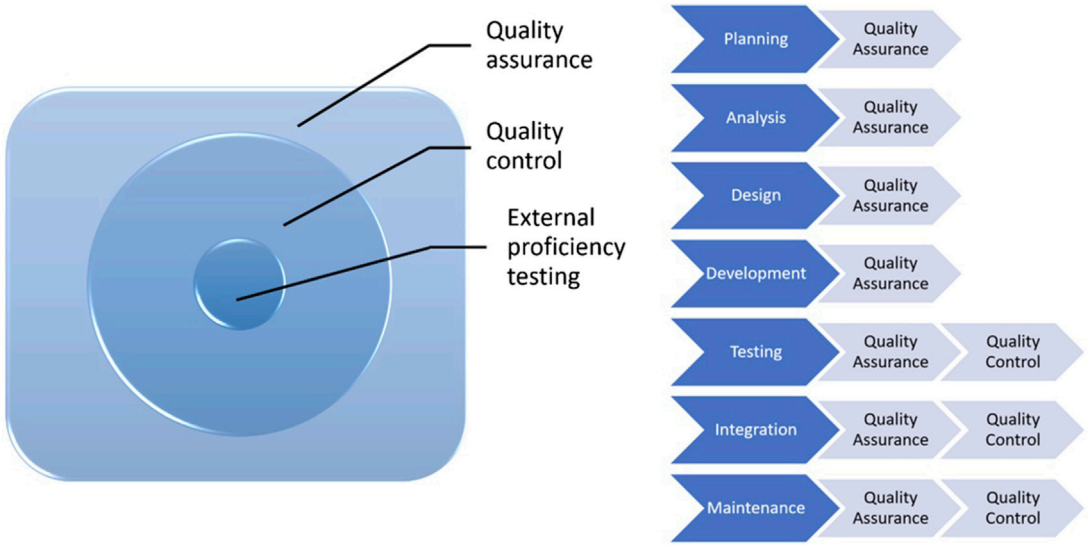

FIGURE 1
Processes from QA to EPT.

clinical guidelines indicate that at least 80% of guidelines, targeting to make a diagnosis or manage a disease, require laboratory tests (Goodman et al., 2005). Laboratories have been aware of this for a long time and try to reduce the risk of misinterpretation of the test results obtained from different laboratories (Shahangian and Cohn, 2000) However, these concepts should be based on well-designed and well-implemented Quality Control (QC) and External Quality Assurance (EQA) systems (Badrick, 2003; Badrick, 2021).

Quality assurance (QA) is a subgroup of quality management (Figure 1). It is proactive, concerns the whole process, includes a series of activities and procedures, that occur during operations and help in providing a high quality analysis, and prevents errors. All health institutions should establish QA policies for laboratories to meet these standards for each analysis. It should be kept in mind that quality control is a part of quality assurance.

QC involves establishment of a quality standard or specifications for each aspect of a test procedure, specification of how the test procedure complies with quality standards and taking the necessary corrective measures to bring up the procedures to standard. It is an active and team-wide process that identifies the errors related to all outputs during the procedure and after the procedure.

Internal Quality Control tests have been designed to control if a test or procedure will produce the same result in case of in-laboratory variations or when performed by varying technicians.

External Quality Control (EQC) is defined as an evaluation study performed by an exteral provider using samples with known or unknown content or concentration with the objective of providing or improving the reliability of laboratory test results. EQC programmes are conducted by independent institutions and comparatively evaluate the performances of test results and reports of laboratories. With EQC programmes, laboratories' performances are compared with the performances of other laboratories and evaluated on an international scale.

Demonstrating compliance of Tissue Typing laboratories with good practices in providing clinical transplantation services has gained importance, and there are many legal and regulatory requirements targeting to provide appropriate review and documentation of services. Therefore, laboratories should provide assurance related to the quality of the services they give in line with regulatory objectives (Harmer et al., 2018).

The increase in the number of laboratories and methods in years proved the necessity of meeting a high standard for the results reported by different laboratories. In this context, standardization studies and survey programmes were established many years ago.

In a study which summarized the studies, in which HLA standardization by way of international cell exchange was performed, tissue typing performed using a blind design in 468 people in a 12-year period was examined. The number of participant laboratories was reported to be increased from 85 in the beginning to 285. It has been emphasized that these standardization studies help to standardize typing reactives of tissue typing laboratories globally, to determine new specificities and to evaluate the status of improvement in tissue typing in renal transplant practices (Loon et al., 1987) The Histocompatibility Survey Programmes was organized in 1982 by American Society for Histocompatibility and Immunogenetics (ASHI) and College of American Pathologists (CAP) as a joint project to evaluate laboratory performance in HLA typing, lymphotoxicity crossmatch and antibody analysis (Marrari and Duquesnoy, 1994).

The risk of post-transplant complications is reduced by way of detailed analysis of the patient's anti HLA profile and appropriate donor-recipient matching. Precise characterization of alloantibodies in sensitized patients and complete HLA typing at the allelic level are mandatory at the time of transplantation (Morath et al., 2012). Furthermore, knowledge of HLA sensitivities and identification of

TABLE 1 EPT provider and EFI regions (based on data from European Federation of Immunogenetics, 2023).

EFI region	Regions	EPT
Region 2	Blux	• Eurotransplant (ET), Leiden • High Resolution EPT, Maastricht
Region 3	United Kingdom and Ireland	• United Kingdom NEQAS for H&I, Pontyclun • United Kingdom NEQAS for Leucocyte Immunophenotyping, Sheffield
Region 4	Germany	• INSTAND e.V., Düsseldorf • DZA, Munich
Region 5	Central Europe	• CET, Vienna • HLA Proficiency Testing for Central and East Europe, Wroclaw • HLA, Prague
Regions 6 + 11	France + Switzerland	• LNRH, Geneva • SFHI, Hôpital Saint-Louis, APHP, Paris
Region 7	Italy	• IT EPT, Rome • EPT Milan
Region 8	Balkans + Israel	• Sofia (Balkan External Proficiency Testing FCXM, CDCXM, PRA) • Istanbul (Balkan External Proficiency Testing FCXM, CDCXM, HLA)
Regions 9 + 10	Iberia	• GECLID-SEI, Valladolid
Other Regions	outside Europe	• UCLA International DNA Exchange, Los Angeles

EFI, european federation for immunogenetics; EPT, external proficiency testing; HLA, human leukocyte antigen; FCXM, flow cytometry crossmatch; CDCXM, Complement-dependent Microcytotoxicity Crossmatch; PRA, panel reactive antibody.

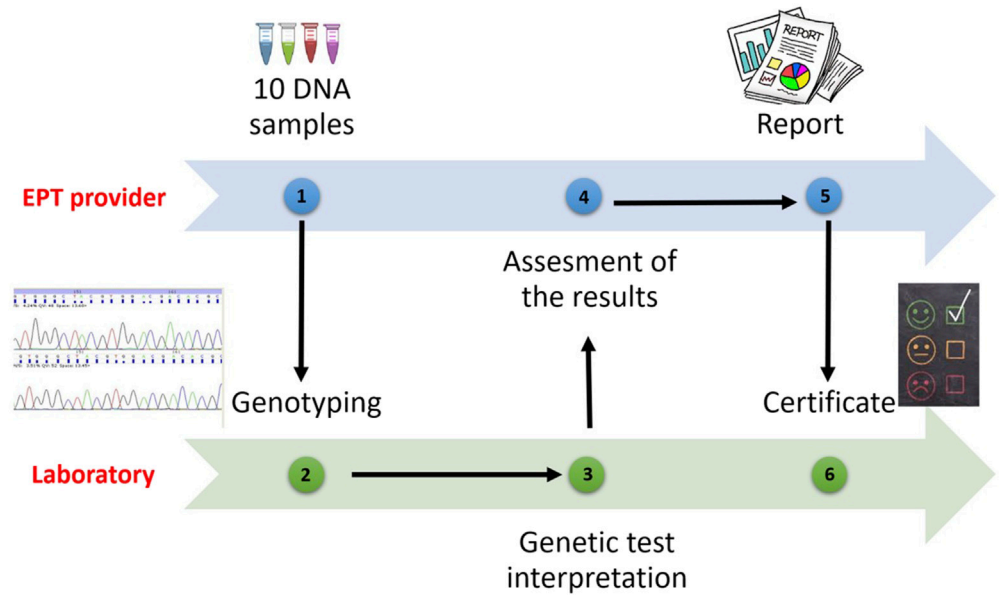

FIGURE 2
EPT Programmes process.

anti-HLA antibodies among potential kidney recipients is essential to control graft loss (Duquesnoy et al., 2016).

Appropriate techniques should be used to increase the reliability of the tests performed for histocompatibility which is accepted to be effective in graft loss, and a meticulous quality control system should be implemented. With this objective, various EPT programmes were established globally (Table 1). Successful performance in EPT was accepted as a prerequisite for accreditation of a laboratory (Doxiadis et al., 2000; Bogunia-Kubik et al., 2006; Bogunia-Kubik and Lange, 2008; Bogunia-Kubik and Lange, 2010; Balza et al., 2023; European Federation for Immunogenetics, 2023).

TABLE 2 EPT methods and samples (based on data from European Federation of Immunogenetics, 2023).

Methods	Minimum number of samples for EPT per year
Serological typing	10 samples
Each low resolution DNA-based typing technique	10 samples
Each high resolution DNA-based typing technique	10 samples
Each allelic resolution DNA-based typing technique	10 samples
HPA/HNA/KIR/MICA typing	10 samples
HLA antibody detection	10 samples for HLA class I and 10 samples for HLA class II
	The same samples can be used for the detection of both classes
HLA antibody identification by CDC	10 samples
HLA antibody identification by solid phase assays	10 samples
HPA/MICA antibody detection and identification	5 samples
Crossmatching	20 tests of different donor/recipient combinations of each accredited cell
Haematopoietic chimaerism and engraftment monitoring	10 tests of different donor/recipient mixtures in the range 0%–100% excluding the reference donor and recipient samples

EPT, external proficiency testing; HLA, human leukocyte antigen; HPA, human platelet antigen; HNA, human neutrophil antigens; KIR, Killer Cell Immunoglobulin-Like Receptors; MICA, Major histocompatibility complex class I chain-related genes A; CDC, Complement-dependent Microcytotoxicity.

All laboratories applying to receive accreditation from the European Federation for Immunogenetics (EFI) or wishing to resume their accreditations, are obliged to participate in EPT programs related to laboratory practices involving the categories for which they will be accredited (HLA typing, antibody screening and detection, cross match, etc.) (European Federation for Immunogenetics, 2023) (Figure 2).

If there are no programmes specified for a certain category, the laboratory should participate in an EPT workshop or trial provided by an EPT provider or be involded in an inter-laboratory sample exchange programmes. The laboratory shall have a predetermined policy for testing EPT specimens, documenting relevant EPT programmes or workshops prospectively on an annual basis. Thus, participation in external proficiency testing workshops will give the opportunity to validate HLA typing results. It will contribute to the training of laboratories by making comparisons with other participants. It is also expected that the error rates of participating laboratories will decrease over the years.

According to the EFI standards, EPT samples should be tested and interpreted one by one or in association using the techniques used routinely for clinical samples. If the same sample is being tested for multiple accreditation categories, the results should be analyzed independent from each other. The annual minimum sample number for EPT is shown in the table (Table 2). If the same sample is being tested with multiple techniques in the same accreditation category, the laboratory should give the provider only one report, but keep the results obtained with different techniques ready for inspection (European Federation for Immunogenetics, 2023).

In recent years, experimental transplant models have shown that mechanisms other than T-lymphocyte anti-donor responses could be effective (Oberbarnscheidt et al., 2014; Dai et al., 2017). In addition, a few translational genetic association studies have showed that incompatibilities originating from interaction of two different genomes could lead to complex immune responses in solid organ transplants and non-HLA antibodies could also be effective in rejection (Sankaran et al., 1999; Grinyó et al., 2008; Menon et al., 2015; Zhang et al., 2021; Jethwani et al., 2022).

Numerous studies in solid organ transplantation provide evidence that high levels of donor-derived cell-free DNA (DD-cfDNA) correlate with clinically relevant endpoints. Increased DD-cfDNA has been associated with episodes of graft injury and rejection. Efforts are ongoing to further improve sensitivity and specificity. DD-cfDNA could be used as a biomarker in the near future as it is quantitative and has the potential to be cost-effective. Although there are EPT programs on cell-free DNA in different fields, EPT programs in transplantation are not yet available (Samoila et al., 2020; Edwards et al., 2022)

Multi-center studies have shown that specification of non-HLA loci with long-term allograft results and identification of non-HLA antibodies in patients might enable sensitive matching of organs in patients who have multiple potential donors. Performance of routine tests in HLA laboratories addressing these parameters will undoubtedly contribute to successful organ transplantation to a great extent. When EPT programmes are examined, it is observed that there is currently no study dedicated to these analyses.

Conclusion

The role of the Histocompatibility and Immunogenetic laboratories in stem cell and organ transplants has expanded to provide HLA antibody detection and tracking for selection of compatible donors and monitoring desensitization therapies.

In the future, they will request new programmes in accordance with clinical needs in order to perform new routine tests successfully

in parallel with accreditation categories and to evaluate and improve laboratory performance.

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Analysis of immunogenetics interlaboratory comparisons' success rates. External quality assurance system of the Spanish Society for Immunology GECLID-SEI

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Background: For many years, transplantation outcomes were uncertain and not hopeful, until histocompatibility testing spread. Common criteria for histocompatibility assays and communications' improvement allowed an efficient organ sharing system. The possibility of organ exchanges is closely linked to the importance of interlaboratory comparisons for histocompatibility and immunogenetics methods. The external proficiency testing (EPT) systems are the most powerful quality assurance tools. They help achieve harmonization of analyses, set a standard of performance, and a common interpretation.

Methods: The external quality assurance program for diagnostic immunology laboratories (Garantía Externa de Calidad para Laboratorios de Inmunología Diagnóstica, GECLID) program nowadays runs 13 external quality assurance (EQA) histocompatibility and immunogenetics schemes, with the first of them from 2011 to date: serological and molecular: low- and high-resolution human leukocyte antigen (HLA), human platelet antigen (HPA), and killer inhibitory receptor (KIR) typing(HLA-B*27, HLA-B*57:01, and coeliac disease-related HLA), cell-dependent cytotoxicity (CDC) and flow cytometry (FC) crossmatches, anti-HLA and anti-HPA antibodies, and chimerism.

Results: A total of 85 laboratories participated in this subprogram in the last 12 years reporting over 1.69 M results: 1.46 M for anti-HLA and anti-HPA antibodies, 203.810 molecular typing data (HLA, HPA, and KIR genes), 2.372 for chimerism analyses, and 39.352 for crossmatches. Based on the European Federation for Immunogenetics (EFI) standards for EPT providers, the mean success rates ranged from 99.2% for molecular typing schemes and antibodies and 94.8% for chimerism, was 96.7% regarding crossmatches, and was 98.9% in serological typing. In 2022, 61.3% of the participating laboratories

successfully passed every HLA EQA scheme, although 87.9% annual reports were satisfactory. Most penalties were due to nomenclature errors or misreporting of the risk associated to HLA and disease.

Conclusion: This EQA confirms the reliability of HLA and immunogenetics assays in routine care. There is little heterogeneity of results of different assays used by participating laboratories, even when in-house assays are used. Reliability of test results is reasonably granted.

KEYWORDS

human leukocyte antigen, immunogenetics, proficiency testing, molecular genetics, crossmatch, chimerism, killer inhibitory receptor, quality assurance

1 Introduction

The Spanish Society for Immunology founded in 1975 is a professional non-profit organization in Spain and is dedicated to promote and support excellence in research, scholarship, and clinical practice in immunology. The external quality assurance (EQA) program for diagnostic immunology laboratories (Garantía Externa de Calidad para Laboratorios de Inmunología Diagnóstica, GECLID) program was first run in 2011 (Martín et al., 2011), and more than 130 laboratories all around the world take their interlaboratory comparisons for proficiency testing nowadays.

The European Federation for Immunogenetics (EFI) in their standards for external proficiency testing (EPT) provides the criteria required for organizations providing EPT services to conform to the accreditation procedures of the EFI (European Federation for Immunogenetics, 2021). The standard comprises recommendations on the organization of an EQA scheme, the requirements for EQA test samples and their evaluation guidelines for an EQA scheme. They propose a scoring system and the mandatory contents of EQA reports, as well as information to participants. ISO 17043 (International Organization for Standardization, 2023) states the general interlaboratory comparison rules to ensure their quality, and ISO 15328 (International Organization for Standardization, 2022) gives advice on how to analyze reported data and calculate assigned values to parameters in EPT exercises.

Proficiency testing provides information on the accuracy of reported results, and subsequently on the clinical performance and on the correct interpretation of results. This is especially relevant regarding laboratory-developed or laboratory-modified tests (LDTs). The EU *in vitro* Diagnostic Device Regulation (European Parliament, 2017) requires appropriateness evaluation for LDTs. They can be evaluated by comparison with published biological variation estimations, and by participation in PT schemes or by comparison with their published results (Comins et al., 2023)

There are plenty of methods and variants for human leukocyte antigen (HLA) testing, but laboratories have to yield homogeneous results to grant patient's safety. Methodological differences together with environmental, personal, or eventual issues can affect laboratories' performance. Pre- and post-analytical issues should be noticed as well. The last aim of a clinical laboratory is to ascertain that their results are true in spite of all handicaps, and this is where EQA plays a key role.

EPT programs are committed to design useful schemes resembling routine work as much as possible. Samples should be representative of the variety of clinical ones; participants should perform their analysis as they usually do and report parameters following their practice. Once all results have been recorded, the assigned values are determined by consensus of participants and ratified by the steering committee. Global reports containing every single lab results (properly anonymized) together with private individual summaries are published. Appeals are as well evaluated by the steering committee.

In the present work, participation, success rates, and mistakes of the last 12 EPT years are described and analyzed to identify relevant factors that might influence error sources or changes in performance over time, within analysis families or by scheme.

2 Materials and methods

The GECLID program nowadays runs 13 external quality assurance (EQA) HLA and immunogenetics schemes (the first of them from 2011 to date): serological and molecular typing (low and high resolution HLA, human platelet antigen (HPA), killer inhibitory receptor (KIR), HLA-B*27, HLA-B*57:01 and coeliac disease-related HLA), anti-HLA and anti-HPA antibodies, crossmatches and chimerism (Table 1). Molecular typing schemes, antibodies, and crossmatch schemes are ISO 17043 accredited by our national accreditation body (ENAC, Entidad Nacional de Acreditación) from 2023 summer. Schemes are grouped in five analytical families: serology, molecular typing, antibodies, chimerism, and crossmatches.

Values are assigned according to EFI rules (European Federation for Immunogenetics, 2021). Success rates are calculated as the total number of error-free results over valid data (not inconclusive or unevaluable items).

The principal objective of the program is to assess the performance of participants by comparing results from the full range of analytical methods presently used in the immunology laboratories of histocompatibility and immunogenetics. Participation is not restricted by the method; laboratories should perform their protocols as they routinely do.

Eighty-five laboratories have participated in any scheme of the subprogram in the last 12 years (Table 2). All schemes but serological typing have increased their participation over time. Spanish laboratories are the most frequent ones, but Portuguese, Czech, Serbian, and Chilean ones have been participating to date as well. Laboratories from Israel and Kazakhstan have formerly participated.

Informed consents were obtained from any individuals included in this study. Samples within the histocompatibility GECLID subprogram are always of human origin, with minimal handling, so that they are as similar as possible to the usual practice of diagnostic laboratories. The methods employed in the preparation and distribution of samples have

TABLE 1 Interlaboratory comparisons provided by the GECLID program and short names.

Scheme	Short name	Analytical family
Serological typing of HLA class I	**SER**	**Serology**
HLA-B27	**B27**	**Molecular typing**
HLA*B57:01	**B57**	**Molecular typing**
Coeliac disease–related HLA	**CD**	**Molecular typing**
Cytotoxicity crossmatch	**XM**	**Crossmatch**
Cytometry crossmatch	**XMFC**	**Crossmatch**
Detection and specificity of anti-HLA antibodies	**ALO**	**Antibodies**
Low-resolution HLA DNA typing	**LOW**	**Molecular typing**
High-resolution HLA DNA typing	**HI**	**Molecular typing**
Chimerism	**CHM**	**Chimerism**
KIR typing	**KIR**	**Molecular typing**
HPA typing	**HPA**	**Molecular typing**
Anti-HPA antibodies	**AHPA**	**Antibodies**

TABLE 2 Groups of schemes by descending mean number of participants in the last year.

		2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Molecular typing	B27	25	25	24	29	29	33	38	37	37	40	41	47
	CD	—	17	20	24	26	33	37	39	42	42	44	45
	LOW	33	33	34	36	35	37	38	38	39	42	43	44
	B57	—	12	14	18	23	26	29	31	33	36	40	40
	HI	28	28	28	28	28	30	31	29	34	33	33	34
	KIR	—	—	—	—	—	16	21	22	23	22	22	25
	HPA	—	—	—	—	—	—	5	4	6	6	7	6
Crossmatch	XM	23	22	22	22	22	23	24	22	23	24	25	27
	XMFC	—	7	10	8	9	10	13	14	15	16	18	17
Antibodies	ALO	27	29	29	30	31	30	34	33	33	34	36	41
	AHPA	—	—	—	—	—	—	—	—	5	6	7	7
Chimerism	CHM	—	—	7	10	10	12	14	14	18	15	16	16
Serology	SER	18	18	16	16	12	11	12	11	9	9	6	5
	Total	40	43	45	50	54	58	64	64	64	65	71	75

been shown to be suitable to ensure uniformity and stability in the conditions listed. Samples are peripheral blood (buffy coats) and sera. All the manipulation is performed under sterile conditions. Most of the samples included in this subprogram come from the Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Also, patients' samples can be obtained from different blood banks and clinical services of the Spanish territory in accordance with current legislation on the subject. The GECLID program has the approval of both the Scientific Committee of the Biobank and the Valladolid Este Ethics Committee.

All samples are distributed in suitable packaging, in accordance with the International Air Transport Association (IATA) standard and are accompanied by documentation (pdf documents sent by e-mail for the sake of a better sustainability) with at least the sample number and lot, additives and/or preservatives included, and analytical tests expected to be carried out on each sample by participant laboratories. All samples included in the schemes have a documented traceability system: origin, serology, staff involved in handling and packaging, date of extraction and shipping, etc. GECLID-SEI keeps a part of each batch of samples for at least 1 year, so that laboratories can acquire on request extra volumes and can reanalyze them, if necessary.

Laboratories' results can be reported exclusively by means of the web results forms available at https://www.geclid.es/ according to

TABLE 3 Data reported by year and scheme.

	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	Total
ALO	14,681	66,200	68,425	69,220	68,699	74,893	73,101	130,260	170,496	187,605	210,847	329,698	1,464,125
LOW	3,161	3,659	3,683	3,482	4,002	3,257	5,508	6,636	7,980	6,840	7,980	6,660	62,848
HI	2,915	3,503	3,403	3,387	3,674	3,234	4,869	6,488	7,705	6,380	7,364	6,160	59,082
XM	1,973	1,112	1,078	1,133	1,077	1,168	1,838	3,382	3,780	3,612	4,368	2,352	26,873
KIR	0	0	0	0	2,383	0	3,541	4,016	4,200	3,538	4,500	3,833	26,011
XMFC	0	831	787	684	892	582	1,551	1,442	1,568	1,230	1,792	1,120	12,479
CD	0	756	690	445	914	345	1,665	1,408	1,600	1,170	1,760	1,095	11,848
SER	1,250	773	976	1,026	579	1,199	296	541	560	640	400	760	9,000
B57	0	942	320	200	846	170	1,050	797	1,240	775	1,540	724	8,604
B27	199	282	278	187	297	191	705	497	750	370	890	380	5,026
HPA	0	0	0	0	0	0	698	600	600	400	720	500	3,518
CHM	0	0	168	152	110	190	281	320	290	260	321	280	2,372
AHPA	0	0	0	0	0	0	0	0	166	175	139	240	720

the guidelines in the HLA prospectus. The prospectuses from every EPT year are publicly available at the website. The analysis of frequencies and robust mean calculation from all results reported by the participants [an algorithm described in Annex C of ISO 13528 (International Organization for Standardization, 2022)] are performed to assign correct values to each parameter.

3 Results

Along these 12 years, 1.69 M results have been recorded, analyzed, and evaluated: 1.46 M for anti-HLA and anti-HPA antibodies, 203.810 molecular typing data (HLA, HPA, and KIR genes), 2.372 for chimerism analyses, and 39.352 for both cell-dependent cytotoxicity (CDC) and flow cytometry (FC) crossmatches (Table 3).

Scheme design, that includes evaluation of results, is based on the EFI Standards for EPT providers (European Federation for Immunogenetics, 2019).

The mean success rates are above 99.0% for low-resolution HLA and KIR molecular typing, anti-HLA and anti-HPA antibodies detection and identification, and HLA-B*57:01 determination (Table 4). PCR-SSO (sequence-specific oligotyping) was the preferred method for HLA-B*57:01, coeliac disease–related HLA, low-resolution DNA HLA typing, KIR typing, and HPA typing, whereas PCR-SBT (sequencing-based typing) was the elected method for high-resolution HLA DNA typing until 2020, when it became the second elected choice. Next-generation sequencing (NGS) has been preferred from then to date. Sequence-specific primer PCR (PCR-SSP) alone or together with PCR-SSO was the second most used method for low-resolution KIR, HLA, and HPA typing. Real-time PCR (rtPCR) was the second most used molecular method for related disease HLA (B27, B*57:01, and coeliac disease-related HLA). The most frequent tests for antibodies were Luminex-based assays—single antigen regarding anti-HLA antibodies (Table 5).

The only scheme under 95% success rate (94.80%) was that of chimerism (Table 4), with most participants performing short tandem repeat (STR) analyses (Table 5) as the most performed method, followed by rtPCR alone or in combined with the STR analysis.

Within the interval: 95%–99% were serological typing, HLA-B27, HLA related to coeliac disease, chimerism, and crossmatches (Table 4). Serological typing was performed by most laboratories on peripheral blood mononuclear cells (PBMCs), while some others worked with T lymphocytes, which were magnetically isolated. Flow cytometry was the elected method for HLA-B27 until 2013, which was as frequent as molecular biology methods in 2014 (most frequently PCR-SSO, followed by rtPCR), and these were preferred from 2015 as shown in (Table 5).

All the low-resolution DNA, HLA, HPA, and KIR typing overall error rates (of 12 years) were under 1% together with anti-HLA and anti-HPA antibodies, and HLA-B27 and HLA-B*57:01 (Table 4). Up to 40.1% of KIR typing errors were due to allele variants (Supplementary Table S1). High-resolution HLA typing had a 1.1% error rate, with 60.1% of its errors attributable to improperly excluding null alleles (Supplementary Table S1). The poorest performance was that of chimerism (4.7%), where 62.2% errors were found in quantification and the second worst performance was of FC crossmatch, with a 4.22% error rate.

With regard to typing, homozygosity-reporting errors were common (Supplementary Table S1). Double writing of an allele should be used only when evidence of heterozygosity exists (EFI, 2019). Any other allele mismatch is here designated as random (wrong allele calls mostly), excluding ones originated by an improper or lacking null allele. All ambiguities that encompass a null allele must be resolved, wherever the polymorphism is located, unless it is evidenced that an expressed antigen is present on the cells (European Federation for Immunogenetics, 2019).

In the high-resolution HLA typing scheme, concordant results were accepted and therefore not penalized, regardless of whether

TABLE 4 Success rates by year and group of schemes.

		2011 (%)	2012 (%)	2013 (%)	2014 (%)	2015 (%)	2016 (%)	2017 (%)	2018 (%)	2019 (%)	2020 (%)	2021 (%)	2022 (%)	Mean (%)
Molecular typing	HPA							100.0	100.0	99.0	99.5	100.0	100.0	99.8
	KIR						98.9	99.4	99.3	99.7	99.8	99.7	99.9	99.5
	LOW	99.0	98.9	99.0	99.5	99.2	99.3	99.2	99.8	99.8	99.6	99.8	99.9	99.4
	B57		98.8	99.5	99.7	100.0	98.1	100.0	98.8	99.0	99.9	99.2	99.8	99.4
	HI	99.0	99.0	99.1	99.5	99.3	99.6	98.7	99.1	99.1	99.0	98.0	98.6	99.0
	B27	99.5	99.5	95.7	99.3	98.9	99.3	98.7	98.4	99.8	99.5	99.2	99.9	99.0
	CD		96.8	99.1	99.3	99.3	95.3	98.9	97.8	98.1	98.8	98.4	99.6	98.3
Antibodies	AHPA									100.0	98.3	99.3	100.0	99.4
	ALO	97.7	98.7	99.1	98.7	98.3	98.6	99.8	99.4	99.3	99.5	99.3	99.7	99.0
Serology	SER	96.2	96.0	98.9	99.6	99.4	99.8	99.7	99.5	99.3	99.3	99.3	100.0	98.9
Crossmatch	XM	96.2	97.2	97.4	97.5	97.3	95.0	99.2	99.3	98.3	99.6	96.6	99.2	97.7
	XMFC		94.9	94.7	97.3	95.1	94.1	95.2	94.5	95.4	97.1	95.3	97.8	95.6
Chimerism	CHM			88.2	92.1	97.0	95.8	96.4	94.6	93.8	96.6	95.7	97.8	94.8

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TABLE 5 Most performed method by year and group of schemes.

		2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Molecular typing	HPA	—	—	—	—	—	—	SSO	SSP	SSP	SSO	SSO	SSO
								40.00%	50.00%	66.67%	50.00%	57.14%	50.00%
	KIR	—	—	—	—	—	SSO	SSO	SSO	SSO	SSO	SSO	SSO
							91.67%	75.00%	73.68%	60.00%	70.00%	73.68%	59.09%
	LOW	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO
		66.67%	64.70%	66.67%	72.22%	77.14%	70.59	67.57	71.05	72.5	67.5	70.45	70.73
	B57	—	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO
			45.45%	46.15%	52.94%	47.83	41.64%	40.91%	48.28%	48.28%	51.61%	50.00%	54.55%
	HI	SBT	SBT	SBT	SBT	SBT	SBT	SBT	SBT	SBT	NGS	NGS	NGS
		33.33%	48.14%	46.87%	50%	44.44%	50.00%	51.85%	42.86%	34.38%	46.15%	69.70%	80.65%
	B27	FC	FC	FC	Mol. gen	Mol. gen	Mol. gen	Mol. gen	Mol. gen	Mol. gen	Mol. gen	Mol. gen	Mol. gen
		42.31%	56%	52%	50%	60.00%	66.67%	70.27%	66.67%	76.47%	68.42%	75.00%	76.92%
	CD	—	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO	SSO
			66.67%	68.42%	68.18%	64.00%	73.33%	82.76%	65.71%	62.16%	72.97%	70.73%	71.05%
Antibodies	AHPA	—	—	—	—	—	—	—	—	Luminex	Luminex	Luminex	Luminex
										75.00%	80.00%	80.00%	100.00%
	ALO	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)	Luminex (SA)
		85.71%	88.89%	93.33%	93.33%	89.66%	93.10%	90.91	93.94	90.91	90.91	90.91	95
Serology	SER	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs
		64.71%	68.42%	81.25%	81.25%	83.33%	80.00%	89.00%	86.00%	71.00%	71.00%	60.00%	75.00%
Crossmatch	XM	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs	PBMCs
		73.91%	77.27%	76.19%	85%	72.73%	70.00%	71.43%	76.19%	61.90%	59.09%	54.55%	51.85%
	XMFC	—	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph	Total lymph
			100%	100%	100%	100%	100%	100%	100%	92%	92%	100%	56%
Chimerism	CHM	—	—	STR	STR	STR	STR	STR	STR	STR and q/rt PCR	STR	STR	STR
				80.00%	42.86%	50.00%	60.00%	57.14%	53.85%	50%	57.14%	46.67%	53.33%

SSO, PCR-SSO (sequence-specific oligotyping); SSP, PCR-SSP (sequence-specific PCR); SBT, PCR-SBT (sequencing-based typing); NGS, PCR-NGS (next-generation sequencing); PBMCs, peripheral blood mononuclear cells; STR, short tandem repeats; q/rt, quantitative or real-time PCR.

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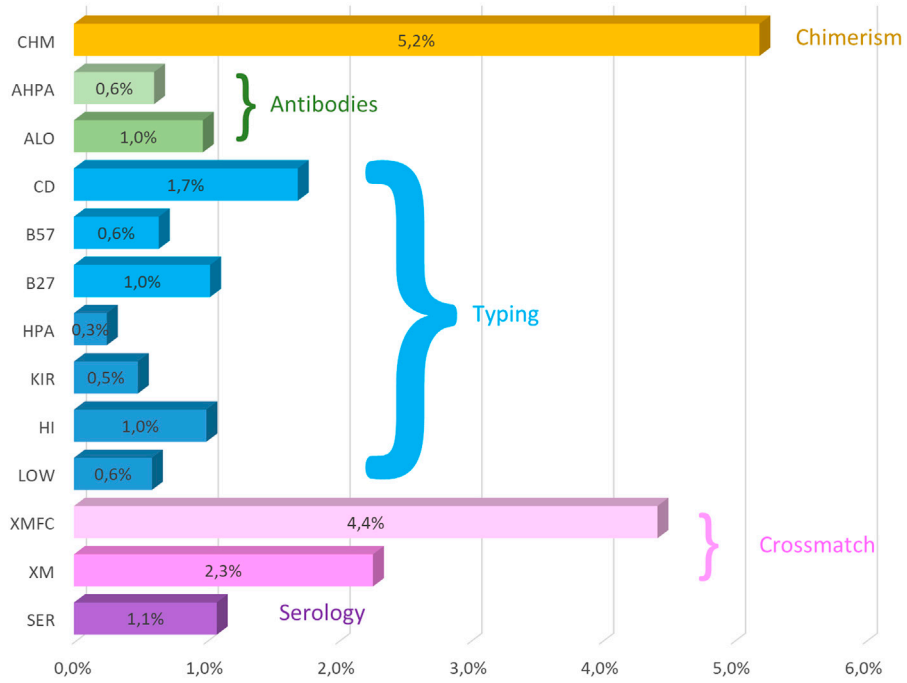

FIGURE 1
Mean (2011–2022) overall error rates of the GECLID EPT program. CHM, chimerism; AHPA, anti-HPA antibodies; ALO, anti-HLA antibodies; CD, coeliac disease–related HLA-B57, HLA-B*57:01 detection; HLA- B27, HLA-B27 status; HPA, HPA typing; KIR, KIR typing; HI, high-resolution DNA HLA typing; Low, low-resolution HLA DNA typing; XMFC, flow cytometry crossmatch; XM, CDC crossmatch; SER, HLA serological typing (O).

allelic resolution was reached or not. The success rate in our series is similar to that reported by international next-generation sequencing–based workshops (Osoegawa et al., 2019)

Crossmatches can be reported as whole results when no separation of cells is performed or as lymphocyte T (LT) or B (LB) separately. This separation was not implemented in CDC crossmatch until 2016. Crossmatch errors are very similar in both CDC and FC crossmatches (Figure 1).

In antibody schemes, both screening or detection and identification of specificities were evaluated by CDC and single antigen bead methods.

No differences in error rates could be evidenced when comparing different time periods but in the case of null alleles in high-resolution HLA DNA typing scheme from 2017 onward, when the steering committee decided not to admit generic annotations such as “every null allele excluded” and every excluded null allele had to be listed.

In 2022, 61.3% of the participating laboratories successfully passed every HLA EQA scheme they had taken, although 87.9% annual reports were satisfactory.

4 Discussion

Recent analyses of interlaboratory comparisons attribute a principal role to laboratory policies (Hicklin et al., 2023), HLA typing being the keystone for donor–recipient matching for both solid organ and hematopoietic stem cell transplantation. Many different laboratories perform HLA typing in the same country or region, and their results must meet the same standards so as to be reliable. Annual user meetings are held every single year where results are discussed and feedback, proposals, and complaints are received. A total of 12 user meetings were carried out from 2011 to 2022.

For all DNA-based EPT, the HLA typing results of the organizer are considered as correct results (European Federation for Immunogenetics, 2021). Our steering committee (integrated by five EFI-accredited laboratories) acts as the multicentric reference laboratory; however, due to the highly concordant results of participants to date, there is scarcely any need to consult them.

HLA-B*57:01 determination has high success rates in our EPT, in accordance with Meini et al. (2016), with a near absolute concordance irrespective of the method. HPA typing presented absolute concordance among participants as has been previously reported in other EPTs (Goldman et al., 2003). This is a low-complexity highly standardized test with minimum error rates, with therefore little improvement options regarding analytical performance.

Regarding high-resolution penalties, we should notice that null alleles are uncommon but not extremely rare, and they affect a significant number of unrelated donor searches regarding bone marrow transplantation (Smith et al., 2005). We occasionally observed unexpected results that were likely due to sample mix-ups, and pre-analytical or post-analytical issues rather than due to technical performance. Most typing penalties were due to nomenclature errors (null alleles not explicitly excluded) according to other EPTs reporting up to 12% nomenclature errors (Kekik Cinar et al., 2020). Automated analyses sometimes ended up in an incorrect HLA allele assignment. It is critical to

review and validate HLA genotypes. It may be especially relevant regarding highly automated systems as the ones in the NGS HLA genotyping software (Meini et al., 2016). Error rates after making it mandatory to explicitly exclude any encompassed null allele indicate that this is the main source of error. Anyway, it is undistinguishable whether the laboratory did exclude the allele or not record it.

The HLA-B27 detection mean error is very similar to that found in a survey by the College of American Pathologists with molecular methods, even as 20%–25% of our participants reported using flow cytometry, which accounts for higher error rates (Peña et al., 2023). Some HLA-B27 alleles are now known to have a stronger association with the development of ankylosing spondylitis (AS), such as HLA-B*27:05, whereas HLA-B*27:06 and HLA-B*27:09 would not be related to AS risk. Perhaps high-resolution typing and risk assessment should be considered in the midterm.

Interpretation of HLA-DQ associated to coeliac disease and its risk were also a recurrent source of penalties. A key feature of coeliac disease is its strong dependence on the presence of susceptibility alleles encoding for HLA-DQ. Some EPT (Horan et al., 2018) revealed inconsistencies with current coeliac disease–reporting guidelines for genotype and clinical interpretation of the genotype data. Evidence-based guidelines are in this case not being consistently adhered to, in the same line as in our experience. Guidelines have been discussed in several of our meetings, even those specifically addressed to this topic, but adherence is not granted.

Regarding KIR typing, a former Spanish workshop (Planelles et al., 2016) found error rates slightly higher than the ones presented here. Success rates have improved over time from the beginning of the EPT as well, indicating that consolidated protocols and probably experienced interpretation account for better performances in this case. This fact may indicate that newer tests can specially benefit from the EPT results, allowing laboratories to improve their performance.

Whole blood crossmatches were as well somehow more prone to errors than LT or LB alone, possibly due to an overinterpretation of results (some participants alleged to have reported whole blood crossmatch positive when either LT or LB crossmatches were positive instead of performing it before lymphocyte separation). Although other EPTs (Putheti et al., 2022) report a decreasing number of laboratories performing CDC, the number of GECLID users of the scheme has grown in these 12 years. Some studies (Putheti et al., 2022) report a higher number of errors in LT than in LB in FC crossmatches, but we only found more LT errors in the first years of our EPT. This might point out that laboratory experience affects LT to a higher extent than LB. FC crossmatches may be too sensitive and lead to excessive wait times or to excessive excluding (Wrenn et al., 2018). Combined exercises of real and virtual crossmatches might help in establishing accurate cutoffs in order to improve efficiency.

The presence/absence of antibodies in each sample will be determined by consensus of at least 75% of the participating laboratories. When participants fail to report a specificity, 95% of the laboratories not reporting the specificity as positive are required to consider it as a negative consensus (European Federation for Immunogenetics, 2021). The specificities that are negative by omission between 75% and 95% of the participants will be included in the report as non-assessable negatives and will not be penalized. Most errors (89.7%) are due to identification of specificities, whereas screening of anti-class I/-class II antibodies accounts for 1.4%–1.9% errors and interpretation of anti-HLA-DQ or anti-HLA-DP from two-antigen coated beads would represent 5.4%. The overall mean error rate is 0.65% for anti-HLA and 0.60% regarding anti-HPA, indicating a good performance irrespective of the method or vendor (Israeli et al., 2015). The anti-HPA error rates in published workshops (Goldman et al., 2003; Bessos et al., 2005; Allen et al., 2013) are far higher than ours, but those exercises were performed on samples selected by their difficulties (differently to routine ones) and their assigned values were not that of consensus, and comparison to these EPTs is therefore useless.

Chimerism has the highest error rate in our EPT as some studies could not predict disease relapse by analyzing chimerism (Beck et al., 2006). This discrepancy could not only be caused by different patient populations or different underlying diseases but also by technical differences of the analysis. It is noticeable that this is also the only quantitative scheme in the HLA subprogram, and is therefore an added handicap. In fact, most penalties are due to deviant quantifications.

The common criteria for histocompatibility assays are the keystone for an efficient organ sharing system. The possibility of organ exchanges is closely linked to the importance of interlaboratory comparisons for histocompatibility and immunogenetics in order to build trustful networks. Most typing issues are due to nomenclature errors rather than technical errors. Manual validation by experienced immunologists is therefore a key point to get error-free results. Interpretation is anyway an error source regarding antibodies or risks, therefore has to be carefully performed only by qualified personnel. Recently incorporated tests obtain the highest profit of EPT participation. There is little heterogeneity of results of different assays used by participating laboratories, even when in-house assays are used. We have evidence enough to ascertain that HLA and immunogenetics assays in routine care in our area are highly reliable.

Data availability statement

The data analyzed in this study are not subject to any licenses/restrictions. Requests to access these data sets should be directed to responsable@geclid.es.

Ethics statement

The studies involving humans were approved by the Ethics Committee Valladolid Este. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MM: Conceptualization, data curation, formal analysis, investigation, manuscript writing–original draft, review, and editing.

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Conflict of interest

MM is the GECLID program's Manager.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1268728/full#supplementary-material

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Harmonisation of the HLA tests for the diagnosis of coeliac disease: experiences from the Czech external proficiency testing program

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Coeliac disease (CD) is an autoimmune disorder caused by the ingestion of gluten-containing grains. One of the prerequisites for the development of the disease is the presence of specific combinations of HLA alleles at the DQA1 and DQB1 loci. The HLA test is a supportive diagnostic test. In the Czech Republic, approximately 3,500 HLA tests for CD diagnosis are performed annually in almost three dozen laboratories. The HLA Department of the Institute of Haematology and Blood Transfusion in Prague has been offering the EPT "Detection of HLA Alleles Associated with Diseases" for more than 10 years. The results are evaluated in terms of the correct determination of predisposing alleles/allelic groups and clinical interpretation. Every year, we notice some problems with the detection of CD-associated alleles and the interpretation of results. Annual workshops are part of this EPT, and they also include recommendations for the interpretation of results. This interpretation is evolving based on the current knowledge in the field. The current recommendation for interpretation was adopted in 2023, dividing HLA-DQA1/DQB1 genotypes into three categories: 1) detected HLA genotype is associated with predisposition to coeliac disease; 2) coeliac disease could not be excluded based on the detected HLA genotype; 3) coeliac disease could be excluded with high probability based on the detected HLA genotype. The quality of examination is increasing but still needs improvement. Correct results and accurate interpretation can inform clinicians' decisions about the diagnosis of coeliac disease in appropriate patients.

KEYWORDS

coeliac disease, HLA, external proficiency testing, disease association, genetic susceptibility

Introduction

Coeliac disease (CD) is an autoimmune disorder triggered by the ingestion of gluten, primarily affecting the small intestine (Catassi et al., 2022). The average prevalence of CD is approximately 1% in the populations of developed countries. The clinical manifestations of CD are highly variable and may combine a wide range of gastrointestinal and extraintestinal symptoms, including those of malabsorption. Diagnosis is based on clinical suspicion, serological parameters (positivity for anti-transglutaminase 2 and anti-endomysial antibodies), and, if necessary, a biopsy of the small intestine (Husby et al., 2020).

Despite current efforts to develop an effective tolerance-inducing therapy for CD (Sollid, 2024), a lifelong gluten-free diet remains the essential tool for disease control and, importantly, preventing CD complications such as growth retardation, anaemia, osteoporosis, and intestinal lymphoma.

The pathogenesis of CD is based on the interplay between the genetically predisposed individual and (mostly unknown) environmental factors (Levescot et al., 2022). Accordingly, CD is a complex disease with a strong genetic component in which specific disease-associated HLA-DQ variants present deamidated gluten peptides to the specific $CD4^+$ T lymphocytes that finally trigger the disease (Sollid, 2024). Historically, the first report of an association between the HLA system and CD was published as early as 1973 (Evans, 1973), although this study only identified the HLA marker (HLA-B8) in linkage disequilibrium and did not identify a causal HLA variant for CD (Mrazek, 2023). In the following decades, studies using new methods of HLA testing based on DNA analysis made it possible to precisely specify the HLA variants necessary for the development of CD, namely HLA-DQ2 (heterodimers DQ2.5 and DQ2.2) and DQ8. Furthermore, it has been clearly shown that not only the presence of these CD-predisposing HLA variants but also their genotype status (gene-dose effect) and/or mutual homologous combinations contribute to the overall risk of the disease (Megiorni and Pizzuti, 2012; Erlichster et al., 2020).

Identification of the associated HLA-DQ variants has been widely incorporated into the diagnosis of CD, although it is not currently mandatory for diagnosis (Husby et al., 2020). Current approaches to certified laboratory HLA tests in CD aim to detect the HLA allelic combinations that predispose people to the disease. Importantly, these tests are characterised by a high negative predictive value—an absence of the predisposing HLA variants in the subject has a high probability of excluding CD.

The aim of this article is to briefly summarise the long-term experience with the external proficiency testing program (EPT) "Detection of HLA Alleles Associated with Diseases" organised by the HLA department of the Institute of Haematology and Blood Transfusion in Prague. Therefore, presented and discussed here are the characteristics of the EPT program, the development of national guidelines for the interpretation of the HLA testing in CD in the context of international recommendations, the main problems in the HLA genotyping and interpretation of the results by the EPT participants, and the current directions in genetic testing for CD.

External proficiency testing program "Detection of HLA Alleles Associated with Diseases"

Approximately 3,500–4,000 HLA tests for CD diagnosis are performed annually in almost three dozen laboratories in the Czech Republic; details of the system of HLA tests for CD have been published elsewhere (Vraná et al., 2017). CE-IVD diagnostic kits for typing HLA genotypes predisposing for CD are used by the laboratories in the majority of reported results.

The HLA department of the Institute of Haematology and Blood Transfusion has organised the EPT "Detection of HLA Alleles Associated with Diseases" since 2010. The EPT program is open to all willing laboratories and diagnostic kit providers/vendors. Any DNA-based method can be used by the EPT participants. Tests for the detection of HLA alleles associated with CD have always been part of this EPT program. Since 2012, we have organised and evaluated this EPT on a regular basis. The results have been evaluated in terms of the correct determination of predisposing HLA alleles/allele groups and their clinical interpretation. The correct detection of HLA-DQA1*02, *05 and *03; DQB1*02, and *03:02 is required for the EPT. The organising laboratory has provided reference DQA1 and DQB1 genotypes based on SBT/NGS high-resolution typing. Clinical interpretation is scored according to the current recommendations (see "Recommendations" section for the interpretation of HLA testing in the diagnosis of coeliac disease). Scoring details are specified in Supplementary Image S1.

A total of 13 Czech laboratories participated in the EPT in 2012, while 34 laboratories from six countries participated in 2023. Since 2013, the EPT has been divided into two rounds of five samples each; labs may choose to participate in one or both rounds. All participants are listed in Supplementary Data Sheet S1. Laboratory techniques used for the detection of associated HLA variants have changed over the years. Polymerase chain reaction with sequence-specific primers (PCR-SSP) has been the most commonly used method throughout the whole period. Details of the techniques used by participants in the most recent EPT year evaluated (2023) and associated genotyping errors are described below. All tests were performed using CE-IVD commercial kits from different manufacturers.

In 2023, various polymerase chain reaction kits with sequence-specific primers (PCR-SSP) intended for CD detection were used by 19 laboratories, one of which missed DQB1*03:02 in one sample. CD-specific real-time polymerase chain reaction (RT-PCR) kits were used by 13 laboratories, one of which incorrectly reported the presence of DQB1*03:02 instead of DQB1*03:03. Sequence-specific oligoprobe (SSO) CD-specific kits with various configurations, including microarray technology, were used by 12 laboratories, two of which incorrectly reported the presence of DQB1*03:02 instead of DQB1*03:03 (both errors were associated with a strip assay). Several laboratories reported using more than one method. Accordingly, the laboratories participating in both rounds were counted twice for the evaluation, as some of them used different methods in the first and second rounds.

Every year (except 2019), we have noticed some problems with the detection of CD-associated alleles and the interpretation of the results. The number of participants and successful participation in the EPT program over the last 10 years is shown in Figure 1. The critical problem that repeatedly appeared in allele detection is the correct determination of the predisposing DQB1*03:02 variant. This allele is often incorrectly reported in individuals carrying the non-predisposing DQB1*03:03 allele. This error leads to the incorrect clinical interpretation of the results. Importantly, many other errors in HLA typing occur despite the use of CE-IVD diagnostic kits by the participating laboratories.

In our experience, the distinct year-to-year variability in EPT outcomes, as measured by the proportion of discrepancies, is related to the selection of samples for a particular EPT run. For example, the samples with uncommon linkage between the

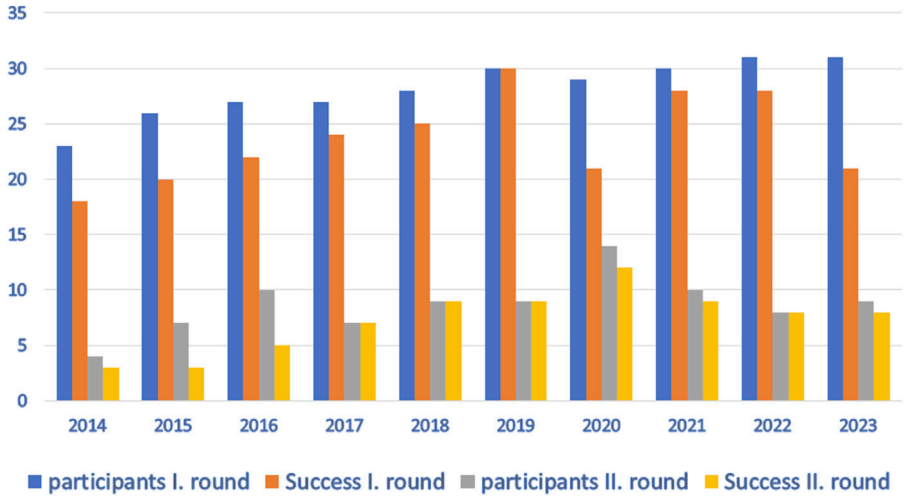

FIGURE 1
Participation and success in EPT in the last 10 years.

variants at HLA-Class II loci (rare haplotypes) tend to be reported with an error more frequently. The proportion of participating laboratories with discrepancies in EPT according to the particular DNA techniques used for testing EPT samples is presented in Table 1. Although we observed more errors among the limited number of laboratories using RT-PCR with one specific kit in the early years of the program, no further evidence of a significant relationship between the success rate in EPT and the techniques used was found.

Recommendations for the interpretation of HLA tests for the diagnosis of coeliac disease

Open annual workshops that are attended by the laboratories (EPT program participants), clinicians, and the representatives of diagnostic kit manufacturers are considered an integral part of the EPT program and also address recommendations for the interpretation of results. The interpretation of HLA results for CD is evolving based on current knowledge in the field.

The main goal of these recommendations is to pinpoint important facts regarding the predictive value of the HLA tests in CD.

- The frequency of HLA haplotypes with confirmed risk of CD in the Czech population is as follows: . . .DQA1*05/DQB1*02 aprox. 9.2%; DQA1*02/DQB1*02 aprox. 8.3%; DQA1*03/DQB1*03:02 aprox. 6.9%. . .. (Zajacova et al., 2016), altogether reaching 24.4%. Accordingly, we estimate that the proportion of individuals carrying at least one of these predisposing HLA-DQ haplotypes in the general Czech population is approximately 43%.
- The prevalence of CD in Western countries is approximately 1% (Catassi et al., 2022; Al-Toma et al., 2019).
- The presence of predisposing HLA alleles/haplotypes cannot be used to confirm CD, as HLA testing as such has low specificity and positive predictive value (Elwenspoek et al., 2021).
- On the other hand, the number of patients with confirmed CD who are not carriers of these alleles/haplotypes is very small, and therefore the examination has a high negative predictive value (Erlichster et al., 2020).

The first of our recommendations was established in 2015 and mainly focuses on consensus on the correct and complete issuance of diagnostic predisposition test results (Vraná et al., 2017). DQ2.5 and DQ8 were determined as the variants associated with the predisposition to CD in accordance with the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition Guidelines for the Diagnosis of Coeliac Disease (Husby et al., 2012). Our recommendation was updated in 2020. Based on HLA analysis of the Czech cohort presented at the 2016 ESPGHAN Congress, DQ2.2 was established as ". . .associated with a rare risk of CD, the diagnosis could not be excluded". In the update in 2022, the consensus was that DQ2.5, DQ2.2, and DQ8 variants are predisposing to CD, and the strength of the association conferred by different variants (e.g., DQ2.5 *versus* DQ2.2) is not considered for the interpretation of the tests. Recently, the genotype containing HLA-DQA1*05 without DQB1*02 or DQ8 was detected in two unrelated Czech individuals with the unequivocally confirmed diagnosis of coeliac disease. Both cases were presented in detail at the 2023 EPT annual workshop. This finding, together with the previous reports describing rare CD cases that were negative for established predisposing HLA variants, namely DQ2.5, DQ8, and DQ2.2, but positive only for DQA1*05 led to much discussion about the relevance of such genotypes for disease prediction. To reflect these observations, in 2023, we finally modified the recommendation on the interpretation of HLA tests for CD predisposition to its current version as follows:

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TABLE 1 Overview of methods used by EPT participants and error rate in distinct years.

year→	2014		2015		2016		2017		2018	
method↓	labs (n)	errors (%)	labs (n)	errors (%)	labs (n)	errors (%)	labs (n)	errors (%)	labs (n)	errors (%)
PCR-SSP	13	7.7%	19	15.8%	17	5.9%	18	5.6%	23	8.7%
RT-PCR	1	100%	4	25.0%	5	100%	2	100%	0	N/A
SSO	9	22.2%	11	9.1%	15	33.3%	10	0.0%	17	0.0%
Sanger seq	0	N/A	0	N/A	0	N/A	1	0.0%	1	0.0%
year→	2019		2020		2021		2022		2023	
method↓	labs (n)	errors (%)	labs (n)	errors (%)	labs (n)	errors (%)	labs (n)	errors (%)	labs (n)	errors (%)
PCR-SSP	17	0.0%	20	15.0%	23	4.3%	19	10.5%	19	5.3%
RT-PCR	2	0.0%	5	0.0%	6	0.0%	11	18.2%	13	7.7%
SSO	11	0.0%	16	18.8%	10	0.0%	10	0.0%	12	16.7%
Sanger seq	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A

year→	all years	
method↓	labs (n)	errors (%)
PCR-SSP	188	8.0%
RT-PCR	49	26.5%
SSO	121	10.7%
Sanger seq	2	0.0%

Error rate: 0%, 0.1%-9.9%, 10-19.9%, 20%-99.9%, 100%.

- Identification of HLA-DQ2.5 (DQB1*02 + DQA1*05), DQ2.2 (DQB1*02 + DQA1*02 without DQA1*05), and/or DQ8 (DQB1*03:02 + DQA1*03) should be interpreted as "Detected HLA genotype is associated with the risk of CD. This result cannot be separately interpreted as a confirmation of CD due to low specificity."
- HLA-DQA1*05 solely without any of the other predisposing alleles mentioned in the previous paragraph should be interpreted as "Detected HLA genotype is associated with the occurrence of coeliac disease in rare cases. The diagnosis of CD cannot be excluded."
- Any combined HLA-DQB1, -DQA1 genotype other than those mentioned above should be interpreted as "Detected HLA genotype is not associated with the risk of CD. The result excludes this diagnosis with high probability."

The recommendation is also concerned with the complete issuance of the results, including all information important for clinical use in a form that is easily understandable to the ordering clinician. A complete report should include the following key information.

- Detected HLA-DQA1 and DQB1 alleles/allele groups predisposing to CD.
- Information on the presence or absence of serological equivalents DQ2.5, DQ2.2, and DQ8.
- Clinical interpretation of the detected result as described above.

Discussion

The detection of predisposing HLA variants has become an important part of the laboratory diagnosis of CD. Compared to the majority of other clinically relevant HLA disease associations, susceptibility to CD is conferred by the presence of specific combinations of alleles at two HLA loci (HLA-DQB1 and -DQA1) and therefore requires an appropriate approach for HLA genotyping and relevant interpretation for clinicians (Tye-Din et al., 2015).

The existence of various approaches for the genotyping, interpretation, and reporting of the results of HLA variants associated with CD that did not historically provide sufficient support for CD diagnostics highlights the need for the harmonisation of HLA diagnostics in CD. External proficiency testing (EPT) programs thus represent a crucial tool in the process of standardisation of HLA disease association testing in CD. Based on their ability to monitor methods, reagents (usually commercial kits), ways of reporting results, and errors for participating laboratories, EPT programs provide a valuable source of data and feedback for all entities involved in CD diagnostics and contribute to further overall improvement of HLA laboratory diagnostics in CD. For example, experience from our EPT program has already contributed to the modification of commercial kits (both in terms of technology and interpretation) to improve their ability to correctly assess genetic susceptibility to CD and to improve parameters of the reports from participating laboratories, such as the use of current HLA nomenclature

and unambiguous/harmonised clinical interpretation that can be widely shared among clinicians.

Not surprisingly, due to the abovementioned complex nature of HLA's association with CD and its interpretation, this part of our EPT program is consistently characterised by the highest proportion of errors in the results compared with other HLA tests offered by our program (e.g., HLA-B*27, -B*57:01, and -DQB1*06:02); similar experiences with poorer performance in HLA testing for CD are reported from other EPT programs that provide tests for HLA disease associations. An important example of a frequent and rather systematic error with a potentially detrimental effect on CD diagnosis is the false positive reporting of the predisposing HLA-DQB1*03:02 allele in subjects carrying only DQB1*03:03, which is not associated with CD.

It is important to note that various genetic laboratories (not just "HLA-oriented") perform HLA-based CD genetic susceptibility testing, usually using commercial kits that focus on detecting combinations of CD-predisposing HLA alleles but do not provide a complete HLA genotype. Furthermore, a significant proportion of these laboratories are not involved in HLA testing for other clinical purposes, such as histocompatibility testing for transplantations, which requires the complete genotyping of specific HLA loci. In this context, the question may arise as to whether newer HLA typing techniques that provide a complete HLA genotype at a high-resolution level (e.g., next-generation sequencing) should be preferred to the CD-specific HLA kits (Devriese et al., 2024). Full HLA genotyping is a relevant and promising alternative for disease association testing and offers several clear advantages, such as the possibility of second-level reference typing in "problematic" samples or the assessment of gene dose effect. On the other hand, when properly and accurately used, commercial CD-specific kits can provide sufficient results to assess CD predisposition in the majority of samples in accordance with the above recommendations. However, as our results show, critical examination of these kits is necessary, and further development is needed. In the case of inconclusive results, alternatives should be sought, such as more precise methods for complete genotyping of the relevant loci as mentioned above. EPT, kit improvement, harmonisation of interpretation results, education, and the use of advanced technologies for sample processing and result evaluation (special applications, artificial intelligence, etc.) can contribute to error prevention and a more accurate and correct interpretation.

At present, there is wide agreement on the recommendations regarding the HLA variants predisposing to CD: heterodimers DQ2.5 (DQB1*02, DQA1*05), DQ2.2 (DQB1*02, DQA1*02) and DQ8 (DQB1*03:02, DQA1*03) (Tye-Din et al., 2015; Pritchard et al., 2024). Currently, the main purpose of HLA investigations in CD is to exclude the diagnosis in patients who do not carry the abovementioned relevant HLA-DQ heterodimers regardless of their gene dosage (heterozygosity/homozygosity) or their combinations. In other words, HLA tests in CD are used for their strong negative predictive value—that is, the ability to exclude the disease.

On the other hand, there is no complete consensus on the interpretation of the HLA-DQ genotypes containing only the HLA-DQA1*05 variant without any other predisposing alleles. HLA-DQA1*05, which encodes half of the DQ2.5 heterodimer, is a very common allelic group in Caucasoid populations. Importantly, for instance, the Australian position paper on the appropriate clinical use of HLA typing for CD (Tye-Din et al., 2015) recommends interpreting the genotype containing only HLA-DQA1*05 as being associated with a very low genetic susceptibility to CD, with the consideration of further clinical work-up for CD. Because we are also aware of rare cases of CD associated with genotypes containing HLA-DQA1*05 in the Czech population, our latest national recommendation for HLA testing in CD from 2023 complies with the above-mentioned Australian paper. In contrast, the very recent UK NEQAS and BSHI guidelines on HLA genotyping to support the diagnosis of CD recommend that individuals should be excluded from a diagnosis of CD if they carry only DQA1*05 or any other HLA-DQ alleles other than those encoding the heterodimers DQ2.5, DQ2.2, and DQ8. However, the question of how to deal with the interpretation of HLA-DQA1*05 solely positive individuals is still a matter of intensive debate (Maimaris et al., 2024).

Conclusion

We have presented here our experience in HLA testing for CD from the external proficiency testing program (EPT) "Detection of HLA Alleles Associated with Diseases" organised by the HLA department of the Institute of Haematology and Blood Transfusion in Prague. The main goal of the program and associated annual workshops is to contribute to the standardisation, harmonisation, and improvement of the overall quality of the HLA investigations for the diagnosis of CD.

Author contributions

MV: writing–review and editing, writing–original draft, and conceptualization. JT: writing–review and editing. FM: writing–review and editing and writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Proficiency testing within Eurotransplant

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Eurotransplant is responsible for the international allocation of organs between eight countries in Europe. All HLA laboratories affiliated to Eurotransplant must be EFI or ASHI-accredited and must participate in the Eurotransplant external proficiency testing (EPT) program, organized by the Eurotransplant Reference Laboratory (ETRL). EPT within Eurotransplant has a long tradition, starting in 1978. The current EPT program consists of the following schemes: HLA typing including serology, CDC crossmatching, HLA-specific antibody detection, and identification. Participants enter the results of laboratory tests using a web-based application. Assessed results are visible on the website. An additional component called "patient-based cases" runs since 2016. Results are summarized and published on the EPT website. Furthermore, these results are discussed during the annual extramural tissue typers meeting, which is organized by the ETRL. Thanks to this EPT program, the performance of all HLA laboratories affiliated to Eurotransplant can be monitored and corrected, if necessary. Because all affiliated laboratories are assessed in the same EPT program, where these laboratories show to be consistent in most of their results, Eurotransplant EPT has proven to be an efficient tool to create a more uniform level of quality of histocompatibility testing within Eurotransplant.

KEYWORDS

proficiency test, solid organ transplantation, histocompatibility testing, Eurotransplant, patient-based cases

Introduction

Eurotransplant is an international organ exchange organization founded in 1967 by prof. Dr. Jon van Rood (van Rood, 1967). Currently, Eurotransplant consists of eight member states: Austria, Belgium, Croatia, Germany, Hungary, Luxemburg, Slovenia, and the Netherlands. Histocompatibility plays an important role in the allocation of most types of organs, and in total, 44 tissue typing laboratories affiliated to the transplant centers participate in Eurotransplant. In order to guarantee a uniform level of histocompatibility testing, the Eurotransplant external proficiency testing started in 1978 (Schreuder et al., 1986), with an exchange of cell material for serological HLA typing. Later, EPT on HLA-specific antibody detection and identification and EPT on crossmatching were introduced (Doxiadis et al., 2000).

Nowadays, three different schemes are running: the EPT scheme for HLA typing and crossmatching, with four dispatches per year; and the EPT scheme for HLA-specific antibody detection and identification, for which 12 different sera are shipped. The latest scheme is the patient-based case scheme, which started with a pilot in 2015 and was

TABLE 1 Overview of EPT schemes and their characteristics.

	Scheme 1		Scheme 2		Scheme 3
	HLA typing	Crossmatching	HLA-specific antibody detection	HLA-specific antibody identification	Patient-based cases
Dispatches per year	4	4	1	1	3
Numbers and types of samples per dispatch	3 tubes of blood	3 tubes of blood 3 serum samples	12 vials of serum in one shipment	12 vials of serum in one shipment	n.a
Period until deadline	2 weeks	2 weeks	4–5 months	4–5 months	2 weeks
Entrance of results	Manually on the website	Manually on the website	Manually on the website	Manually on the website	By e-mail
Entrance of results	Eurotransplant match determinants	Positive/negative	Positive/negative	Specificities for HLA-A, -B, -C, -DR, -DQ, and -DP	Answer and motivation related to the case
Time for the assessment of the results	2 weeks; reports are published on the EPT website	2 weeks; reports are published on the EPT website	1 month; reports are published on the EPT website	1 month; reports are published on the EPT website	2 months; the summary is published on the EPT website
Assessment method	Based on 75% consensus	Based on 75% consensus	Detection 75% consensus	Identification CDC: 75% consensus; bead-based methods: 95% consensus	Not assessed; a summary with all the results is published
Maximum yearly discrepancy rate	10%	15%	20%	25%	n.a
Methods assessed	CDC and low-resolution HLA typing	CDC	Bead-based methods, CDC	CDC, single antigen bead assays and complement-dependent SA bead assays	n.a
Number of participants in 2023	68	66	76	76	52

officially introduced in 2016. These EPT schemes cover the most frequently used techniques in laboratory testing for solid organ transplantation (Bontadini, 2012).

All schemes follow the recommendations of the European Federation for Immunogenetics (EFI), as documented in both the EFI standards and the EFI standards for EPT providers (https://efi-web.org/). All laboratories affiliated to Eurotransplant and most of the other participating laboratories are EFI-accredited, for which EPT plays a significant role (Harmer, Mascaretti, and Petershofen, 2018). The Eurotransplant Tissue Typing Advisory Committee (TTAC) has an advisory role for all histocompatibility-related activities within Eurotransplant and has to approve major changes in the EPT scheme.

Additionally, all EPT schemes organized by the ETRL follow the policies of Eurotransplant. The schemes are unique because they give the possibility to compare the laboratories affiliated to Eurotransplant. However, the Eurotransplant EPT is also open for Histocompatibility and Immunogenetics (H&I) laboratories from countries outside of Eurotransplant. In total, 44 Eurotransplant-affiliated laboratories and 36 laboratories from outside the Eurotransplant area are participating in one or more of the schemes organized. All schemes are mandatory for Eurotransplant-affiliated laboratories, while the remaining laboratories can choose to participate in one or more of the schemes. Patient-based cases are also open to participants outside of Eurotransplant, taking part in either the HLA typing and crossmatching scheme and/or in the HLA-specific antibody detection and identification scheme. The results from all EPT schemes are published on the EPT website. Furthermore, during the yearly Eurotransplant extramural tissue typers meeting, the results from all EPT schemes are discussed with the representatives of the different tissue typing laboratories.

Materials and methods

EPT on HLA typing

Every year four sets of three blood samples are shipped to the participants. Characteristics are depicted in Table 1. HLA typing results are to be entered in the format of Eurotransplant Match Determinants, based on the 2008 HLA dictionary (Holdsworth et al., 2009), as well as on serotypes as published by Osoegawa et al. (2022).

Results can be entered manually and authorized on the ETRL-EPT website. The deadline for the submission of the results is 2 weeks from the day of shipment. After the deadline, results are assessed on the basis of consensus, as described in the EFI standards for providers. In case no consensus is reached, reference typing is done by the ETRL.

EPT on CDC crossmatching

For crossmatching, four shipments consisting of three blood samples and three serum samples per year are sent to the participating laboratories. For an overview, see Table 1.

Crossmatching on either T cells or unseparated cells is mandatory for all Eurotransplant-affiliated laboratories. All participating laboratories can perform B-cell crossmatches as well. Results are entered on the ETRL-EPT website. The deadline is 2 weeks from the day of shipment. Crossmatch results are assessed on the basis of a 75% consensus.

EPT on HLA-specific antibody detection

In total, 12 serum samples are sent yearly for the EPT on HLA-specific antibody detection. The characteristics are shown in Table 1. Eurotransplant-affiliated participants must use a CDC-based technique. Additionally, it is possible to use Luminex bead-based, flow cytometric-based, and ELISA-based techniques. Results are to be entered as positive (HLA-specific antibodies present) or negative (absence of HLA-specific antibodies) on the ETRL-EPT website. The period until the deadline is 4 to 5 months from the day of shipment.

EPT on HLA-specific antibody identification

For the EPT on HLA-specific antibody identification, the same 12 sera are used as shipped for the EPT on HLA-specific antibody detection. More information is given in Table 1. For HLA-specific antibody identification, Eurotransplant-affiliated laboratories must use CDC as a method. Last year, 11 laboratories from outside Eurotransplant used CDC as a technique. Next to this, Luminex single-antigen bead (SAB) assays and Luminex SAB complement binding assays are assessed separately. An assessment is done on the basis of consensus. The ETRL uses 75% consensus for CDC and 95% consensus for SAB assays. Results are entered on the ETRL-EPT website. The period until the deadline is 4 to 5 months from the day of shipment.

Patient-based cases EPT

The Eurotransplant patient-based cases EPT started with a pilot in 2015. This was done because the need was felt to not only produce laboratory results of crossmatching, HLA typing, and HLA-specific antibody detection and identification but to also have an EPT that asks for the interpretation of these results in order to assess the (level of) histocompatibility between a patient and a potential donor.

Two pilot cases were sent out. The first pilot case consisted of patient and donor information and laboratory data, such as HLA typing, immunizing events, HLA-specific antibody identification data, and crossmatch results. Participants could interpret the complete dataset and judge whether the donor offer was suitable for the patient and motivate their answer.

The second pilot case was a combination of the regular EPT on HLA typing and crossmatching in combination with the question to select the best suitable patient for a donor. Both crossmatches and donor HLA typing were performed by the participating centers. Other data like recipient HLA types were given.

After this pilot, it was decided to continue with the exercises solely based on the results that were already available, as in the first pilot case. Combined exercises (second pilot case) were stopped because with only few available data (HLA typing and crossmatch results), it was difficult to create realistic scenarios.

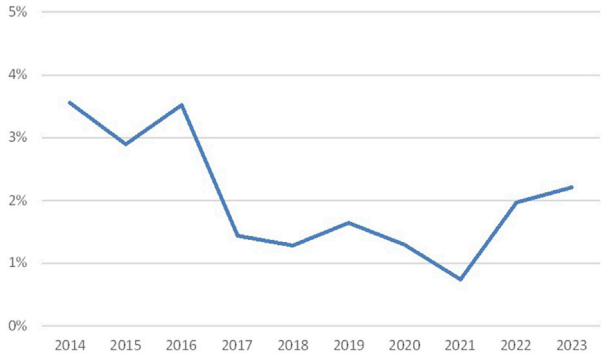

FIGURE 1
Discrepancy rates in HLA typing (percentage discrepancies over the years 2014–2023; source Eurotransplant).

In 2016, the patient-based case EPT was formalized. Since then, this EPT is mandatory for all Eurotransplant-affiliated laboratories, and a yearly certificate of participation is issued. The EPT consists of three patient cases each year. The focus is on transplantation with kidneys from deceased donors as the Eurotransplant-affiliated laboratories have testing for this goal as one of their main tasks.

Over time, other scenarios were added, e.g., to give attention to specific Eurotransplant policies, upcoming changes in Eurotransplant or Eurotransplant programs, such as the AM program (Heidt et al., 2021), and the recently introduced virtual donor crossmatch (Claas and Heidt, 2020).

Certificates

Certificates are issued by the ETRL based on the performance in the EPT for both the HLA typing and crossmatching scheme and for the HLA-specific antibody detection and identification scheme. The following results on the certificate are possible: fulfilled/not fulfilled/participated. The certificate for (not) fulfilling the requirements can only be awarded if a minimum number of 10 participants report results in the respective category. For certificates of successful performance (fulfilled), the participant needs to meet the criteria, as described in the latest version of the Eurotransplant Manual, the latest version of the EFI standards, and the latest version of the EFI standards for providers. When for a given technique less than 10 participants join the EPT, a participation certificate will be issued. For the patient-based cases EPT, a certificate of participation is issued.

Results and discussion

The present results have been partly published in the Eurotransplant Annual Report 2023 (www.Eurotransplant.org).

In general, no significant differences between the eight Eurotransplant member states are observed, and there are no large differences in the results between Eurotransplant-affiliated

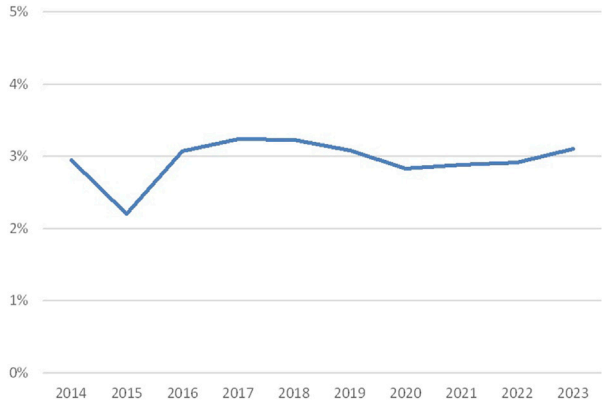

FIGURE 2
Discrepancy rates crossmatching (percentage discrepancies over the years 2014–2023; source Eurotransplant).

laboratories and all other laboratories. Overall, the participants in the Eurotransplant EPT have satisfying results, with occasional exceptions.

EPT on HLA typing

The results of the HLA typing scheme are shown in Figure 1. Since the observation that DNA-based HLA typing is more reliable than serological typing (Opelz et al., 1991), molecular typing has been introduced within Eurotransplant, leading to a diminished discrepancy rate (3.6% in the period 2014–2023). The observed discrepancies are most often clerical errors, such as errors occurring during the manual entrance of the results. Other more often observed errors are mix-up of samples and entering a broad when a split can be detected (e.g., Cw3 instead of Cw10 (Cw3).

The decrease in discrepancy rates until 2021 can partly be explained by the fact that a Eurotransplant policy was introduced to only assign Bw4 together with B-locus antigens. During a few years, the participants have gradually incorporated this. The increase in the following years is probably related to the introduction of the virtual crossmatch. Since that moment DQA1, DPB1, and DPA1 results had to be reported as well.

With the introduction of the virtual crossmatch in Eurotransplant, donor HLA types can be electronically transmitted. A discussion has started whether this may be feasible for the EPT on HLA typing in the future. A possible advantage could be that this way clerical errors will be avoided.

EPT on CDC crossmatching

Overall, approximately 3% discrepancy rates were observed in the period 2014–2023 (Figure 2), with an exception in 2015 (2.2%). There is no clear explanation, such as lower consensus rates, for this lower percentage.

Discrepancies in crossmatching may have various causes. Since for crossmatching fresh blood is shipped, the duration of the shipment can influence the quality of the target cells. Moreover,

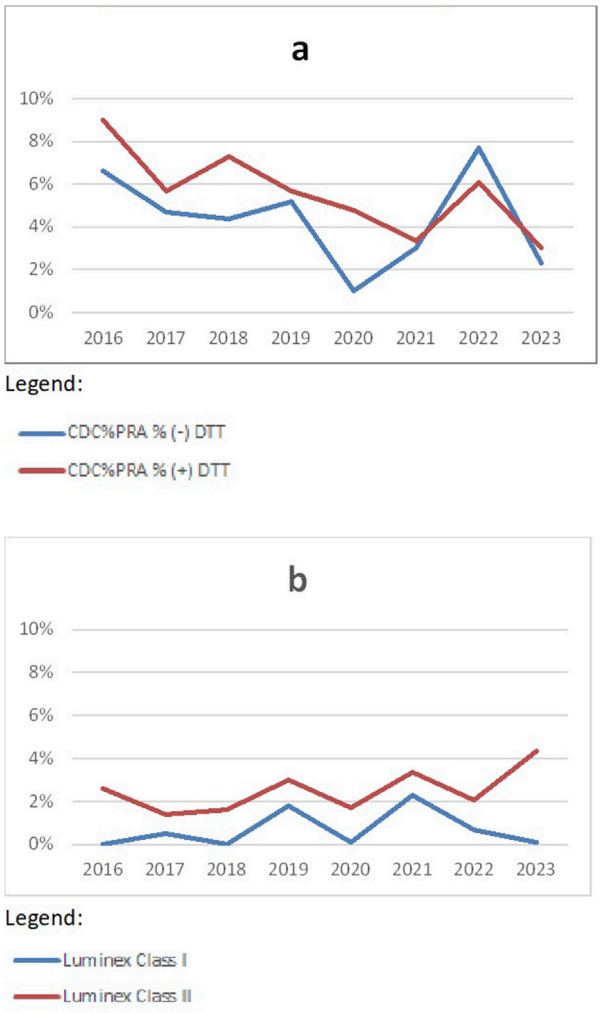

FIGURE 3
Discrepancy rates in HLA-specific antibody detection CDC **(A)** and methods **(B)** over the years 2016–2023 (source Eurotransplant).

crossmatching is done by CDC-based methods, which may vary with respect to the technique itself and the interpretation of the results, depending on the technician and her or his experience. In addition, a difference is seen between the cell types used for crossmatching. The comparison of B-cell crossmatches to T-cell crossmatches and unseparated cell crossmatches is challenging, leading either to more discrepancies or to results that are not in consensus. To this end, B-cell crossmatches are analyzed separately.

Consensus rates (Supplementary Figure S1) were around 90% in the period 2014–2023, with higher consensus rates for unseparated cell and T-cell crossmatching and lower (but usually not below 75%) for B-cell crossmatching.

EPT on HLA-specific antibody detection

When looking at the detection of HLA-specific antibodies (Figure 3), it is obvious that more discrepancies are found in CDC-based methods (Figure 3A, discrepancy rates around 5%) compared to Luminex-based methods (Figure 3B, discrepancy

TABLE 2 Percentages of participants with unsatisfactory results in the period 2019–2023.

	HLA typing (%)	Crossmatching (%)	HLA-specific antibody detection (%)	HLA-specific antibody identification CDC (%)	HLA-specific antibody identification SPA SA (%)
% Participants with unsatisfactory results	3.2	1.2	2.4	9.0	0.3

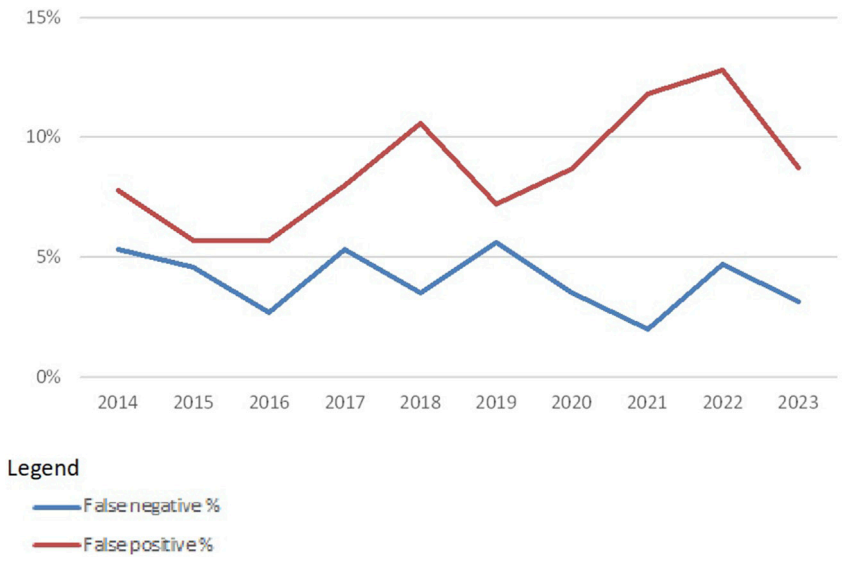

FIGURE 4
False-negative and false-positive rates in HLA-specific antibody identification with CDC-based methods over the years 2014–2023 (source Eurotransplant).

rates around 1.5%). Currently, there are two vendors of Luminex-based antibody screening kits, which differ in performance and sensitivity. When discrepancies are seen in Luminex-based methods, this is often caused by one kit being more sensitive than the other kit. Since separated analysis for CDC and SAB techniques was introduced in 2016, only results from 2016–2023 are shown.

EPT on HLA-specific antibody identification

The CDC-based HLA-specific antibody identification results in more false-negative and false-positive results relative to the total amount of consensus specificities found compared to SAB techniques as long as this is analyzed (over 10 years). The number of consensus specificities in CDC-based HLA-specific antibody identification was between 11 and 26 (average 17 specificities) in the period 2014–2023. In addition, more laboratories with less satisfactory results in CDC-based HLA-specific antibody identification are seen (Table 2). This can be explained by variation in the cell panel size and used HLA types in cell panels. Around 50% of the ET-affiliated laboratories use an in-house panel (information from an ETRL inventory in 2023), and the other half of ET-affiliated laboratories use CDC tests from different companies.

Overall, this leads to false-negative rates of around 4% and false-positive rates of around 10% (Figure 4). False positives are extra specificities found by the participating laboratories. The higher (10%–12%) percentages seen here are mainly due to a few participants reporting several specificities detected via SAB techniques in the CDC proficiency tests. False negatives are missed specificities. This percentage (around 4%) is much lower, which may indicate that most laboratories are indeed capable of detecting relevant specificities with CDC, which shows that this technique is valuable for the future.

The false-negative and false-positive rates in SAB techniques are much lower mainly because more consensus specificities are found in the period 2014–2023. The average number of consensus specificities for HLA Class I is 279 and the average number for consensus specificities for HLA Class II is 55. In 2023, also DQA1, DPA1, and DPB1 specificities could be entered, resulting in 102 consensus specificities for Class II. A second reason why these false-negative and false-positive rates are lower is that every laboratory is using one of the two commercially available kits, whereas CDC cell panels vary from laboratory to laboratory.

False-negative rates and false-positive rates for SAB techniques are usually around 0.6% (Figure 5). The introduction of additional specificities (DQA1, DPA1, and DPB1) led to a higher false-positive rate in 2023. False positives for SAB techniques could be due to low or very low cut-off values used in some of the laboratories. In addition, for SAB tests, the market is dominated by only two vendors. When using identical cut-off values in tests from both vendors, misinterpretation may be a consequence (Karahan et al., 2023).

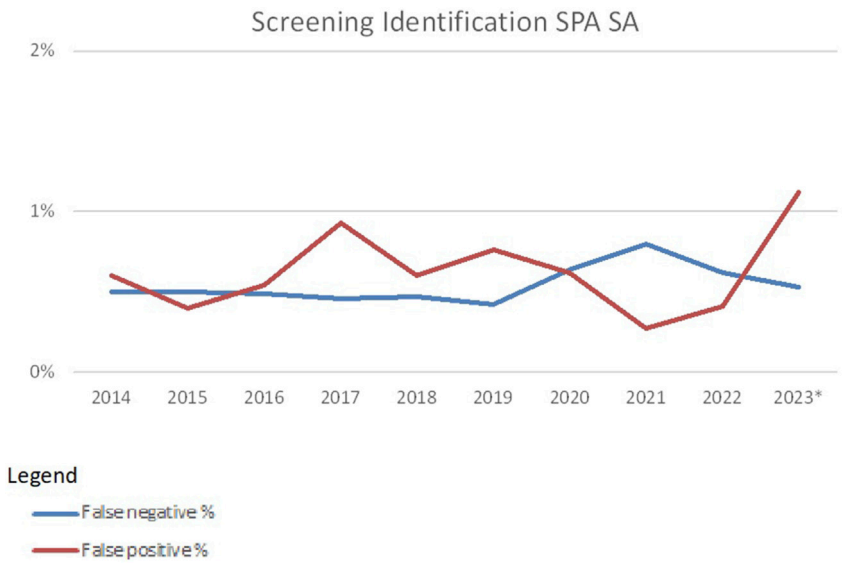

FIGURE 5
False-negative and false-positive rates in HLA-specific antibody identification with SAB-based methods over the years 2014–2023 (source Eurotransplant). *As of 2023 DQA, DPA and DPB specificities were included.

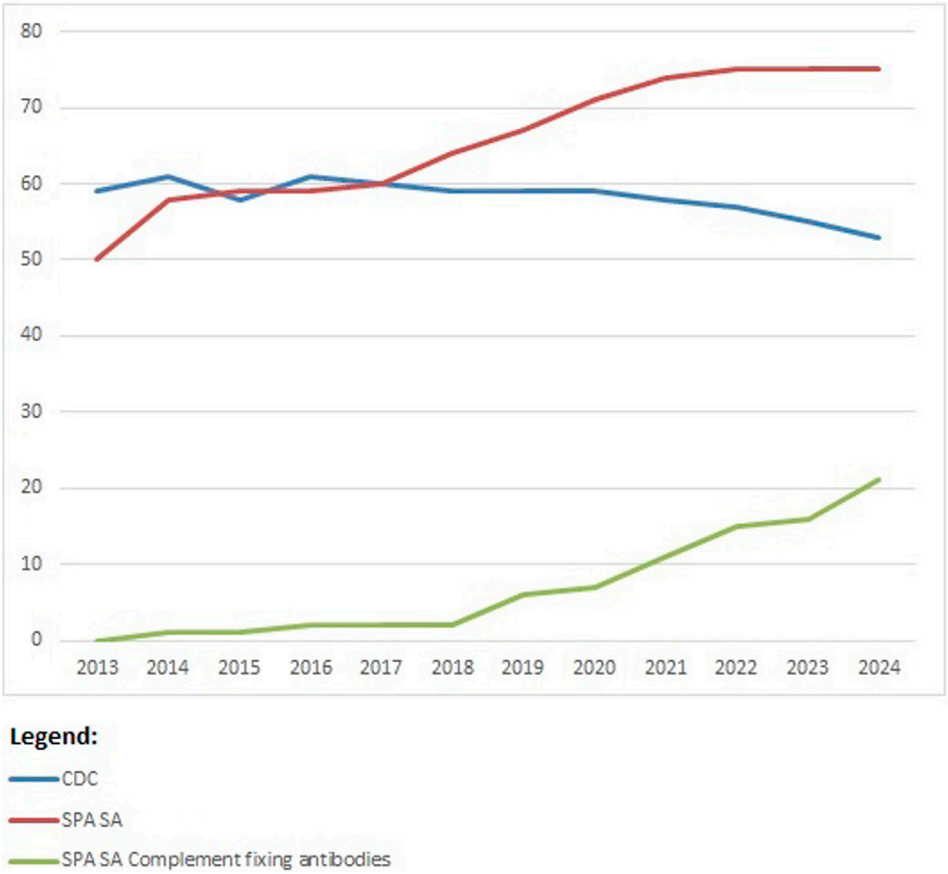

FIGURE 6
Participation per technique for HLA-specific antibody identification over the years 2013–2023; number of participants on the vertical axis.

From 2013 until present, there is a trend toward using SAB methods by almost all participants (Figure 6). Within Eurotransplant, CDC is used together with SAB methods to have more complete information about the impact of the antibodies detected and possibly exclude denatured antibodies found in SAB methods. Therefore, CDC is still a mandatory technique for the Eurotransplant-affiliated laboratories. Outside Eurotransplant, 11 participants were using CDC as one of the techniques used in HLA antibody identification in 2023. The complement-fixing SAB techniques are used more and more, especially outside Eurotransplant (11/16 participants are not affiliated to Eurotransplant).

A future prospect may be the combination of CDC and SAB techniques in order to define acceptable and/or unacceptable antigens.

Patient-based case EPT

The results of the patient-based cases are reported to the participants by means of a summary, which is published on the EPT website. The information in the summaries consists of a short description of the case itself. Next to this, a graph is made to show an overview of the final answer (e.g., transplanting yes, no, or may be). Furthermore, for all the categories of final answers, observations and motivations are sorted, counted, and listed. Then, a list of missing information (according to the participants) and a list of recommendations are given. Finally, an ETRL comment is given, e.g., with the outcome of a transplantation or information about Eurotransplant policies.

All patient-based cases are presented and discussed during the Eurotransplant extramural tissue typers meeting. The same holds true for all other EPT results. This meeting is meant for all staff in the Eurotransplant-affiliated laboratories and is organized yearly by the ETRL together with one of the affiliated laboratories.

The presentation and discussion of the patient-based cases results reveal differences in policies between transplantation centers and affiliated tissue typing laboratories and provide insights why certain decisions are taken. These discussions are very important and create a more solid basis for a proper interpretation of the laboratory results.

Whenever there is a new policy in Eurotransplant, the patient-based case EPT is used to practice with this upcoming policy and is used to familiarize the affiliated transplant centers with the policies. Another practical use of these cases is to give the participants the possibility to practice with ETRL/Eurotransplant tools such as the calculators for donor frequency and virtual panel reactive antibodies (vPRAs).

The results for the patient-based case EPT are not assessed, like in some patient-based cases EPT in clinical chemistry laboratories (Sciacovelli et al., 2003). The main reasons why the cases are not assessed are as follows:

- Centers within Eurotransplant are allowed to have their own policy regarding the acceptance of donors as long as the rules in the Eurotransplant manual are followed. This means that there can be more than one correct answer.
- An assessment of the cases may prevent an open discussion on the pros and cons of different decisions.
- An assessment of such cases is complicated and would require a team judging the cases before and determining the best assessment criteria, which is prone to being subjective.

Certificates

In general, most participants have satisfying results for different ETRL-EPT schemes, which leads to a certificate stating "fulfilled." In the years 2019–2023, 3.2% of the participants had unsatisfactory results for typing, and 1.2% of the participants failed to meet the criteria for crossmatching. In total, 2.4% of the participants had poor results for HLA-specific antibody screening detection, and for HLA-specific antibody identification, the percentages for unsatisfactory results were 9.0% and 0.3%, respectively (Table 2). These percentages are quite stable over this 5-year period. Some fluctuations in CDC HLA-specific antibody identification were observed. This can be explained by the variation in HLA-specific antibody specificities of the serum samples shipped. It is known that not all CDC cell panels allow for the detection of all HLA-specific antibody specificities. In particular, HLA-C specificities are not always detected by all participants, most likely due to the lower expression of HLA-C compared to HLA-A and HLA-B on the cell surface.

Whenever participants have unsatisfactory results and receive a certificate stating "not fulfilled," they have the option to join the extra EPT, which is organized by the ETRL. This extra EPT gives the opportunity to show that unsatisfactory scoring was of a temporary nature. Additionally, this extra EPT serves those participants who could not test all samples of the regular shipment(s) because of problems in the transportation of the material.

Conclusion

The Eurotransplant EPT program has shown its benefit with respect to the performance of the individual laboratories affiliated to Eurotransplant and the interpretation of the results. Laboratory results incidentally are below a satisfactory level but usually return to a satisfactory level in the next year or in the extra EPT exercise at the end of the year organized by the ETRL. The discussion of both the EPT results and the patient cases during the yearly extramural meeting makes all participants aware of the fact that there are still differences in policies with respect to decision-making in different transplant centers. The discussion of these differences is very useful for the critical evaluation of existing policies in the local centers, but it also leads to novel insights, especially with respect to the interpretation of the clinical relevance of HLA antibody reactivities. In particular, the translation of an HLA-specific antibody specificity detected in Luminex SAB assays into an acceptable or non-acceptable HLA antigen will be an important part of the discussion in the coming years, considering the recent introduction of virtual crossmatching within Eurotransplant.

Author contributions

YZ: writing–original draft, writing–review and editing, conceptualization, data curation, formal analysis, funding

acquisition, investigation, methodology, project administration, resources, software, supervision, validation, and visualization. SH: supervision and writing–review and editing. MV: investigation and writing–review and editing. GH: investigation and writing–review and editing. FC: writing–review and editing and supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1451748/full#supplementary-material

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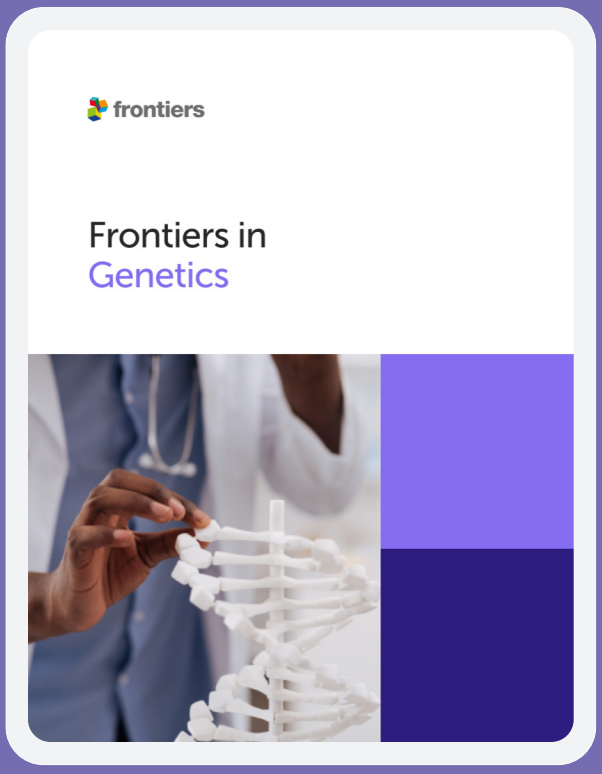

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