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Original article

Results of red cell antibody screening assays from 2,500 laboratories in 10 external quality assessment programs: sensitivity for anti-D

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Background - Red cell antibodies can cause severe or fatal hemolytic transfusion reactions and hemolytic disease of the fetus and newborn. Screening for antibodies, such as anti-D, has been applied over decades for any transfusions and pregnancies. A comparison of sensitivities across laboratories is lacking for such antibody assays.

Materials and methods - We investigated the detection rates for defined antibody concentrations of anti-D, the most common trigger of adverse outcomes. In a concerted effort among 10 providers of external quality assessment and proficiency testing programs, 4 spiked samples were tested by exactly 2,500 laboratories applying their clinical routine procedures, covering 4 test principles, more than 24 test cells, and diagnostic devices from 5 manufacturers.

Results - The sensitivity of the assay depended on the test principle. Detection rates correlated with anti-D antibody concentrations: 0.1 IU/mL and 0.025 IU/mL can reliably be recognized. Some assays enabled detection at 0.01 IU/mL, and only a few at 0.005 IU/mL. Erythrocyte-magnetized technology and solid phase red cell adhesion performed better than various modifications of the column agglutination technology. The conventional test tube technology, depending on visual reading, was least sensitive.

Discussion - The results show options for action to improve antibody detection, may support a practice change to optimize routine strategies of red cell antibody screening, and can guide studies to evaluate the clinical impact.

Keywords: *limit of detection, red blood cell, antibody screening, in vitro diagnostic medical devices, immunohematology.*

INTRODUCTION

Individuals are sensitized by red cell antigens when exposed to blood by transfusion or feto-maternal hemorrhage. The probability of evoking red cell antibodies depends on the immunogenicity of the antigen and the immune competence of the individual[1,2].

Approximately 2-5% of transfused patients become sensitized, as documented by the detection of newly found antibodies[3,4]. Upon feto-maternal hemorrhage, the rate varies between 0.4% and 8.74%[5,6]. Maternal IgG antibodies pass the placenta and react with antigens on fetal erythrocytes or hematopoietic precursors. The ensuing hemolytic disease of the fetus and newborn (HDFN) sometimes causes fetal demise and often requires clinical treatment[7]. Reliable and sensitive methods for antibody detection are critical for patient care and safety.

The immune response to initial sensitization is characterized by slow kinetics and generally low antibody concentrations. Without further antigen exposure, the antibody concentration decreases until it may no longer be detectable[1,4-8]. A hemolytic transfusion reaction (HTR) occurs upon re-exposure to the antigen on foreign red cells in a sensitized patient. Such a secondary immune response is characterized by rapid kinetics and high antibody concentration. No limit for antibody concentrations is known, below which HTR cannot occur.

The detection of specific antibodies in serum or plasma demonstrates the patient's sensitization to red cell antigens. The chance of detecting antibodies, however, depends on the antibody concentration and the sensitivity of the test system. Each diagnostic test has a distinct sensitivity, defined by its limit of detection (LoD), below which an antibody will not be detected. The lack of evidence for an antibody shall not be considered evidence for its absence[9], because the sensitivity of the tests, in difference to their specificity, is hardly monitored in routine practice[1-6,10,11]. The assay sensitivity for low-titer antibodies is particularly important to detect anti-D long after transfusions and for the early detection during pregnancy.

Blood group test systems are *in vitro* diagnostic medical devices (IVD-MD) of the highest risk class, according to European Union (EU) regulations[12]. However, a need to harmonize quantitative results in immunohematology has long been neglected. The potency of red cell antibodies is quantified by titration, if at all. The result of this functional determination is documented by the last positive dilution in a geometric series: for example, a titer 1024 is reported, when a sample has been diluted 1:1024 and reacts in a given test system but fails to react at 1:2048 dilution. Hence, a titer of an antibody is depending on the medical device used. If the potency of red cell antibodies is evaluated at all, it is generally expressed as titer (a dimensionless number) rather than as concentration (IU/mL). An anti-D titer correlates loosely with an anti-D concentration: The threshold of 5 IU/mL, established for HDFN prevention, corresponded to a titer range between 128 and 1024, based on a CAT system. Conversely, a titer of 128 corresponded to concentrations between ~1 and ~11 IU/mL[13], representing a possible 10-fold error.

The potency of an anti-D serum can be expressed in international units (IU). The activity found in 0.95 mg of the International Standard for anti-D Incomplete Blood-Typing Serum[14] has arbitrarily been defined as 1 IU. This standardization enabled documenting that maternal anti-D antibodies in concentrations of greater than 3.5 to 5 IU/mL will cause HDFN[15,16]. The standard was originally established as a reference to ensure appropriateness and consistency in the manufacture and testing of therapeutic anti-D preparations intended to prevent anti D-immunization in Rh D-negative pregnant women. The first International Standard from 1964[14] has been the basis for the second in 2003[17] and third in 2019[18].

Reagents and test cells for determining markers of ABO, Rh and Kell blood group systems were already assigned to the class of highest risk by the 1998 Directive on In Vitro Diagnostic Medical Devices (IVD-MD)[19]. The currently applicable In Vitro Diagnostic Regulation (IVDR) additionally assigns markers for Kidd and Duffy to this class[12]. This regulation uses the terms "marker", "biomarker" and "target marker", but does not define the term "marker" itself. According to constant case law, as rendered by the Court of the European Union, "*in interpreting a provision of EU law, it is necessary to consider not only its wording, but also the context in which it occurs, and the objectives pursued by the rules of which it is part*"[20]. A "marker" can be defined as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*"[21]. Considering the aim of the IVDR to strengthen patient safety and the performance of IVD-MD, the term "marker" can therefore be interpreted as including the detection of blood group antigens and corresponding antibodies.

To address the LoD of antibody test systems, we established a research collaboration among large providers of external

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quality assessment (EQA) and proficiency test programs. The terms "EQA" and "proficiency test" are synonyms and preferentially used in the EU and the US, respectively[22]; for convenience, we apply EQA when referring to either term. Such EQA programs are ideal tools for evaluating the performance of test systems in routine clinical laboratory operations. Most laboratories are required to participate in EQAs. These laboratories utilize standardized applications and their EQA result necessarily represent most test systems, unique reagents or batches of reagents, and devices currently used in clinical routine. In an EQA setting, all participating laboratories will analyze identical aliquots of samples with defined properties, while undisclosed and unknown to any laboratory.

Antibody standards exist for only 4 out of 398 antigens in the 48 blood group systems currently recognized by the ISBT: anti-D, anti-C, anti-E and anti-c[23,24]. Anti-D was chosen for this study because it has the largest known immunogenicity[4,25] and causes severe HTR and HDFN.

MATERIALS AND METHODS

The WHO International Standard Anti-D Immunoglobulin (NIBSC code 16/332; NIBSC, Potters Bar, UK) was used for spiking sample material for this study[26]. Pilot tests showed that common antibody assays detected anti-D antibodies at concentrations of 0.05 IU/mL as positive and 0.025 IU/mL as weakly positive. Some antibody assays gave false negative results at 0.01 IU/mL. We decided to use for this study 1 sample that should yield a clearly positive, 2 samples weakly positive, and 1 sample presumably negative (below the LoD of most test systems). Hence, our panel comprised 4 anti-D samples with 0.1 IU/mL, 0.25 IU/mL, 0.01 IU/mL, and 0.005 IU/mL activity.

The EQA super-challenge

This term has been defined as *concerted activities involving more than one EQA provider* with the condition that *one or more samples with identical properties are used* in their EQA programs *simultaneously* or at approximately the same time[27]. An EQA super-challenge aims to evaluate information inherent to the data collected by an individual EQA program as part of its routine EQA activity. By combining several providers of EQA programs the catchment area is broadened, enabling us to include much larger numbers of participants, a variety of test systems, and different procedures that may mainly be used regionally.

EQA providers and laboratories

We invited more than 50 EQA providers from all 6 continents to participate in an EQA super-challenge through the professional network of the European Organisation of External Quality Assurance Providers in Laboratory Medicine (EQALM). The following 10 EQA providers agreed to join the study (in alphabetical order by country and name, **Table I**): RCPAQAP, Australia (with 125 participating laboratories); Austrian Association for Quality Assurance and Standardization of Medical and Diagnostic Tests (ÖQUASTA), Austria (130); Sciensano, Belgium (144); Biologie Prospective, France (117); Society for Promoting Quality Assurance in Medical Laboratories (INSTAND), Germany (652); Reference Institute for Bioanalytics (RfB), Germany (432); AOU-Careggi, Italy (38); Nasjonalkontrollen, Norway (75); SANBS, South-Africa (303); and UK NEQAS, UK (484). Among these 10 EQA providers, a total of 2,500 laboratories participated.

Preparation and sample shipment

Sample matrix was a defibrated donor plasma blood group O, Rh(D)+, spiked with the WHO International Standard Anti-D Immunoglobulin to achieve the 4 target concentrations of anti-D[26]. Samples were produced by a commercial provider (ANTITOXIN, Bammental, Germany), then stored and distributed to all EQA providers at +4°C, from where samples were shipped to the participating laboratories under ambiAll 4 samples were shipped by 9 EQA providers, while UKNEQAS chose to ship in its program 2 of the study samples (0.25 and 0.01 IU/mL). The EQA providers decided the order and designation of the samples, whether they were distributed all in 1 or over consecutive cycles of their 2024 EQA programs. Participating laboratories were informed by their EQA providers to analyze study samples in the same way as patient samples, typical for any EQA approach.

Anti-D quantification

Anti-D antibodies in our 4 samples were quantified using anti-D titration (CNRHP)[28]. This semi-quantitative titration assay used a column gel agglutination technique with papain-treated D positive red cells (D+C-E-c+e+ phenotype, RH:1,-2,-3,4,5) and serial 2-fold dilutions of the samples in comparison to a 0.12 IU/mL polyclonal anti-D standard (calibrated by the WHO International Standard Anti-D Immunoglobulin)[26]. Concentrations of anti-D were calculated by multiplying the inverse of the highest

Table I - *Anti-D antibody detection rates for the participating laboratories among the 10 EQA providers*

EQA provider[1]	Anti-D concentration (IU/mL)											
	0.1			0.025			0.01			0.005		
	Pos	Total	%	Pos	Total	%	Pos	Total	%	Pos	Total	%
RCPAQAP, Australia	125	125	100%	102	127	80.3%	33	126	26.2%	1	126	0.8%
ÖQUASTA, Austria	130	130	100%	122	126	96.8%	43	126	34.1%	3	130	2.3%
Sciensano, Belgium	143	144	99.3%	140	144	97.2%	51	144	35.4%	17	144	11.8%
Biologie Prospective, France	117	117	100%	113	113	100%	74	114	64.9%	41	115	35.7%
INSTAND, Germany	579	586	98.8%	555	586	94.7%	186	575	32.3%	44	572	7.7%
RfB, Germany	431	432	99.8%	413	430	96.0%	114	398	28.6%	22	432	4.9%
AOU-Careggi, Italy	37	37	100%	38	38	100%	12	35	34.3%	1	38	2.6%
Nasjonalkontrollen, Norway	71	71	100%	69	71	97.2%	23	72	31.9%	0	71	0%
SANBS, South Africa[2]	234	313	74.8%	149	341	43.7%	0	303	0%	1	303	0.3%
UK NEQAS, United Kingdom[3]	not shipped			483	484	99.8%	305	482	63.3%	not shipped		
Total[4]	1,867	1,955	95.5%	2,184	2,460	88.8%	841	2,375	35.4%	1,301	1,931	6.7%

[1]Detection rates differed significantly among the EQA providers at each anti-D concentration (p<0.0001; Chi square test, two-tailed). [2]Excluding the SANBS data, the detection rates did not differ significantly among the remaining 8 EQA providers at 0.1 IU/mL (p>0.05). [3]UK NEQAS shipped only 2 samples. Hence, data from its 484 participating laboratories are lacking for 0.1 IU/mL and 0.005 IU/mL. [4] Totals do not add up to 2,500, because some participating laboratories did not test their samples or did not report their data.Pos: positive.

reactive sample dilution by the concentration of the anti-D standard with the same agglutination strength.

Anti-D adjustment

The anti-D concentrations measured versus the concentrations calculated based on the dilution factor were 0.11 *vs* 0.1 IU/mL, 0.026 *vs* 0.025 IU/mL, and 0.011 *vs* 0.01 IU/mL, respectively. In our 4th sample, anti-D was detected with a concentration below the limit of quantitation (LoQ) at 0.0075 IU/mL. Assuming our dilution procedure and precision were comparable, we released this sample without verification, because a suitable method for quantification was lacking.

Homogeneity and stability testing

Vial-to-vial homogeneity of the samples was determined according to applicable international standards[29]. From the more than 10,000 aliquots, 12 aliquots for each of the 4 different anti-D concentrations were randomly selected and measured twice. The homogeneity of all samples was deemed adequate based on the mean and coefficient of variation (CV) of the 24 results per anti-D concentration. The anti-D concentrations were determined for a second time after the last EQA samples had been shipped to the participating laboratories. Our results confirmed the stability of the measurand until the study period had ended.

Data collection and evaluation

The EQA providers collected data generally in their routine way and reported their participants' results to our study database as Excel files in a predefined format. The information requested included pseudonyms (numbers) of the participant laboratories, the methods (visual or instrument), test cells and consumables (e.g. gel cards), name and manufacturer of the device if applicable, and the test principle. The results were reported as red cell antibody screening "positive" or "negative". We requested data on the type of participating laboratories and the reagents used, such as screening test cells and their antigen expression, enhancement media and antiglobulin reagents. Compliance with reporting this additional information was variable, often precluding its evaluation (**Figure 1**).

Our EQALM database was used to compile data submitted by the EQA providers. Routines transferred the data semi-automatically from report forms into the database. Data management procedures made the data comparable between EQA providers. These procedures included definition of common determination methods and expression of results. The database served to collect information on test systems, collate their results, and to query the dataset upon request.

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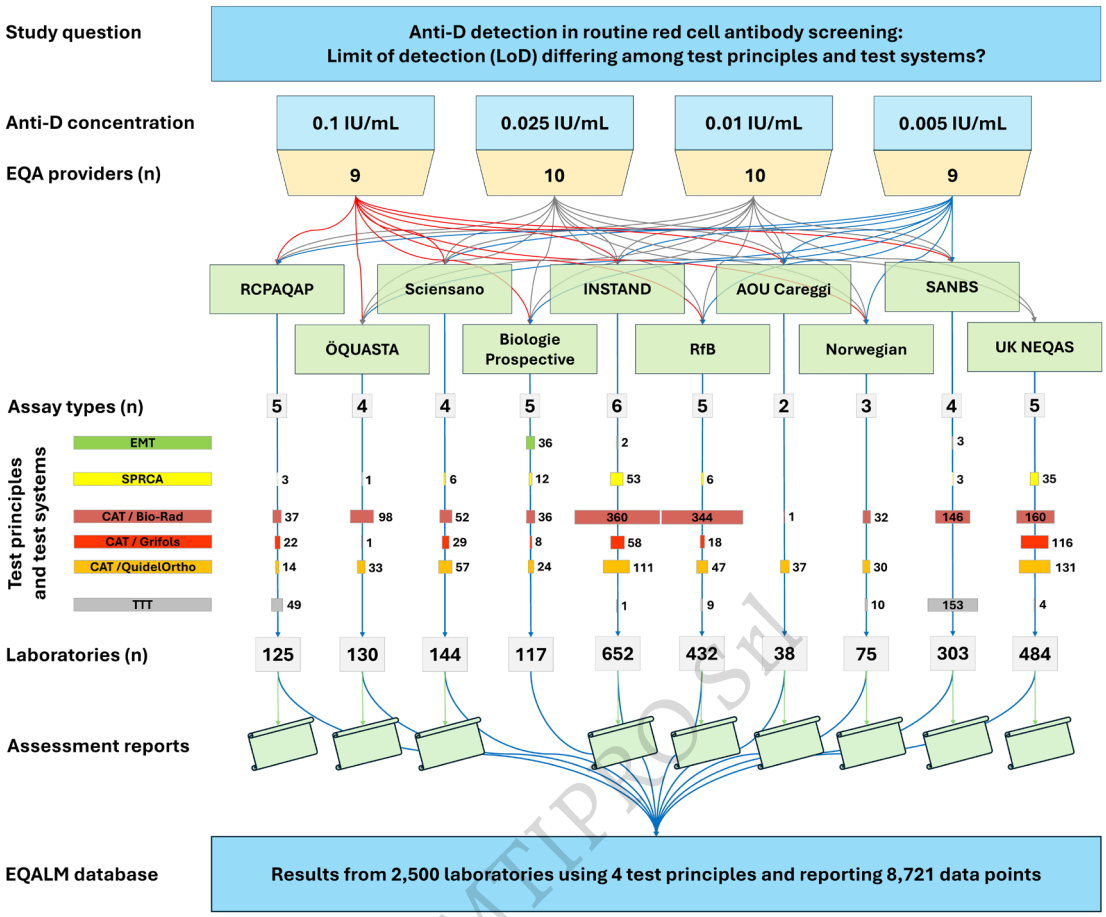

Figure 1 - Workflow of the EQA Super Challenge among 10 EQA providers covering 2,500 participating laboratories
More than 10,000 aliquots with 4 defined polyclonal anti-D concentrations diluted in human serum were distributed to the EQA providers, who shipped the aliquots to the laboratories. Results were collected by the EQA providers. They compiled assessment reports and uploaded their results online to the EQALM database.

Statistics

The proportion of positive responses was compared between techniques using a general linear model with a probit link. Fisher's exact test, two-tailed and Chi square test, two-tailed were performed to compare frequency distributions using a web resource[30]. P-value <0.05 was considered statistically significant. We did not correct for multiple testing. Statistically significant differences should be interpreted with caution, when many comparisons were tested and some cells had a small number of observations.

Manuscript preparation

We used the EQALM guidelines for publishing interlaboratory comparison studies (PubILC)[31] to plan this EQA super-challenge study and to write the manuscript (*Online Supplementary Content*).

RESULTS

We evaluated assay results from 2,500 laboratories comprising 8,721 data points, compiled by 10 national and international EQA providers (**Figure 1**). Test systems with instruments from 5 manufacturers and test cells from more than 24 manufacturers had been used, of which 8 were represented at least 15 times, the minimum number required for evaluation. Only 9 laboratories (<0.4%) reported test systems as Laboratory-developed test (LDT).

Sensitivity of anti-D detection

The overall detection rate for anti-D at the highest concentration of 0.1 IU/mL was 95.5% (**Table I**). If 1 outlier is excluded, the average rate of greater than 99% will be comparable among the remaining 9 EQA providers. However, the rates differed

significantly for 0.025 IU/mL and less (p<0.001, Chi square test, two-tailed). To explore possible causes, we investigated the influence of technical differences among the assays used. Reading methods, test principles, and test systems varied widely for the laboratories across continents.

Reading method

The anti-D antibody detection rates differed significantly between instrument and visual reading (**Figure 2**) based on data from 855 to 1,374 laboratories. For the remaining laboratories that did not report their reading method the anti-D detection trended toward rates typical for an instrument reading (*Online Supplementary* **Table SI**). All test principles allowed visual reading, but the newer ones were more often applied with instrument reading. Instruments with automated reading were generally superior to visual reading and manual methods.

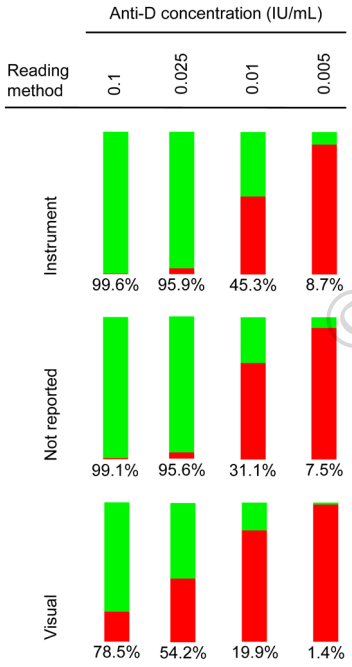

Figure 2 - Detection rates for red cell antibody assays
The WHO International Standard Anti-D Immunoglobulin was tested at 4 concentrations. Exactly 2,500 laboratories participated. The positive (green bar) and negative results (red bar) are depicted. The rates of positive results are calculated (%). The number of assays performed per group varied from 348 to 1,093 for a total of 8,721 assays (see *Online Supplementary* **Table SI** for detailed results). The difference between instrument and visual reading method was statistically significant at each of the 4 concentrations (p<0.0001, Fisher's exact test, two-tailed).

Test principle

The EQA providers reported the use of 4 test principles: test tube technology (TTT), column agglutination technology (CAT), solid phase red cell adherence (SPRCA) technology, and erythrocyte-magnetized technology (EMT)[32]. The anti-D antibody detection rates markedly differed for the 4 test principles (**Figure 3**). TTT was the outlier with 1/3 negative results even at 0.1 IU/mL and the only method dependent on visual reading. The other 3 test principles had comparable results at the 2 higher concentrations but began to differ significantly at 0.01 IU/mL and below.

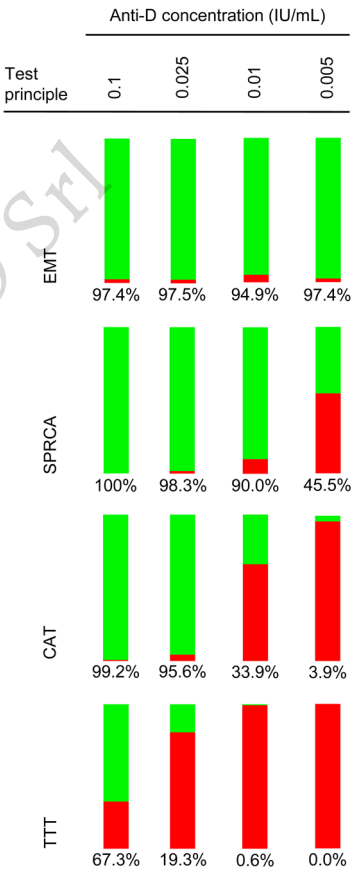

Figure 3 - Anti-D antibody detection rates for test principles
The 4 concentrations of the anti-D were tested with assays comprising 4 different test principles: test tube technology (TTT), column agglutination technology (CAT), solid phase red cell adherence (SPRCA) technology, and erythrocyte-magnetized technology (EMT). The rates are calculated (%) for positive results (green bar) and depicted relative to the negative results (red bar). The number of assays performed per group varied from 39 to 1,935 for a total of 8,061 assays (see *Online Supplementary* **Table SII** for detailed results), because the test principle was not reported for 660 assays (not shown). The difference among the test principles was statistically significant at each of the 4 concentrations (p<0.0001, Fisher's exact test, two-tailed).

CAT failed to detect 0.01 IU/mL and SPRCA at 0.005 IU/mL in most laboratories, only EMT reliably detected even 0.005 IU/mL (**Figure 3**), the lowest concentration in our study. Less than 10% of the laboratories did not report their test principle (*Online Supplementary* **Table SII**).

Utilization of test principles

The traditional TTT remained in use by 7% of the laboratories (*Online Supplementary* **Table SII**). EMT and SPRCA became routinely applied over the last 2 decades from 1 supplier each (Diagast, Loos, France and Werfen, Barcelona, Spain, formerly Immucor, Norcross GA, USA, respectively) and were used by a combined 6% of the laboratories (*Online Supplementary* **Table SII**) with large differences among the EQA providers possibly based on regional availability (*data not shown*). CAT test systems became routinely available in the early 1990s. The 3 CAT suppliers utilize a dextran-acrylamide matrix (Bio-Rad, Hercules CA, USA and Grifols, Barcelona, Spain) or glass beads matrix (QuidelOrtho, San Diego CA, USA, formerly Ortho Clinical Diagnostics), respectively. CAT was utilized in approximately 79% of the participating laboratories (*Online Supplementary* **Table SII**).

CAT test systems

The detection rates at 0.1 IU/mL were perfect in instrument reading for all 3 CAT systems (**Figure 4**). The majority of laboratories failed to detect anti-D below 0.025 IU/mL with any CAT system, although many more laboratories relied on instrument than visual reading (*Online Supplementary* **Table SIII**).

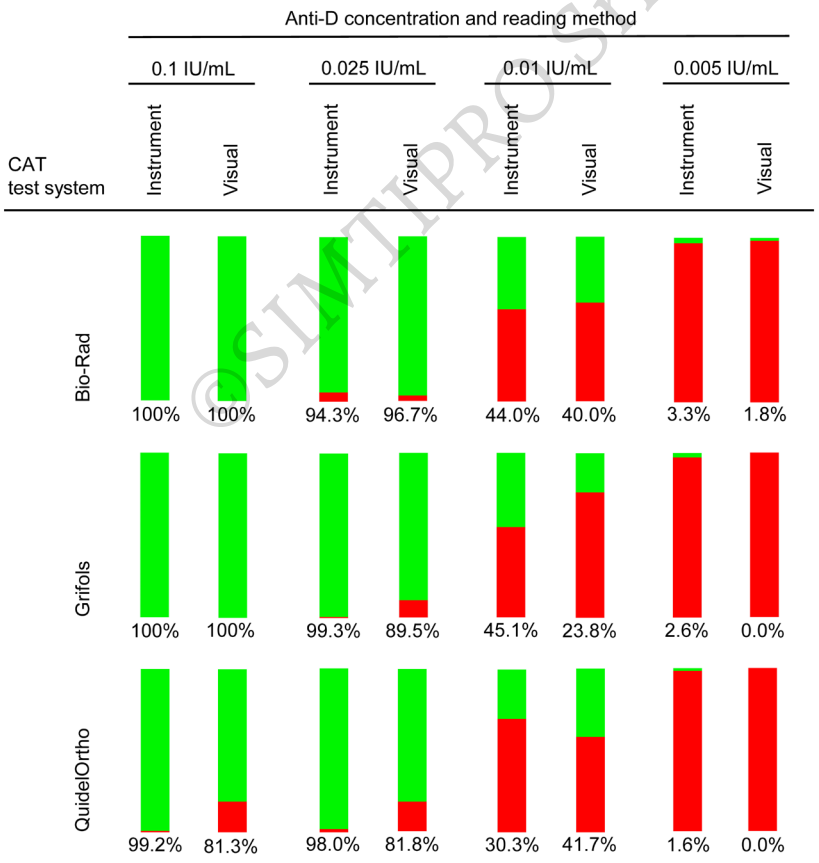

Figure 4 - Anti-D antibody detection rates for column agglutination technology (CAT) test systems
The 4 concentrations of the anti-D were tested with 3 CAT systems. The rates are calculated (%) for positive results (green bar) and depicted relative to the negative results (red bar). The number of assays performed in the 24 groups varied from 16 to 422 for a total of 3,129 assays with visual and instrument reading (see *Online Supplementary* **Table SIII** for detailed results). The reading method was not reported for 3,762 assays (*not shown*) among the total of 6,891 CAT assays in this study.

DISCUSSION

In a collaboration among 10 EQA programs, we documented the sensitivity of anti-D detection in current clinical routine in the areas covered by the EQA providers. Samples with distinct antibody concentrations, traceable to the international standard, were tested in 2,500 laboratories (**Table I**). Our results may be representative of most assays currently used for red cell antibody screening in patients worldwide and revealed options to improve antibody detection in routine clinical settings.

The reading method caused the most striking difference (**Figure 2**). Visual reading had a substantial failure rate even at the highest concentration, significantly different from any instrument reading. However, much of this difference was caused by the analytical principle (**Figure 3**). The traditional tube technique (TTT), still used in 7% of all clinical testing, requires visual reading and had the most failures. TTT results were mostly reported from 1 region, explaining the outlier relative to the remaining 9 EQAs (**Table I**). The other 3 test principles, EMT, SPRCA and CAT, were mostly read by instruments and then, yielded a nearly 100% detection rate at the highest concentration.

CAT, with a utilization reaching 79%, was the evident clinical standard for red cell antibody detection. Overall, the 3 CAT systems performed similarly (**Figure 4**). The glass beads matrix of 1 CAT system may be more challenging for visual reading than the 2 systems with dextran-acrylamide matrix, which however can be overcome by instrument reading (**Figure 4**). These results may be used to support a practice change towards an exclusive use of instrument reading in routine clinical settings for red cell antibody detection independent of the test principle.

Anti-D detection of 0.025 IU/mL was assured by any CAT system. The use of enzyme-treated red cells as reagents, not recorded by the EQA providers, may have decreased the LoD of the CAT systems[33] and explain some differences among laboratories for the 0.01 and 0.005 IU/mL samples. A further increase of the sensitivity for anti-D detection requires test principles other than TTT and CAT. While we collated results from less than 100 laboratories (*Online Supplementary* **Table SII**), we can conclude that SPRCA may detect 0.01 IU/mL and EMT 0.005 IU/mL or less (**Figure 4**). We documented significant differences among the commercially available assays routinely used in many clinical laboratories, which begs the question what sensitivity is minimally required for patient care and optimal patient safety.

All test principles and systems, including TTT or visual reading, reliably detected anti-D at concentrations considered relevant for the development of HDFN (3.5-5 IU/mL). However, this threshold was only established to avoid adverse effects for the fetus and newborn in pregnancy. No such threshold has been defined, if it exists, for sensitized individuals who will mount a secondary immune response upon being exposed once again to the involved antigen. The obligatory testing of all patients after transfusions or pregnancies and documentation of the results in registries would be one way to study antibody sensitization and evanescence[34,35]. Because routine follow-up testing may be neither practical nor cost-effective, antibody detection with higher sensitivity seems to remain the option of choice.

When no antibody is detected, results are commonly reported as "negative", "none" or "-". This kind of reporting ought to be replaced, because 2 critical pieces of information are missing. First, the technically correct wording should read as "(irregular red cell antibodies) not detected". Second, the test principle or medical device should be documented, because the antibody titer and detection threshold depend on the assay. Ideally, this improved reporting should be supplemented by the detection limit of the assay (LoD), for instance "anti-D <1 IU/mL"[9]. This revised practice will raise the awareness among clinicians that negative results cannot be taken lightly: Patients who have been sensitized before can be at risk for secondary immune response with life-threatening HTR or the fetus and newborn could experience HDFN, even when they test negative in all assays.

The sensitivity, as a characteristic of a diagnostic test, must be declared by manufacturers, required per IVDR. Laboratories must have this information available, required per International Standard ISO 15189:2022[36]. Devices and reagents for blood group testing are classified in the highest risk categories of IVD-MD. This classification calls for a thorough verification of the performance characteristics of test systems by their manufacturers. With IVDR having become effective in 2022, while fully enforced by 2031, the CE-certification of IVD-MD

(Class B or higher) is based on the performance assessment by notified bodies, as competent and independent third parties. In addition, the evaluation of Class D products involves a European Union Reference Laboratory (EURL)[37]. Hence, going forward a greater attention will be paid to describe the sensitivity of immunohematologic assays, such as their LoD, particularly when batches of reagents need to be evaluated and approved individually.

Once the detection limit (LoD) is routinely declared, the common "type-and-screen" approach[38] will be refined. In this common practice, the decision to perform a serologic crossmatch before transfusion is delayed or dropped altogether, when no antibodies are detected. Our data documented under routine practice conditions that the LoD varied by a factor of 10 or greater depending on the test principle (**Figure 3** and **4**). The number of patients experiencing secondary antibody booster and HTR, because their sensitization is missed by assays of lower LoDs, remains unknown[39]. Studies could be based on the LoD to ascertain which assays are most resource-efficient without posing avoidable HTR or HDFN risks to patients. More than 3.3 million patients in the EU and 4 million in the US receive red cell transfusions annually, some of whom experience HTR. This clinical evidence could be used to discern a safe LoD, if it exists, and contribute a huge dataset to the long-standing discussion about the existence and possible definition of correlates of protection (CoP) in the closely related field of vaccinology[40]. For red cell antibody detection, the participating laboratories may recognize approaches to evaluate the clinical impact of their practice and options for improvement.

The potency of red cell antibodies is generally expressed as titer (a dimensionless number) rather than as concentration (IU/mL). Both medical laboratory diagnostics and pharmaceutical analysis exemplify how quantitative methods for antibodies can routinely be applied in patient care[13,41]. An anti-D titer correlates only loosely with anti-D concentration: The threshold of 5 IU/mL, established for HDFN prevention, corresponded to a titer range between 128 and 1024, based on a CAT system. Conversely, a titer of 128 corresponded to concentrations between ~1 and ~11 IU/mL[13], representing a possible 10-fold error. An inaccuracy in the range of 1 order of magnitude may not be best for patient safety, and reporting of titers should be replaced by antibody concentrations to enable closer monitoring of pregnancies with red cell antibodies.

The number of participating laboratories was several-fold larger than for any individual EQA provider and covered regions across continents, impossible to achieve by any EQA alone. For instance, the significantly higher LoDs for EMT and lower for TTT (**Figure 3**) were only recognized, because we included 2 providers (Biologie Prospective and SANBS) covering different regions where either EMT or TTT use was widespread (**Figure 1**). EQA super-challenges have been applied in other fields since at least 2017 as recently reviewed (see Table 1 in[27]). These 11 studies revealed information far beyond the usual data from individual EQA providers. For instance, the annual HbA1c EQA super-challenge is now covering test systems from 21 manufacturers used by 5,120 laboratories in 23 countries[42]; a SARS-CoV-2 antibody study included laboratories from 64 countries and compared the performance of 176 test systems from 75 manufacturers[43]; and an avian influenza A H5N1 clade 2.3.3.4b study compared 26 commercial influenza A genome detection assays in 444 laboratories[44]. Creatively designed panels of samples allowed to concurrently evaluate a range of performance characteristics[45], such as analytical sensitivity, analytical specificity, linearity and variability of Ct values, their ability to detect selected variants of pathogens.

Limitations were an imbalance in the number of results for the various test principles and test systems. Some detailed information was not provided by the laboratories, such as D antigen density on the test cells, exact laboratory procedures, and the type and diagnostic focus of the participating laboratories reflecting staff competence.

EQA programs are slowly gaining recognition for the usefulness and significance of their data beyond the assessment of individual participants' results. This novel use of EQA data will allow to evaluate the performance of test principles using real-world data. The manufacturers, notified bodies, and supervisory and regulatory authorities can monitor the continued assay suitability by this post-market surveillance.

CONCLUSIONS

Any EQA provider individually, even the large ones, could not compile a similarly comprehensive overview of anti-D detection in routine clinical practice worldwide,

demonstrating the utility of our EQA super-challenge approach, novel for immunohematology. Our report showed greater 10-fold differences in the sensitivity of anti-D detection among 2,500 laboratories, which could be traced to test principles. Future investigations can evaluate the clinical significance of the statistically significant differences in anti-D sensitivity, address assay performance for red cell antibodies other than anti-D or multiple red cell antibodies occurring simultaneously and compare different reagent and test cell batches. We recommend changes in practice, such as assay selection based on their relative performance and the establishment of EQA performance benchmarks based on defined antibody concentrations. Clinical studies are needed to clarify whether our findings support the definition of minimum sensitivity targets for anti-D detection. Our data documented that assay sensitivity can be improved and standardized, contributing to patient safety.

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Autor contributions

CB conceptualized this study. CB and WC analyzed the data and verified its reproduction in the manuscript. CTN, AM provided methodology and resources for laboratory determinations. CB, GA, IBJ, AB, LB, SG, SG, RH, JK, TN, JPS, NS, and HS acquired data from their EQA programs. CB and WC visualized data. CB, CTN, AM, KH, RB, and GFK wrote the original draft; WAF revised the manuscript substantially; and CB and WAF wrote the final version. All Authors had full access to all data in the study, critically reviewed and edited the manuscript, and accept responsibility for its publication.

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Conflicts of interest

WAF serves as Section Editor in BT but had no role in the peer-review process or editorial decision. GFK and TW receive speaking fees from Bio-Rad Laboratories; the other Authors declare no conflicts of interest.

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ONLINE SUPPLEMENTARY CONTENT

Table SI - *Detection rates for anti-D antibodies*

Reading method[1]	Anti-D concentration (IU/mL)											
	0.1			0.025			0.01			0.005		
	Positive	Total	%	Positive	Total	%	Positive	Total	%	Positive	Total	%
Visual	277	353	78.4%	224	413	54.2%	77	386	19.9%	5	348	1.4%
Not reported	1,083	1,093	99.1%	1,038	1,086	95.6%	335	1,042	32.1%	81	1,076	7.5%
Instrument	507	509	99.6%	922	961	95.9%	429	947	45.3%	44	507	8.7%
Total	1,867	1,955	95.5%	2,184	2,460	88.8%	841	2,375	35.4%	1,301	1,931	6.7%

[1]Detection rates differed significantly between visual and instrument reading at each anti-D concentration (p<0.0001; Fisher's exact test, two-tailed).

Table SII - *Anti-D antibody detection rates of different test principles*

Test principle	Anti-D concentration (IU/mL)											
	0.1			0.025			0.01			0.005		
	Pos	Total	%	Pos	Total	%						
Erythrocyte-magnetized technology (EMT)	38	39	97.4%	39	40	97.4%	37	39	94.9%	38	39	97.4%
Solid phase red cell adherence (SPRCA)	75	75	100%	113	115	98.3%	99	110	90.0%	30	66	45.5%
Column agglutination technology (CAT)	1,527	1,539	99.2%	1,850	1,935	95.6%	640	1,886	33.9%	60	1,531	3.9%
Test tube technology (TTT)	107	159	67.3%	35	181	19.3%	1	156	0.6%	0	151	0.0%
Test principle not reported	120	143	83.9%	147	189	77.8%	64	184	34.8%	128	144	88.9%
Total	1,867	1,955	95.5%	2,184	2,460	88.8%	841	2,375	35.4%	1,301	1,931	6.7%

Pos: positive.

Blood Transfus 2026; doi: 10.2450/BloodTransfus.1277

Table SIII - *Anti-D antibody detection rates of CAT test systems*

CAT test system	Reading method	Anti-D concentration (IU/mL)											
		0.1			0.025			0.01			0.005		
		Positive	Total	%	Positive	Total	%	Positive	Total	%	Positive	Total	%
Bio-Rad	Visual	112	112	100%	119	123	96.7%	46	115	40.0%	2	110	1.8%
	Not reported	1,039	1,044	99.5%	1,141	1,203	94.8%	408	1,169	34.9%	45	1,040	4.3%
	Instrument	274	274	100%	398	422	94.3%	182	414	44.0%	9	274	3.3%
Grifols	Visual	16	16	100%	17	19	89.5%	5	21	23.8%	0	15	0.0%
	Not reported	136	137	99.3%	248	254	97.6%	86	247	34.8%	9	131	6.9%
	Instrument	40	40	100%	151	152	99.3%	64	142	45.1%	1	38	2.6%
QuidelOrtho	Visual	13	16	81.3%	18	22	81.8%	10	24	41.7%	0	16	0.0%
	Not reported	346	352	98.3%	454	473	96.0%	126	462	27.3%	4	353	1.1%
	Instrument	128	129	99.2%	250	255	98.0%	76	251	30.3%	2	129	1.6%
Total	Visual+Instrument	583	587	99.3%	953	993	95.9%	383	967	39.6%	14	582	2.4%
	Not reported	944	952	99.2%	897	942	95.5%	257	919	30.0%	46	949	4.9%
	All	1,527	1,539	99.2%	1,850	1,935	95.6%	640	1,886	35.9%	60	1,531	3.9%

EQALM guidelines for publishing interlaboratory comparison studies (PubILC) (Item)

Description of activity (1)	
(1a)	Description of the activity - The description of the activity should be consistent and unambiguous to make the nature of the activity and the publication reporting on it clear. Activity is described in Materials and Methods section.
(1b)	Additional information on the activity reported on - More detailed information on the activity and the local regulations for participation in EQA/PT should be described. n/a
(1c)	Initiator (and executor, if different) of the activity reported on; business purpose of the initiator - The initiator of the ILC activity should be named and their business purpose should be declared. This study was initiated by the Immunohematology Working Group of EQALM, the umbrella organization of EQA providers for laboratory medicine; executors were EQA providers identified via the professional network of EQALM;
(1d)	Purpose of the activity - Purpose of the activity should be clearly defined and reflected in the publication. If an activity is reported with a purpose different from what is ultimately intended (incidental finding), the original purpose and the unexpected outcome should be stated. The purpose of the study was to evaluate and compare the sensitivity for anti-D antibodies of different RBC antibody screening systems in routine use;
(1e)	Time span of activity - The time span within which the samples were analysed should be provided; this provides information about the prevailing knowledge at the time of the study; the usual methodical performance of the analysis, or the predominant strains and variants of infectious agents that were used as sample material at the time of the study. Dates when samples were shipped to EQA participants are given in Materials and Methods.
(1f)	Number of items (samples) included in the sample panel - Information on the number of samples used in the activity reported on should be provided. The sample panel consisted of four samples positive for anti-D; results obtained in analyses for characterization of the samples are given in Materials and Methods section.
(1g)	ISO 17043 accreditation status of EQA provider / scheme / parameter - Accreditation ensures that the organizer of the study has a universally accepted and peer-reviewed management system for providing EQA/PT services. Not applicable for a super-challenge, as each EQA provider obtains samples and assesses results according to their procedures.
Information on items (samples) used (2)	
(2a)	Type, origin and manufacturer – type of sample (serum, plasma (with detailed information, e.g., EDTA, lithium heparin), urine, any other substance); If commercial material is used, its manufacturer and detailed information on the product(s) should be provided. Items (samples) used in ILC activities may also be data (e.g., virtual microscopy, data sets). The manufacturer, matrix and material used for spiking are reported in Materials and Methods section.
(2b)	Ethics for samples with human origin - Result of the referral to the competent ethics committee if substances of human or animal origin were used. The basis for this can be found in the Declaration of Helsinki. n/a
(2c)	Detailed specification and justification for selecting each sample - It should be stated, for which purpose each sample was included in the sample panel. Samples 1-4 had decreasing concentrations of anti-D and were intended to identify detection rates of test systems at different concentrations.
(2d)	Matrix and additives - Information on components in sample materials except the measurand should be provided. Matrix is described in Materials and Methods.
(2e)	For quantitative analyses: Range of concentration of measurand(s) - It should be reported whether a measurand is in the low - medium/normal - high range and/or around clinical decision limits; report quantitative results preferably in SI units. It is explicitly stated that the samples have very low concentrations of anti-D antibodies and that the purpose of the study is to analyze such samples.
(2f)	For qualitative analyses: Identity of examinand - The identity of examinands in samples for qualitative analyses should be reported. The identity of the analyte is specified (anti-D antibodies)
(2g)	Attestation of sample homogeneity - Adequate homogeneity of the measurand(s) in samples is required so that all participants receive the same starting materials for their analyzes and thereby equal opportunities to obtain correct results. Vial-to-vial homogeneity was determined according to applicable international standards and were found adequately homogenous; see Materials and Methods section.
(2h)	Attestation of sample stability - Adequate stability of the measurand(s) in samples is required so that all participants receive the same starting materials for their analyzes and thereby equal opportunities to obtain correct results. Stability testing procedures are described in. Stability of the measurand was determined according to applicable international standards and were found adequately stable; see Materials and Methods section.
(2i)	Physical properties of samples / conditions during shipment to participants - Environmental conditions during transportation can affect the properties of materials; measures to prevent this should be described. Storage and transport conditions are described in Materials and Methods section.
(2j)	Activities required to prepare samples prior to analysis should be described. None; see Materials and Methods.

(2k)	Classification of samples according to the requirement to submit correct results for them to pass the activity - Depending on the design of the EQA/PT activity, it may be possible that reporting correct results for some samples is required, but incorrect results are accepted for other samples to still pass the round. If applicable, the reasons should be stated. It was left to the participant EQA providers to decide whether results reported for S1-S4 were to be assessed and what target they used.
(2l)	Commutability of sample materials if applicable - If commutability of samples was evaluated, details should be provided [60]. If commutability was not evaluated, it should be stated that no definitive conclusions on accuracy can be made from the comparisons of results obtained with various methods. n/a
(2m)	Volume per sample - If relevant for the results presented, the volume of each sample provided to the participants should be presented. The samples were filled in the usual quantities of the EQA providers.
	Information and instructions to participants (3)
(3a)	Statement how participants were instructed to analyse samples - Participants should use their unmodified routine procedures when analysing EQA/PT samples (as far as practicable). It should be stated that participants were made aware of this. However, in some challenges it might be required to deviate from routine procedures and for example analyse materials in triplicate. Information is given in Materials and Methods section.
	Information on participant entities (4)
(4a)	Number of participant entities / respondents - The number of entities registered for the challenge and the rate of respondents should be provided. Given in Results section.
(4b)	Types of participant entities - Information on the participant laboratories should be provided. n/a
(4c)	Location (state(s), country, region) - The geographical place where the EQA/PT activity took place should be described. Given in Table I.
	Information on participating measurement procedures (5)
(5a)	Trade name of devices and reagents used (measurement system) - Participant test systems (devices and reagents) should be identified; regarding an international readership, ideally including Unique Device Identifiers (UDI) Manufacturers of antibody screening test systems are named in the text.
(5b)	Methods / techniques applied by the measurement systems - If relevant for the results presented, methods applied by test systems (5a) should be described. Methods are described in the text.
(5c)	Lot numbers of reagents - If relevant for the results presented, Lot numbers of reagents should be reported. n/a
	Submission of results (6)
(6a)	Possible ways provided by the EQA/PT organizer to report results - It should be described how results are reported to the EQA/PT provider/organizer. Contact remained with the individual EQA providers, who collected and reported the data to the study centre.
(6b)	Format, unitage (for quantitative results) and - if applicable - factorized results - It should be described whether results are reported by selecting from a dropdown menu, entering free text (incl. numeric results) and whether quantitative results are to be reported in a specified unit or by selection of units (e.g. from a drop-down menu); preferably SI units should be used. n/a
(6c)	Applicable limitations for acceptance of results - If applicable, limitations for submission of results should be reported. n/a
	Evaluation and assessment of results (7)
(7a)	Determination of the target /assigned value (including uncertainty of the target value) - The traceability of target values should be described, e.g., reference measurement procedure, certified reference materials, overall mean/median, peer group, consensus values, expert laboratories; including - if applicable - uncertainty of the target value. The specified methods for determining the targets / target values correspond to requirements in ISO 13528:2022. If the targets are determined by one or several laboratories, the methods used by them should be described (they may differ from the methods used by participant laboratories). A WHO Standard was used to spike EQA samples. Concentration of measurand was measured in an expert laboratory.
(7b)	Acceptance criteria - The basis for deciding which results are accepted (pass) and which are not (fail); analytical performance specifications (APS) and - if applicable - APS per intended use of the test should be described. Determination of acceptance criteria and assessment of results remained with the individual EQA providers.
(7c)	Outlier removal procedures employed - It should be described how the statistical model deals with outliers. n/a
(7d)	Statistics applied - Basic and advanced statistics applied in routine assessment of results. Statistical methods used are described in the Materials and Methods section.

Reporting of assessment results (8)	
(8a)	***to participants*** - The way participants are informed about their performance should be described. Remained with the individual EQA providers.
(8b)	***to other interested parties*** - If applicable, the way of notifying other interested parties should be presented. Remained with the individual EQA providers.
Findings (9)	
(9)	Information gained according to the purpose of the activity - Study results, answering the study question, observed variability between laboratories and potential clinical impact. Study objectives and outcome are clearly described; the potential clinical impact needs to be evaluated.
Limitations (10)	
(10)	EQA/PT related limitations - A limitation of EQA/PT studies may be that results can only be analysed as they were reported by participants. It must be trusted that they were generated properly and with the test system specified. Included in the text.
Impact of the outcome of the activity (11)	
(11a)	Educational aspects for participants, clinical impact of the findings - Educational aspects can be an integral part of ILC activities and publications about them. If the activity being reported has such aspects, they should be mentioned in the publication. The entire publication is educational.
(11b)	Knowledge gain for other interested parties - If applicable, information revealed by the study and relevant for other interested parties should be made clear. Authorities and manufacturers could become aware of the current disregard of the limits of detection of red cell antibody screening tests.
(11c)	Identified areas for improvement (for the EQA/PT provider) - If applicable, improvement potentials revealed by the study should be reported. Attention of European Reference Laboratories, which will be responsible for the approval of individual lots of red cell antibody screening tests.