Heterophilic antibody interference in commercial immunoassays; a screening study using paired native and pre-blocked sera

Nils Bolstad[1,*], David J. Warren[1], Johan Bjerner[2], Gunnhild Kravdal[3], Lutz Schwettmann[4], Kari H. Olsen[1], Pål Rustad[2] and Kjell Nustad[1]

[1] Department of Medical Biochemistry, Oslo University Hospital, Radiumhospitalet, Oslo, Norway

[2] Fürst Medical Laboratory, Oslo, Norway

[3] Unit of Medical Biochemistry, Division for Diagnostics and Technology, Akershus University Hospital, Lørenskog, Norway

[4] Department of Medical Biochemistry, Ålesund Hospital, Ålesund, Norway

Abstract

Background: Heterophilic antibodies are still an important source of interference in immunoassays. We have conducted a screening study for interference in a panel of commercially available assays using two sera known to contain high titer Fc-reactive heterophilic antibodies.

Methods: The sera were distributed to laboratories participating in the Nordic External Quality Assessment cooperation (EQANord). Duplicate samples pre-blocked with aggregated murine monoclonal MAK33 were also supplied. Discrepancies ($>50\%$) between the results for native and blocked samples were used to classify the tested assays as susceptible to interference. A total of 170 different assay kits covering 91 analytes were tested.

Results: We found that 21 assays, covering 19 different analytes, were susceptible to interference from the heterophilic antibodies in the two sera. Many of these are clinically and commercially important assays. Some of the false results were grossly elevated and could have been detrimental to patient care in a clinical setting.

Conclusions: Heterophilic antibodies with Fc-reactivity remain a threat. A more widespread use of antibody fragments and aggregated immunoglobulin could potentially improve the heterophilic antibody resistance of assays intended for clinical use.

Keywords: HAMAs; heterophile antibody; heterophilic; interference; immunoassay.

Introduction

Immunoassay techniques have revolutionized the determination of clinically relevant protein and peptide analytes. However, these methods do not always give the ''correct'' result (1–4), and extreme caution is needed when clinical findings and assay results are discordant. The unnecessary diagnostic and therapeutic interventions that often follow such discordance can be costly to both patients and hospitals (5–11). In these situations troubleshooting is complicated by the proprietary nature of most information relating to the assay kits.

Immunometric assays are particularly sensitive to interference by multivalent antibody-binding moieties that can bridge the reagent antibodies. Such cross-linking results in the generation of positive assay signals in the absence of analyte. Heterophilic or human anti-mouse antibodies (HAMAs) present in patient sera are the usual culprits (1, 3, 4). Several approaches can be effective in limiting heterophilic antibody interference including sample pretreatment with heterophilic blocking tubes (HBT) (12), polyethylene glycol (PEG) precipitation (13), affinity chromatography on protein A (14) or size exclusion chromatography (15). These methods are however not well suited to the high-throughput assays used in clinical laboratories. Indeed, optimal reductions in assay interference can most probably only be achieved by focusing on this problem during the assay design phase (16).

In a previous study, we found that the frequency of interference in our in-house immunometric assay for carcinoembryonic antigen (CEA) was 4.0% (1). The addition of a heat-aggregated irrelevant murine monoclonal antibody, MAK33, to the assay buffer reduced the frequency to 0.86%. Significantly, the use of F(ab')$_2$ fragments as assay solid phases was found to reduce the frequency of interference to 0.1% even in the absence of irrelevant mouse immunoglobulin. This is in agreement with previous studies which indicated that most interfering immunoglobulin target the Fc portion of assay antibodies (17, 18).

Most commercial immunoassays have irrelevant animal immunoglobulin added to the assay reagents in order to limit interference. Some manufacturers aggregate the immunoglobulin by heat or chemicals, but this is rarely detailed in package inserts. To our knowledge, only a limited number of commercial assays are constructed using F(ab')$_2$ or Fab' fragments.

Herein we describe the results from a screening study of heterophilic antibody interference in commercially available immunoassays using two high titer sera originally referred to

our laboratory for testing. Both sera, shown to contain Fc-reactive heterophilic antibodies, were distributed to 18 clinical laboratories through the Nordic External Quality Assessment cooperation (EQAnord). The sera were supplied in both native form and pre-blocked by the addition of heat-aggregated MAK33 non-specific immunoglobulin.

Our aims were firstly to investigate if selected sera, supplied as paired native and pre-blocked specimens, can be used as screening tools for assay interference and secondly, how well a panel of commercial assays were protected against Fc-reactive heterophilic antibodies.

Materials and methods

Human test sera used for assay screening

Serum 1 was from a man in his fifties. Interference was suspected when analysis of soluble transferrin receptor (Tina-quant® sTfR on the Cobas Integra 800; Roche Diagnostics, Mannheim, Germany) gave an elevated result without corresponding laboratory results or clinical symptoms. The result was normalized after addition of aggregated MAK33 to the sample. Approximately 6 months after he donated serum to our study, he was diagnosed with rheumatoid arthritis.

Serum 2 was from a woman in her thirties. Interference was suspected when analysis of β-hCG (total β-hCG on the Architect i2000 SR; Abbott Diagnostics, Abbott Park, IL, USA) gave an elevated result without concomitant pregnancy or malignancy. The result was normalized after addition of MAK33. Re-analysis of β-hCG with a different method (hCG + β on the Cobas e601; Roche) confirmed the normal result. Tragically, prior to our identification of heterophilic antibody interference, this donor endured unnecessary chemotherapy and three inappropriate surgical procedures, including the laparoscopic removal of a fallopian tube.

Both sera were obtained with informed consent following national and institutional guidelines. The study has been approved by the Oslo university hospital privacy office.

Characterization of heterophilic antibodies

All assays used to characterize the sera were manual 3-step methods performed using streptavidin-coated DELFIA® microtitration strips. The wells were coated with biotinylated antibodies, washed, and then incubated with the serum samples. Following additional washing, the assays were developed using europium-labeled tracer antibodies. Methodological details are given in a supplemental file (web only) and in Bjerner et al. (19).

To detect heterophilic antibodies, and characterize their reactivity to murine immunoglobulin, assays were established using intact IgG, F(ab')$_2$, and Fc fragments of the IgG1 monoclonal antibodies K57 (anti-α-fetoprotein) and T84.66 (anti-carcinoembryonic antigen) as solid phase reagents and K57-IgG as tracer. These are nonsense immunometric assays since they use non-complementary assay pairs. A positive signal indicates the cross-linking of the solid phase and tracer antibodies in the absence of analyte.

Species specificity of the heterophilic antibodies was determined using polyclonal murine, rabbit, ovine, equine, bovine, and human IgG as solid phase antibodies and K57-IgG as tracer antibody.

The size of the interfering antibodies was estimated by gel-permeation chromatography on a pre-calibrated Superdex S200 column. Column fractions were assayed using a non-sense method (solid phase K57-IgG, tracer K57). Isotyping was performed using K57-IgG as solid phase antibody and commercially available rabbit F(ab')$_2$ antibodies to human heavy and light chains as tracers.

Selection of tested assays and participating laboratories

EQAnord provided invaluable assistance in selecting representative laboratories and methods. Particular focus was directed at including the assays most widely used in the Nordic countries. Where possible, assays performed on different instrument models from the same manufacturer were tested. Of the 19 laboratories invited to participate in our study, only one laboratory declined, citing reorganization of laboratory services. The participating laboratories were invited to include immunoassays at will if they had surplus test sera. For this reason, some in-house assays and non-immunometric (competitive) assays were included in the study. A total of 170 commercial immunoassay kits were tested.

Heat treatment of MAK33

Murine monoclonal IgG1κ antibody MAK33 (Roche Molecular Biochemicals, Mannheim, Germany) was stored at a concentration of 2 g/L in 0.15 mol/L NaCl, 0.01 mol/L Na$_2$HPO$_4$, pH 7.4, at −30ºC. Aliquots were heat-treated by incubation in a 60ºC water bath for 10 min (1). The change in absorption at 595 nm from approximately 0.02 to approximately 1.0 was used to monitor aggregation.

Screening of commercial immunometric assays

Aliquots of the two patient sera with known interference from heterophilic antibodies were distributed to participating laboratories. Duplicate samples pre-blocked with 180 μg/mL aggregated murine monoclonal MAK33 were also supplied. The participating laboratories were informed about the purpose and design of the study, but were not informed about which aliquots were blocked with MAK33. Laboratories were instructed to perform analyses and report results as for routine samples.

Prior to the study, we set a cut-off limit of 50% for the difference between the results from native and pre-blocked sera to indicate if the method tested is vulnerable to heterophilic antibody interference. As assays are subject to analytical variation, such differences may occur by chance. The probabilities for an observed 50% difference between native and pre-blocked sera by chance are p = 0.001 at an analytical CV of 11.4%, p = 0.01 at a CV of 15.1% and p = 0.05 at a CV of 21.4%. The three smallest differences considered to be significant in the study were in the SHBG and BNP assays from Abbott laboratories, with observed differences of 60% and 107%, and analytical CV of 10% and 12% as stated by the manufacturer, and the AutoDELFIA TSH assay from Perkin Elmer Life Sciences, with an observed difference of 60%, where analytical CV has been reported as 2.8% (20). This corresponds to p < 0.001 for all the three assays. Thus, all differences reported in the study correspond to p < 0.001. Our screening method might not be suitable for some of the included assays, e.g., competitive assays or assays using non-murine antibodies. As we have little experience with interference in competitive assays, we have not classified these assays based on our test. However, results from all tested assays are presented in Table 2 in the electronic supplement that accompanies this paper.

Results

Characterization of sera

Both sera gave grossly elevated responses in non-sense assays indicating the presence of heterophilic antibodies with

affinity for whole IgG and the Fc fragments of IgG1 antibodies. They also displayed high titers and could be diluted 1:300 (serum 2) and 1:3,000 (serum 1) before a positive assay signal was lost. Responses were normalized after the addition of heat-aggregated MAK33 immunoglobulin to the sera. Very little binding to F(ab')$_2$ fragments was observed (Figure 1).

In addition to their strong reactivity to murine IgG1-antibodies, the heterophilic antibodies in serum 1 showed some cross-reactivity to rabbit IgG, but minimal cross-reactivity to human, bovine, and equine IgG, while the heterophilic antibodies in serum 2 showed some cross-reactivity to bovine IgG (Figure 2).

Gel-permeation chromatography indicated that the size of the heterophilic antibodies was >650 kDa suggesting that they are most likely IgMs (data not shown). Isotyping using a modified non-sense assay gave strong signals for μ and κ but comparatively low signals for λ light chain. However, we were unable to detect monoclonal components using the routine methods available at our hospital: capillary zone electrophoresis with immunotyping and immunofixation electrophoresis.

Screening of commercial immunoassays

Analysis of one or both sera showed interference in 21 assay kits covering 19 different analytes (Table 1). As expected, interference was not limited to assays from one or a few manufacturers. False results were seen for both test sera in 13 assays, while 8 assays gave false results for either serum 1 (6 assays) or serum 2 (2 assays). The degree of false elevation varied between assays. In 15 assays, results for one or both native samples were increased at least five-fold compared to corresponding blocked samples. In 11 assays, the difference between native and blocked sera was >10-fold. No assays displayed negative interference in this study. The
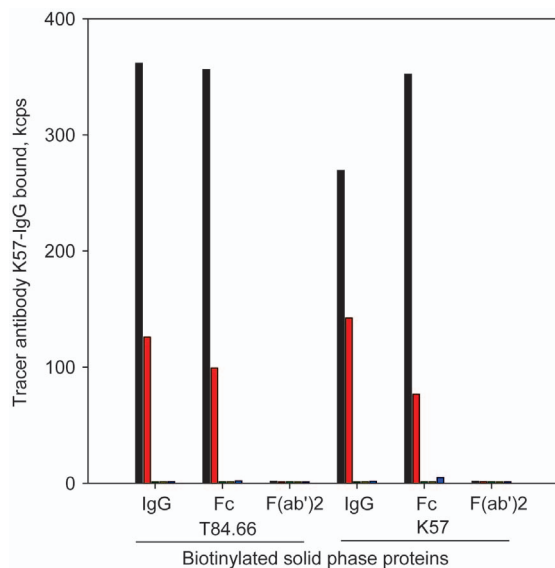

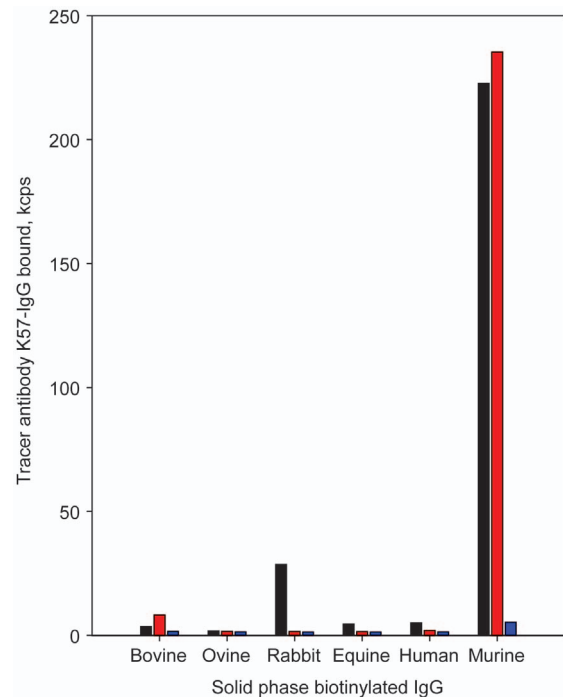**Figure 2**   Reactivity of heterophilic antibodies to IgG from different animal species.

Figure 1   Reactivity of heterophilic antibodies to murine IgG1 fragments.

results for the pre-blocked samples in vulnerable assays were comparable to corresponding results for the native samples in resistant assays (Table 2, electronic supplement).

For one assay, CA125 (CA125II, Abbott) on the Architect platform, our results could indicate a significant variation between lots with respect to vulnerability from interference (lot numbers are reported in the electronic supplement). For the other interference-positive assays where different lots were tested, such as β-hCG (total β-hCG, Abbott) and soluble transferrin receptor (Tina-quant® sTfR, Roche), inter-batch variation was not observed in this study.

CA125 on Abbott Architect models i2000 and ci8200, and β-hCG on Abbott Architect models i2000SR, ci8200 and ci16200 showed falsely elevated results for both sera tested, with little or no difference between instruments. All results were normalized when adding heat-treated MAK33. The results obtained for CA125 and β-hCG on the Architect instruments using pre-blocked sera are comparable to results from assays negative for interference.

D-dimer (STA®-LIATEST® D-DI) on STA-R Evolution and STA Compact; (Diagnostica Stago, Gennevilliers, France, www.stago.com) showed grossly elevated results for both sera tested. Although significantly lower than in the native sample, results after addition of heat treated MAK33 in serum 1 differed from results obtained with assays negative for interference. This is most likely due to the addition of inadequate amounts of aggregated immunoglobulin to completely block interference in this sample in this particular assay. Results for serum 2 with MAK33 are comparable to those from assays negative for interference. The values obtained in serum samples are higher than in a plasma sam-

Table 1 Assays vulnerable to inference in the present study[a].

Analyte, unit	Manufacturer	Instrument	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2
AFP, µg/L	Diasorin	Liaison	2.6	**4.2**	0.6	**2.8**
BNP, pmol/L	Abbott	Architect i2000SR	<2.9	**6.0**	<2.9	<2.9
		Architect ci16200	<2.9	3.4	<2.9	<2.9
CA125, kU/L	Abbott	Architect i2000	8	**256**	6	**96**
		Architect ci8200	7	**278**	6	**84**
		Architect i2000	7	**20**	6.7	**20**
	Diasorin	Liaison	5.5	7	4.2	**74**
CA19-9, kU/L	Diasorin	Liaison	17	18	7.3	**89**
CEA, µg/L	Diasorin	Liaison	<1	**1.9**	<1	**3.6**
Crosslaps, ng/L	Roche	Modular E 170	75.6	**1838**	11.5	**32.8**
CRP[b], mg/L	Roche	Cobas Integra 800	1.1	**3.4**	<0.6	0.7
		Modular P	<0.6	**4.3**	<0.6	<0.6
D-dimer[b], mg/L	Diagnostica Stago	STA-R Evolution	1.8	**>4.0**	0.6	**>4.0**
		STA Compact	2.5	**>20**	0.5	**2.3**
		STA Compact	2.1	**>20**	0.6	**2.2**
GH, µg/L	PerkinElmer	AutoDELFIA	0.1	**1.5**	0.1	**0.5**
hCG, U/L	Abbott	Architect ci16200	<1.0	**42**	<1.0	**113**
(Total β)		Architect i2000 SR	<1.2	**59**	<1.2	**147**
		Architect ci8200	<2	**36**	<2	**117**
Insulin, pmol/L	Siemens	Immulite 2000	351	**1191**	22	**131**
Interleukin 1β, ng/L	Bio-Rad	Bio-Plex 200	<0.13	**134**	<0.13	**2.8**
Interleukin 2, ng/L	Bio-Rad	Bio-Plex 200	<0.13	**685**	<0.13	**4.8**
Interleukin 6, ng/L	Bio-Rad	Bio-Plex 200	18.8	**232**	1.6	**11**
PAPP-A, U/L	PerkinElmer	AutoDELFIA	0.003	**0.16**	0.002	**0.21**
SHBG, nmol/L	Abbott	Architect i2000	32.8	**52.6**	97.3	98
	Beckman Coulter	UniCel DxI 800	29	**99.5**	73.7	85
sTfR[b], mg/L	Roche	Modular P	3.5	**26.5**	1.7	1.8
		Cobas Integra 800	4.4	**19.2**	1.9	**3.1**
		Hitachi 917	2.7	**25.7**	1.7	1.8
TNF-α, ng/L	Bio-Rad	Bio-Plex 200	1.2	**95.5**	4.8	5.3
TSH, mU/L	PerkinElmer	AutoDELFIA	1.5	**2.3**	1.6	2.1

[a]Results indicating interference (difference >50% between native and blocked sample) in bold. [b]Immunoturbidimetric assays. Assays resistant to interference in our study (listed by manufacturer): Abbott: AFP, anti-CCP, anti-CMV IgG, anti-HAV IgG, anti-HAV IgM, anti-HBcII, anti-HBs, anti-HCV, anti-HTLV I/II, anti-rubella IgG, anti-toxo IgG, anti-toxo IgM, anti-TPO, B12, CA15-3, CA19-9, CEA, CRP, ferritin, FSH, FT3, FT4, HBsAG, HIV Ag/Ab combo, LH, prolactin, PSA, PSA(free), PTH, transferrin, tTroponin I, TSH. Beckman Coulter: anti-TPO, BNP, CA125, CEA, estradiol, ferritin, FSH, hCG (total β), LH, prolactin, PSA, PTH, testosterone, troponin I, TSH. Biomerieux: anti-CMV IgM. Bio-RAD: anti-toxo IgG, anti-toxo IgM, anti-mycoplasma IgM. Biotest: anti-EBNA IgG. Biotrin: anti-parvo B19 IgG, anti-parvo B19 IgM. Brahms: AFP, CA19-9, procalcitonin, TgAB**.** Dade Behring (Siemens): anti-borrelia IgG, anti-borrelia IgM, anti-HSV IgG, anti-syphilis, anti-varicella IgG. Diasorin: CA15-3, NSE. Instrumentation Lab: d-dimer. Novitec (Orion): anti-EBV VCA IgG, anti-EBV VCA IgM. Ortho: BNP, CRP, troponin I. PerkinElmer: α-1-antitrypsin, LH, FSH, β-hCG (free), PSA, PSA (free). Roche: AFP, ApoA1, ApoB, CA15-3, CA19-9, CA72-4, CA125, CEA, cortisol, C-peptide, cyfra 21-1, D-dimer, DHEAS, estradiol, ferritin, FSH, FT3, FT4, haptoglobin, hCG (hCG+β), IgA, IgG, IgM, insulin, LH, myoglobin, NSE, NT-proBNP, progesterone, prolactin, PSA, PTH, S-100, SHBG, testosterone, transferrin, troponin T, troponin T hs, TSH, TSH-receptor-Ab. Siemens (Advia-platforms): anti-TPO, B12, CRP, ferritin, FSH, hCG (ThCG), LH, prolactin, PSA, PTH, TSH. Siemens (Immulite-platforms): ACTH, calcitonin, CDT (%), C-peptide, GH, hCG, IGF1, interleukin 1β, interleukin 6, interleukin 8, interleukin 10, NT-proBNP, sIL-2R, TgAB, Tg (thyroglobulin), TNF-α.

ple due to the expected formation of some d-dimer fragments during the coagulation phase prior to sample centrifugation.

Insulin on Immulite2000 (Siemens Healthcare Diagnostics, Erlangen, Germany, www.medical.siemens.com) showed falsely elevated results for both sera tested. Results after addition of heat-treated MAK33 are very similar to the results from another insulin-assay (Roche).

Interleukins 1β, 2, and 6 analyzed on Bio-Plex 200 (Bio-Rad Laboratories, Hercules, CA, USA, www.bio-rad.com) all gave falsely elevated results for both sera tested. TNF-α gave a falsely elevated result for the native serum from donor 1. For all four samples, duplicate results were reported for each analyte. All results are given in the electronic supplement, while only the first result for each analyte is given in the printed table. It should be noted that for serum 1 (both native and blocked), the Bio-Plex instrument gave a warning signal with the result, possibly due to bead aggregation.

Discussion

Characterization of the sera used in this study indicated that they contained heterophilic antibodies belonging to the IgM class with principle reactivity to the Fc domain of murine

IgG1. In our non-sense assays this reactivity was effectively blocked by the addition of heat-aggregated MAK33 immunoglobulin. These observations permitted the use of the sera, as paired native and pre-blocked samples, to test if commercial immunoassays are sufficiently protected against interference from Fc-reactive heterophilic antibodies.

The fact that such a large number of the tested assays are vulnerable to heterophilic antibodies with classic Fc-reactivity is a cause for concern. We have previously shown that this interference could probably have been avoided by removing the Fc fragment from the solid phase assay antibodies (2). It is therefore surprising that this approach is not used more frequently.

In the rare event that heterophilic antibodies bind the $F(ab')_2$-region of the assay antibodies, the inclusion of blocking immunoglobulin in the assay buffers provides a final, but important, line of defense. As demonstrated herein, and in a number of previous studies, aggregated antibodies are potent blockers of interference (1, 21, 22). This efficacy is most probably related to the stable binding of low-affinity interfering IgMs (23) to the reiterative epitopes displayed on aggregated immunoglobulin. MAK33 is a good choice for blocking reagent since IgG1 monoclonal antibodies are commonly chosen as capture antibodies to prevent consumption of the solid phase through complement activation (24).

As long as immunoassays are vulnerable, it is important that clinical laboratories implement strategies for identifying samples with a high probability of interference. To identify samples, Ismail et al. (25) suggest a probabilistic approach, i.e., elevated results in assays known to have a low rate of true positive results should be retested for interference. We agree with this probabilistic approach, but we also think that the impact of the assay result should guide which samples to retest for interference. A false-positive HIV-1, hCG or troponin I result probably has more impact than a falsely elevated interleukin 6, although interference may be equally probable in all these assays. An optimal strategy would be based on detailed knowledge of (and experience with) the assay, analyte, and interference tests in question (26, 27). We stress this because interpretation of interference tests is rarely as simple and straightforward as we would like. An extensive discussion on this subject has been published previously (28). A general rule is that a negative interference test does not exclude heterophilic antibody interference. A positive test, given appropriate controls and correct interpretation, can normally be trusted as a proof of interference.

In this study, we relied on the ability of the commercially available immunoglobulin MAK33 to block heterophilic antibodies when added to sera prior to assay. This approach was chosen because in a previous study, using a panel of 11,261 sera, we demonstrated that this reagent was able to reduce the level of interference to <1% (1). We believe that re-assay after blocking with aggregated MAK33, or other commercially available heterophilic blocking reagents (HBRs) (29) for that matter, may prove a good testing alternative when interference is suspected in the routine diagnostic laboratory. Not only is aggregated MAK33 commercially available in a form that has undergone stringent quality con-

trol, but also it is easy to use and interpretation is relatively simple. It should, however, only be used with assays containing murine antibodies and, since MAK33 is an antibody to CK-MB, its use is inappropriate with assays for this particular analyte.

Herein we show that a surprising number of immunoassay kits (21 out of 170 tested) are vulnerable to Fc-reactive heterophilic antibodies. Had more sera with heterophilic antibodies been included, or more assays been tested, it is likely that additional vulnerable assays would have been identified. The fact that 149 assays proved resistant to Fc-reactive heterophilic antibodies in our study should not lead to a false sense of security when using these particular assays. As with other interference tests, negative results do not exclude the possibility of interference.

Based on this study and our previous findings (1, 30), we argue that some immunoassay kits need to be better protected against Fc-reactive heterophilic antibodies. This could be accomplished using either $F(ab')_2$, Fab' or scFv assay antibodies and adequate concentrations of aggregated irrelevant IgG in the assay reagents. The added blocking immunoglobulin should be similar to the assay antibodies with respect to species and subclass.

A potential limitation of this study, given the marked heterogeneity of heterophilic antibodies, is the small number of sera used. However, in a prior investigation, using 198 interference-positive sera (selected from 11,261 tested specimens) we observed that 194 demonstrated Fc-reactivity (2). Thus, the two sera we used in this study contained heterophilic antibodies with the reactivity most commonly associated with antibody interference.

In conclusion, this article describes a simple way of screening immunoassays for interference using small panels of native and pre-blocked sera. Using this method we demonstrate that some commercial assays are poorly protected against heterophilic antibodies with Fc-reactivity.

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