

## ORIGINAL ARTICLE

## Laboratory science

# Factor VIII/IX inhibitor testing practices in the United Kingdom: Results of a UKHCDO and UKNEQAS national survey

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**Abstract**

**Introduction:** Inhibitor formation is the greatest challenge facing persons with haemophilia treated with factor concentrates. The gold standard testing methodologies are the Nijmegen-Bethesda assay (NBA) for FVIII and Bethesda assay (BA) for FIX inhibitors, which are affected by pre-analytical and inter-laboratory variability.

**Aims:** To evaluate inhibitor testing methodology and assess correlation between self-reported and actual methodology.

**Methods:** Methodology was evaluated using a survey distributed alongside a UK National External Quality Assessment Service Blood Coagulation external quality assurance (EQA) exercise for FVIII and FIX inhibitor testing.

**Results:** Seventy four survey and EQA exercise responses were received (response rate 63.2%), with 50 paired survey/EQA results. 47.1% (33/70) reported using the NBA and 42.9% (30/70) the BA for FVIII inhibitor testing. Review of FVIII inhibitor assay methodology demonstrated discrepancy (self-reported to actual) in 64.3% (BA reporting) and 27.6% (NBA reporting). Pre-analytical heat treatment was used by 32.4%, most commonly 56°C for 30 minutes. Assay cut-offs of 0.1-1.0 BU/mL were reported. EQA samples (acquired FVIII and congenital FIX) demonstrated titres and coefficients of variation (CV) of 3.1 BU/mL (0.7-15.4 BU/mL; CV = 43%) and 18.0 BU/mL (0-117 BU/mL; CV = 33%), respectively. No significant assay or laboratory factors were found to explain this variance, which could have resulted in change in management for 6 patients (5 misclassified high-titre FVIII inhibitors and 1 false negative for a FIX inhibitor).

**Conclusions:** Heterogeneity was seen at each stage of assay methodology. No assay-related factors were found to explain variation in inhibitor titres. Further standardization is required to improve inhibitor quantification to guide patient care.

**KEYWORDS**

Bethesda, haemophilia, inhibitor, NEQAS, survey

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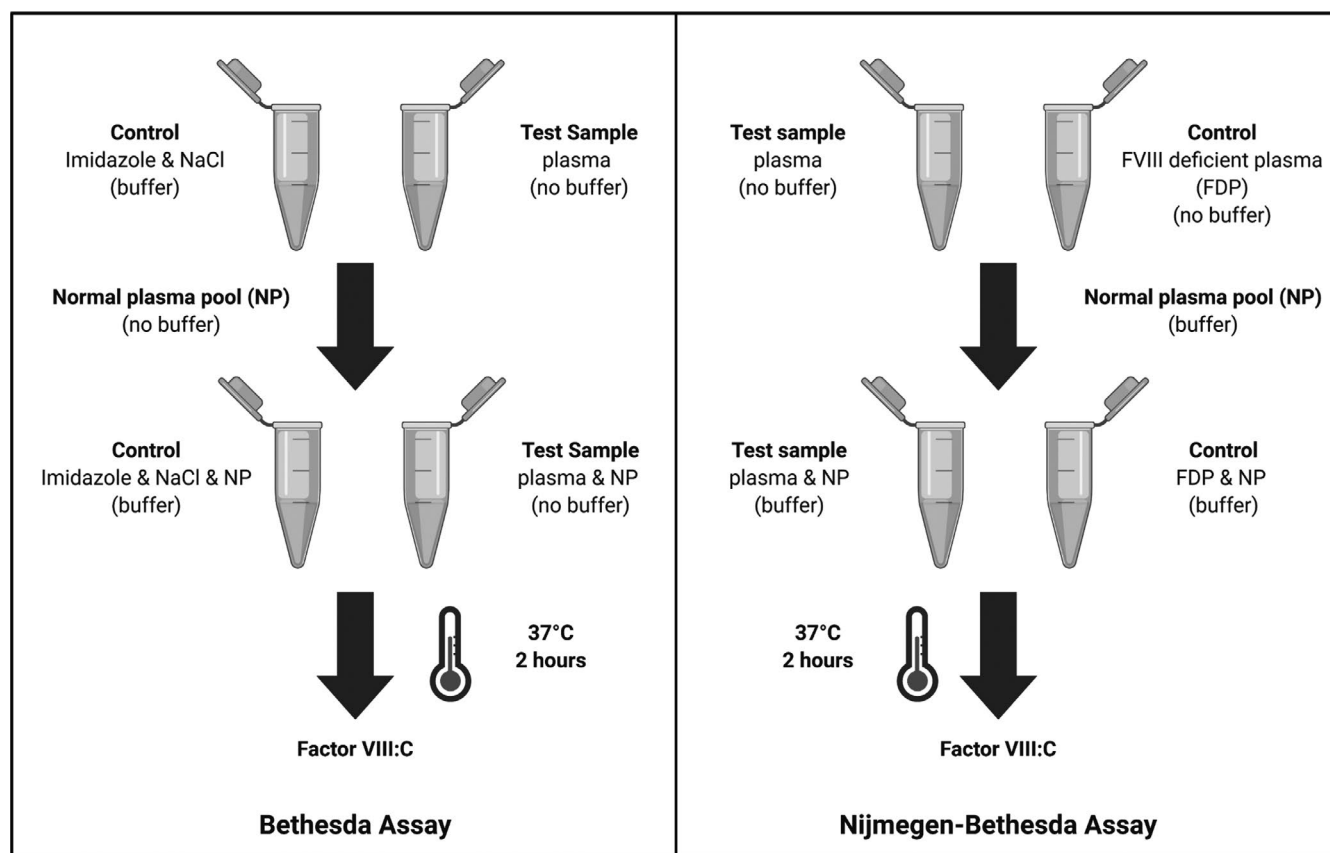


## 1 | INTRODUCTION

Inhibitor formation and detection are one of the greatest challenges for haemophilia centres and laboratories. Laboratory assays currently used for inhibitor diagnosis and quantification are functional clotting-based assays. These were developed in the 1970s (Bethesda assay; BA) to standardize reporting of inhibitor results in patients with severe haemophilia A.<sup>1</sup> In the Bethesda assay, a patient's plasma sample (without buffer) and a control sample (imidazole buffer) are mixed with pooled normal plasma and incubated in parallel for 2 hours at 37°C (Figure 1). FVIII coagulant activity (FVIII:C) is measured for both samples, and the inhibitor titre is calculated based on the proportion of FVIII:C in the patient's compared to the control sample. This assay was reported to have low sensitivity due to differences in protein content and pH between test and control samples. A modification to this assay (Nijmegen-Bethesda assay, NBA) has since been reported using FVIII-deficient plasma as a control and buffered normal plasma (test and control) to adjust for these factors (Figure 1).<sup>2</sup> United Kingdom Haemophilia Centres Doctors' Organisation (UKHCDO) and European Medicines Agency (EMA) guidance recommend the NBA as the gold standard assay for inhibitor quantification.<sup>3,4</sup> Additional modifications to these assays have been reported, including substitution of FVIII-deficient plasma

with bovine serum albumin and measurement of FVIII:C using chromogenic methodology (reviewed in<sup>5</sup>). Although inhibitor assays have provided standardization of reporting in clinical studies, these assays are affected by numerous pre-analytical variables,<sup>6,7</sup> including residual FVIII:C or FIX:C. Methodology to adjust for presence of FVIII:C and FIX:C, includes using a washout period for the patient or an assay modification (mathematical or in vitro) where this is not feasible. An alternative approach utilizes pre-analytical heat treatment (PHT) to denature residual FVIII:C or FIX:C in samples, prior to testing.<sup>8-12</sup>

Accurate detection and quantification of inhibitors are important in the management of persons with haemophilia. Previous external quality assurance (EQA) exercises have demonstrated inter-laboratory variability in inhibitor testing potentially affecting clinical decision-making.<sup>13,14</sup> However, no consistent factors have been found to explain this variability.<sup>13-16</sup> We evaluated inhibitor testing methodology using a survey distributed alongside a United Kingdom National External Quality Assessment Service—Blood Coagulation scheme (NEQAS BC) supplementary exercise for FVIII and FIX inhibitor testing. The aim of this was to evaluate current inhibitor testing practices, to identify factors resulting in variance from consensus in inhibitor testing results and provide additional information to help improve standardization of practices.



**FIGURE 1** Inhibitor assay methodology. Comparison of the Bethesda (BA) and Nijmegen-Bethesda (NBA) assays. FDP, Factor VIII deficient plasma. NP, normal plasma. FVIII:C, FVIII coagulant activity

## 2 | MATERIALS AND METHODS

### 2.1 | Survey of inhibitor assay methodology

A survey of inhibitor practice was performed by the UKHCDO inhibitor working party and NEQAS BC between October and December 2016. This consisted of 24 questions relating to indication/timing, assay methodology and reporting of inhibitor results (Appendix S1). Paper copies were distributed centrally to regular NEQAS BC participants ( $n = 117$ ), with responses being returned by post or electronically. Responses were linked to centres by a unique identifier, meaning that only one response could be received per centre. Responses were centrally coded and de-identified, ensuring anonymity to the research team. Potential coding errors were checked against the original de-identified survey responses. The primary outcome measure was to describe current FVIII inhibitor assay methodology and to assess correlation between self-reported and actual inhibitor testing methodology. Secondary outcome measures were to evaluate inhibitor assay timing, cut-offs and practices accounting for residual pre-analytical FVIII:C.

### 2.2 | NEQAS BC samples

Lyophilized plasma samples were prepared with consent from donations from two patients (FVIII inhibitor: acquired haemophilia A and congenital severe haemophilia B with FIX inhibitor) using previously described UKNEQAS BC ethical approval processes.<sup>13,17</sup> Samples were centrally distributed alongside the questionnaire and centres were asked to perform inhibitor testing using their standard operating procedures and report the inhibitor titre.

### 2.3 | Statistical analyses

Data are presented as mean, standard deviation (SD), median, range and frequencies. Coefficient of variation (CV) corresponds to the ratio between the mean and SD. Inter-rater agreement (Fleiss Kappa) was rated as poor  $<0$ , slight  $=0-0.2$ , fair  $=0.21-0.4$ , moderate  $=0.41-0.6$ , substantial  $=0.61-0.8$  or almost perfect  $=0.81-1$ . Factors influencing inhibitor titre results were analysed using univariable linear regression, with  $P < .05$  taken as significant. Analyses were performed using IBM SPSS version 25 (IBM Corp.), Stata version 13.0 (StataCorp LLC) and NVivo qualitative data analysis software version 11 (QSR International Pty Ltd).

## 3 | RESULTS

A total of 98 responses were received to either the inhibitor testing survey and/or EQA exercise. Of those completing one or more

TABLE 1 Factor VIII and IX inhibitor assays and screens performed

	N (%)
<b>FVIII inhibitor assay (n = 70)</b>	
Bethesda	30 (42.9%)
Nijmegen-Bethesda	33 (47.1%)
Both (BA + NBA)	2 (2.9%)
Other	3 (4.3%)
Inhibitor screen only	2 (2.9%)
<b>FIX inhibitor assay (n = 69)</b>	
Bethesda	31 (44.9%)
Nijmegen-Bethesda	27 (39.1%)
Both (BA + NBA)	2 (2.9%)
Other	2 (2.9%)
Inhibitor screen only	4 (5.8%)
Refer or None	3 (4.6%)
<b>FVIII inhibitor screen (n = 29)</b>	
Dilution (n = 22) <sup>a</sup>	
50:50	18 (81.8%)
50:50 and 80:20	3 (13.6%)
Other	1 (4.5%)
Incubation time (n = 23) <sup>a</sup>	
1 h	8 (34.8%)
2 h	12 (52.2%)
1 & 2 h	1 (4.3%)
1 & 4 h	1 (4.3%)
2 & 4 h	1 (4.3%)
Interpretation (n = 13) <sup>a</sup>	
>4-10 s difference	7 (53.8%)
10%-12% difference	2 (15.4%)
Rosner index	3 (23.1%)
Other	1 (7.7%)
<b>Porcine assay (n = 64)</b>	
Yes	7 (10.9%)
No	55 (85.9%)
In development	2 (3.1%)

<sup>a</sup>Number of responses where additional information was provided in these categories for analysis of inhibitor screen methodology.

survey question ( $n = 74$ ), paired EQA data were available from 50 participants. A further 24 unpaired responses were received from the EQA exercise alone. The survey response rate was 63.2% (74/117) and the complete data set (survey and EQA) response rate 42.7% (50/117). Respondent sources were as follows: 22 (29.7%) comprehensive care centres, 20 (27.0%) treatment centres, 23 (31.1%) non-UK centres, 4 (5.4%) UK centres not listed, 3 (4.1%) UK private laboratories and 2 (2.7%) UK research facilities. Surveys were completed by a laboratory scientist ( $n = 45$ , 70.3%), laboratory scientist and consultant haematologist ( $n = 14$ , 21.5%), consultant

TABLE 2 Inhibitor testing assay methodology

	N (%)
<b>Pre-analytical factors</b>	
Washout (n = 40)	20 (50%)
Trough	8 (20%)
Pre-analytical heat treatment (n = 71)	23 (32.4%)
<b>Analytical factors</b>	
NP source (n = 66)	
Commercial frozen	11 (16.7%)
Commercial lyophilized	42 (63.6%)
Local donor pool	13 (19.7%)
Usage of buffered NP (n = 64)	27 (42.2%)
Addition of imidazole (n = 37)	16 (43.2%)
Buffered inhibitor assay (n = 64)	29/64 (45.3%)
Control tube composition (n = 68)	
NP + Factor VIII-deficient plasma	34 (50%)
NP + Buffer	21 (30.9%)
Other	13 (19.1%)
Sample diluent (n = 67)	
Buffer	35 (52.2%)
Factor VIII-deficient plasma	22 (32.8%)
Other	10 (14.9%)
Methodology for sample dilution (n = 67)	
Doubling dilutions	57 (85.1%)
Sample dependent	4 (6.0%)
1/2, 1/5, 1/10, 1/20, 1/40	3 (4.5%)
Other	3 (4.5%)
<b>Assay cut-off</b>	
Assay cut-off (n = 48)	
<1.0	5 (10.4%)
<0.7	2 (4.2%)
<0.6	11 (22.9%)
<0.5	7 (14.6%)
<0.4	12 (25.0%)
<0.3	6 (12.5%)
<0.1	2 (4.2%)
0	3 (6.3%)
Source of assay cut-off (n = 26)	
Literature	18 (69.2%)
Laboratory based	4 (15.4%)
Historical	2 (7.7%)
Not known	1 (3.8%)
Other	1 (3.8%)

Note: Owrens veronal buffer [OVV] or Owrens-buffered saline [OVS]. Abbreviations: BSA, bovine serum albumin; FDP, Factor VIII-deficient plasma.

haematologist (n = 2, 3.1%), laboratory scientist and haemophilia nurse specialist (n = 2, 3.1%) or haemophilia nurse specialist (n = 1, 1.5%). As a result, a laboratory scientist was involved in completion of 95.3% (61/64) responses.

### 3.1 | Inhibitor assay methodology

For FVIII inhibitor testing, similar proportions of laboratories reported using the BA (42.9%, 30/70) to the NBA (47.1%, 33/70) (Table 1). Three laboratories reported using other assays (in-house, chromogenic BA and South-Mimms inhibitor assay).<sup>18,19</sup> A FVIII inhibitor screen was used by 29 respondents, with most incubating samples in a 1:1 ratio with NP for 2 hours at 37°C. Two laboratories reported using an anti-FVIII ELISA (Immunocor). For FIX inhibitor testing, 39.1% (27/69) used an NBA and 44.9% (31/69) a BA-based assay.

The methodology and reagents used for FVIII inhibitor testing are shown in Table 2 and Table S1. The majority (80.3%, 53/66) of laboratories use normal plasma (NP) from a commercial source. For laboratories using in-house donor pools (n = 13, 19.7%), most use ≥20 donors, with no laboratories using a single donor. Just under half of laboratories state that they use buffered normal plasma (27/64, 42.2%), with 14 of these adding varying concentrations of imidazole to their NP. Two further laboratories add imidazole buffer to unbuffered normal plasma, resulting in 45.3% (29/64) laboratories self-reporting using buffered NP (Table 2). Half of respondents use a control tube containing NP and FVIII-deficient plasma and the remainder NP diluted in buffer or another diluent. The diluent used when a positive result is found in the neat sample tube was buffer based in 52.2% (35/67) and FVIII-deficient plasma in 32.8% (22/67). All respondents who provided details on their sample incubation conditions (n = 69), reported incubating samples at 37°C for 2 hours. A quality control sample was included within the inhibitor assay by 46 and 19 laboratories when performing FVIII or FIX inhibitor assays, respectively. Within this response, only laboratories that use a QC sample in their assay provided details, with no respondents stating that they did not use a QC sample, due to additional missing data in this category a lack of response was not assumed to represent a laboratory did not use QC, so these data are reported as a number of responses only.

Evaluation of the FVIII inhibitor assay that laboratories perform was made by three blinded assessors based on compositions of the NP, control sample and diluent. This analysis included 65 laboratories (BA = 29, NBA = 33 and Other = 3) where sufficient assay detail was provided for review. Assays were categorized as: **A**: Bethesda (unbuffered NP + imidazole control); **B**: Bethesda hybrid (unbuffered NP + FVIII-deficient plasma control); **C**: Nijmegen-Bethesda (buffered NP + FVIII-deficient plasma control); **D**: Nijmegen-Bethesda hybrid (buffered NP + imidazole control); or **E**: other.<sup>14</sup> There was moderate (Kappa = 0.43) overall inter-rater agreement on assay type performed (Assay Kappa: **A** = 0.33, **B** = 0.40, **C** = 0.70, **D** = 0.27 and **E** = 0.31). All raters agreed on the assay type for 38.5% (25/65), two agreed in 53.8% (35/65), and there was no consensus for 7.7% (5/65). For the laboratories where no expert consensus on assay type was made, laboratories self-reported using the NBA (n = 4) or BA (n = 1). These were excluded from the subsequent analysis. For laboratories self-reporting using the BA, 35.7% (10/28) use a BA type assay (BA = 6, hybrid-BA = 4), 25% (7/28) an NBA type assay (NBA = 2, hybrid-NBA = 5) and 39.3% (11/28) another assay. For laboratories

self-reporting using the NBA, 72.4% (21/29) use an NBA-based assay (NBA = 17, hybrid NBA = 4), 20.7% (6/29) a BA type assay (BA = 2, hybrid-BA = 4) and 6.9% (2/29) another inhibitor assay. For laboratories reporting using another type of assay (n = 3), two described a non-BA/NBA type of inhibitor methodology and one an NBA type assay methodology. As a result, 55% (33/60) laboratories accurately reported the inhibitor assay type that they were using.

### 3.2 | Methodology to adjust for residual FVIII:C

Twenty centres (20/40, 50%) use a washout prior to inhibitor testing, with most using 48-72 hours (15/20). Eight centres test at the time of a trough and 12 reported not using a washout. PHT was used by 32.4% (23/71) laboratories, most frequently incubating samples at 56°C for 30 minutes (n = 9). There were, however, 11 different combinations of incubation temperature (56-60°C) and times (30-90 minutes) reported. Two respondents listing incubation conditions of 37°C for 2 hours were categorized as not using a PHT step, as these likely represented inhibitor assay incubation conditions. One laboratory reported using different PHT incubation times prior to testing for FVIII (90 minutes) and FIX (30 minutes) inhibitors. Seven laboratories use PHT for all samples and 16 based on the FVIII:C result, which was most frequently  $\geq 0.01$  IU/mL (n = 8). One laboratory reported only using PHT when testing acquired haemophilia A samples. For laboratories that did not use a washout, 41.7% (5/12) use PHT. Four laboratories reported using neither a washout nor PHT. Seven laboratories reported using both PHT and a washout prior to testing. Overall, 36 centres' practice (washout or PHT) accounts for residual FVIII:C within samples.

### 3.3 | Inhibitor assay reporting and Cut-Off

The most frequently reported assay cut-offs were  $<0.4$  BU/mL (n = 12) and  $<0.6$  BU/mL (n = 11). Cut-offs were predominantly derived from published literature (n = 18), with only four laboratories using in-house testing to define or validate cut-offs. Five centres use different cut-off for positive and negative results, allowing the potential for reporting of borderline results. Six centres, reported not using a cut-off, with all results being reported. Approximately half of centres (65.5%, 36/55) report a negative inhibitor assay as being 'negative'.

### 3.4 | Indication for inhibitor testing

Timing and indications for inhibitor testing were evaluated from 67 survey respondents (Table 3). The most commonly listed reason for inhibitor testing related to timing during follow-up (n = 47, 70.1%). This was most frequently stated as 6 monthly (n = 31), annually (n = 12) or more frequently at initiation of treatment (3-5ED = 6 and 3 monthly = 6). Other reasons included ineffective treatment

(n = 26, 38.8%), following intensive treatment (n = 13, 19.4%), surgery (n = 12, 17.9%) or unexpected pharmacokinetic data (n = 12, 16.4%). Specific indications for patients with non-severe haemophilia or acquired haemophilia were listed by 17.9% (n = 12) and 10.4% (n = 7), respectively.

### 3.5 | NEQAS BC samples

Response rates for the FVIII and FIX EQA exercise were 77.9% (74/95) and 67.4% (64/95), respectively. The median FVIII inhibitor titre was 3.1 BU/mL (range 0.7-15.4 BU/mL), with all centres reporting this sample as positive (Figure 2A). Most centres (93.2%, 69/74) provided results consistent with a low-titre ( $<5.0$  BU/mL) inhibitor, with the remainder reporting results consistent with a high-titre inhibitor ( $\geq 5.0$  BU/mL). The median FIX inhibitor titre was 18.0 BU/mL (range 0-117 BU/mL), with 63 centres reporting this as positive (high-titre inhibitor) and one negative (Figure 2B). Excluding outlying results (FVIII n = 1, 15.4 BU/mL and FIX n = 1, 117.54 BU/mL), both obtained from a single laboratory, the coefficients of variation (CVs) were 43% (FVIII) and 33% (FIX). Although most laboratories report inhibitor titres to the nearest integer (FVIII 14/74, FIX 19/64) or 1 decimal place (FVIII 34/74, FIX 36/64), some reported results to 2 decimal places (FVIII 26/74, FIX 9/64).

Univariable linear regression was performed to evaluate factors affecting the results of the FVIII NEQAS BC inhibitor result (Table 4). There was a trend towards a small difference in inhibitor titre, relating to imidazole addition (imidazole 3.67 BU v no imidazole 3.60 BU, coefficient 0.07,  $P = .06$ ). No other factors (laboratory or assay methodology) were found to explain variability of the FVIII EQA exercise results.

## 4 | DISCUSSION

Significant heterogeneity was seen within this survey at each stage of inhibitor assay methodology. Although similar proportions of laboratories report using the BA or NBA for FVIII inhibitor testing, review of methodology demonstrated only 55% perform an assay following these principles. It is notable that there was only moderate agreement from three blinded experts in laboratory haematology, regarding the assay type used by survey respondents. Although assay heterogeneity or laboratory type might explain variability seen within the EQA exercises, this was not seen on more detailed analysis. Inhibitor assay CVs seen in this survey are similar to those reported previously<sup>13,14</sup> and differences in management might have occurred in 4.3% (6/138) patients if these results had been used to direct clinical care.

### 4.1 | Adjustment for residual FVIII:C

Residual FVIII:C in samples may affect inhibitor detection when using coagulation-based assays. Although trough levels may be undetectable in young boys with severe haemophilia commencing



TABLE 3 Inhibitor testing timing and indications

Indication	Responses (%)
<b>Timing</b>	<b>47 (70.1%)</b>
6 monthly	31
Annual	12
3-5ED	6
3 monthly	6
UKHCDO guideline	3
Other	3
3-6 monthly	2
Post 1st dose	2
Every 5ED 20-50ED	1
2 monthly	1
Clinic visit	1
1 monthly	1
<b>Ineffective treatment</b>	<b>26 (38.8%)</b>
<b>Intensive treatment</b>	<b>13 (19.4%)</b>
<b>Surgery</b>	<b>12 (17.9%)</b>
Pre-surgery	8
Post-surgery	3
Pre- and post surgery	1
<b>Non-severe haemophilia</b>	<b>12 (17.9%)</b>
After all treatment	5
After intensive treatment	3
Annual	2
6 monthly	1
Fall in baseline FVIII:C	1
During initiation of treatment (<6ED)	1
<b>Pharmacokinetic</b>	<b>12 (16.4%)</b>
Poor recovery	7
Low trough	3
Low half-life	2
<b>Bleeding</b>	<b>9 (13.4%)</b>
<b>Product switching</b>	<b>7 (10.4%)</b>
<b>Acquired haemophilia</b>	<b>7 (10.4%)</b>
<b>Clinician request</b>	<b>7 (10.4%)</b>
<b>Diagnosis</b>	<b>4 (6.0%)</b>
<b>Guide treatment</b>	<b>3 (4.5%)</b>
<b>New patient</b>	<b>2 (3.0%)</b>
<b>Other</b>	<b>5 (7.5%)</b>
Change in clinical picture	1
Clinical trial	1
NEQAS	1
Not routinely test	1
Trauma	1

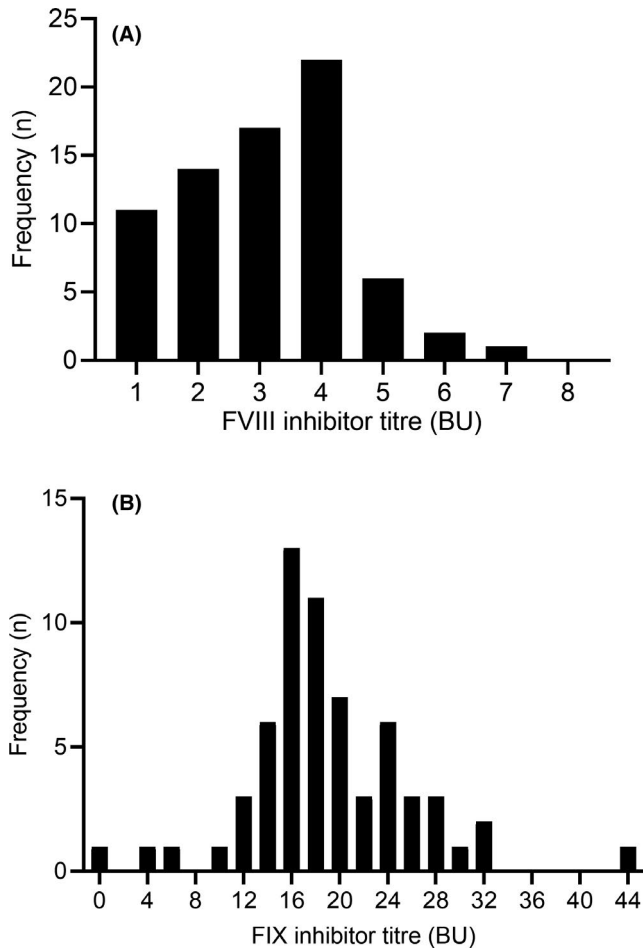
Note: Figures in bold text represent the number of individuals stating one or more reason for inhibitor testing within each category. As multiple responses were given by each respondent within the survey, subcategories responses may not equal category total.

prophylaxis, this may not be the case for older patients using pharmacokinetic guided prophylaxis. A washout period is currently advised prior to inhibitor testing in severe haemophilia.<sup>4</sup> Significant heterogeneity and uptake of usage of a washout was seen, similar to previously unpublished UKNEQAS BC findings (Jennings, I., personal communication). There appears to be increased usage of PHT since a 2012 UKHCDO acquired haemophilia survey (1/26)<sup>20</sup> and unpublished UK NEQAS BC data from 2013 (23/110) (Jennings, I., personal communication). Similar uptake of PHT has been reported by the Royal College of Pathologists of Australasia (RCPA).<sup>16</sup> The most commonly reported incubation condition was 56°C for 30 minutes, which differs from the World Federation of Haemophilia recommendations (58°C for 120 minutes).<sup>21</sup> These conditions are likely derived from a centralized inhibitor testing study, which demonstrated fall in FVIII:C and FVIII:Ag to <1 IU/dL following incubation at 56°C for 30 minutes.<sup>8</sup> Differences have been reported in reduction in residual FVIII:C relating to different incubation conditions (time and temperature).<sup>12,22</sup> There are also conflicting data on the effect of PHT on loss of inhibitor detection using different incubation conditions using either an IgG4-specific assay<sup>22</sup> or anti-FVIII IgG ELISA and NBA.<sup>12</sup> There are likely differences in thermostability of FVIII concentrates from different sources.<sup>23</sup> Further evaluation of incubation conditions is required and laboratories should evaluate the effect of PHT conditions within their inhibitor assays.

## 4.2 | Inhibitor assay methodology

International guidance advises usage of the NBA as the 'gold standard' FVIII inhibitor assay.<sup>3,4</sup> Just under half of laboratories self-reported using the NBA within this survey. This figure is higher compared to previous UKHCDO (31%) and European Concerted Action on Thrombosis (ECAT) surveys (25%-30%).<sup>13,14</sup> Interestingly, as most respondents use commercial NP (which may contain buffer), this may underestimate the number of laboratories performing NBA-like assays. Very few laboratories reported using ELISA-based techniques (n = 2). This methodology demonstrates high specificity for inhibitor screening, and potential applications have been outlined in previous UKHCDO guidance, eg in the presence of a lupus anticoagulant or non-inhibitory antibody affecting FVIII clearance.<sup>4</sup> This approach may not have found application due to associated additional costs and uncertain implications from detection of non-inhibitory antibodies.

Previous surveys have attempted to identify factors to explain inter-laboratory variability, with inconsistent results. Higher CVs were reported comparing inhibitor samples from acquired to congenital haemophilia A patients in one NEQAS BC EQA exercise,<sup>13</sup> although these findings were not seen in a similar RCPA exercise.<sup>16</sup> Lower inter-laboratory CVs were seen in an RCPA wet-laboratory exercises comparing the BA (37.9%-67.5%) to NBA (22.3%-53.3%) and for buffered or non-buffered NP usage.<sup>16</sup> However, no difference in CV was seen between assay type (BA, BA + FVIII-deficient plasma, NBA + imidazole buffer and NBA) in an ECAT exercise.<sup>14</sup> No difference in variance was seen comparing methodologies (control



**FIGURE 2** Histogram of inhibitor titres from the Factor VIII (A) and Factor IX (B) NEQAS BC EQA exercise

mixture, NP and buffering of the NP) by ECAT and North American Specialized Coagulation Laboratory Association (NASCOLA), similar to our reported findings.<sup>14,15</sup> Although no significant factors were found to explain variance within this EQA exercise, this analysis was limited by sample size and more detailed study is required.

Recent changes in the UK have seen approvals of porcine recombinant FVIII (Susoctocog alpha, Obizur<sup>®</sup>, Takeda) and an antifactor IXa/X biphenotypic antibody (Emicizumab, HemLibra<sup>®</sup>, Roche). Centres using Obizur<sup>®</sup> to manage acquired haemophilia A will require assays for detection and quantification of antiporcine FVIII inhibitors. At the time of this survey, these assays were available in only seven laboratories, which has increased to 18/27 comprehensive care centre laboratories in January 2020. Future EQA exercises will need to include antiporcine inhibitor samples, as inter-laboratory variance was similar for antihuman and antiporcine inhibitors, when plasma-derived porcine FVIII was previously used.<sup>13</sup> Use of non-factor replacement therapies including emicizumab, antithrombin siRNA and TFPI monoclonal antibodies in patients with congenital haemophilia with inhibitor will provide additional challenges to laboratories. These developments are beyond the scope of this study and discussed in more detail in the following recent publications.<sup>24,25</sup> Greater standardization of inhibitor assay methodology and usage of

chromogenic methodology will become more important as the landscape of novel haemostatic agents evolves.

### 4.3 | Cut-off used for inhibitor detection

A range of inhibitor assay cut-offs (0-1 BU) was reported similar to previous studies.<sup>13,15</sup> Although variation in cut-offs reflects a lack of clear experimental data, this range is surprising given most laboratories derive these values from published literature. This range is potentially problematic in patients with low-titre inhibitors (eg 0.6-0.9 BU), where some centres would define these as being positive and some negative, which will impact on bleed treatment choices. The largest study evaluating cut-offs studied titres in patients with ( $n = 56$ ) and without an inhibitor history ( $n = 588$ ), reporting a cut-off  $\geq 0.5$  as demonstrating higher sensitivity than  $\geq 0.6$ .<sup>8</sup> It is possible that this study and consensus guidance advising a cut-off  $\geq 0.6$  BU/mL has affected practices seen within this survey. There is lack of consensus how inhibitor results should be reported, whether as positive, negative or by giving a titre alone (even if negative). It is interesting to note, given the inherent imprecision of these assays, some laboratories reported titres to 2 decimal places. This most likely results from automated laboratory analyser readouts interfacing directly to patients' digital clinical record systems. This level of reporting implies the assay to be more sensitive than it is, especially for higher titre inhibitors where a difference of 1-2 IU in the residual FVIII:C after incubation has even greater effect on the final inhibitor assay titre. More consideration is required in titre reporting where marginal changes could be over-interpreted as clinically relevant by multi-disciplinary team members or patients/families.

### 4.4 | Timing of inhibitor screening

Inhibitor screening guidance in severe haemophilia has focussed on active, intense screening during early treatment when inhibitor incidence is greatest.<sup>4</sup> This allows prompt inhibitor detection to avoid anaphylaxis in haemophilia B<sup>26</sup> and early commencement of immune-tolerance induction in haemophilia A.<sup>27</sup> Testing indications within this survey generally matched these recommendations. Guidance for screening in non-severe haemophilia A predates publication of inhibitor risk factors from the INSIGHT study.<sup>28,29</sup> This advised annual testing in patients treated with FVIII concentrate within the preceding year or following intensive exposure ( $\geq 5$ ED), surgery or following any FVIII treatment in those with 'high-risk' F8 mutations. There remains uncertainty as to what constitutes high-risk treatment in these patients and data presented by our group has demonstrated low uptake of proactive convalescent inhibitor screening.<sup>30</sup>

There are some limitations and benefits for using a survey and EQA-based approach to evaluate variance in inhibitor testing. Traditional paper/electronic-based surveys often result in complex



TABLE 4 Univariable analysis of factors affecting FVIII inhibitor assay results

Variable	n	Mean BU	Regression coefficient	SE	CI	P-value	% R squared
<b>Control tube</b>							
NP and Buffer	13	3.19					0.1%
NP and FVIII	24	3.18	0.00	0.53	-1.07-1.05	.99	
Other	9	4.49	0.09	0.66	-1.25-1.42	.90	
<b>Diluent used</b>							
Buffer	22	3.80					1.8%
FVIII-deficient plasma	15	2.85	-0.42	0.50	-1.43-0.59	.41	
Other	8	3.24	-0.03	0.62	-1.2-1.21	.96	
<b>Pre-analytical heat treatment</b>							
No	30	3.23					0.2%
Yes	17	3.09	-0.14	0.46	-1.06-0.78	.76	
<b>Buffered assay (reported)</b>							
No	24	2.78					5.6%
Yes	20	3.47	0.69	0.43	-0.19-1.57	.12	
<b>Imidazole added</b>							
No	14	2.60					13.0%
Yes	13	3.67	1.07	0.55	-0.07-2.21	.06	
<b>NP source</b>							
Commercial frozen	7	3.93					4.9%
Commercial lyophilized	28	3.00	-0.93	0.64	-2.21-0.36	.15	
Local donor	10	3.06	-0.87	0.74	-2.37-0.63	.25	
<b>FVIII assay (reported)</b>							
BA	18	3.28					10.3%
NBA	25	3.36	0.08	0.72	-1.37-1.54	.91	
Both	2	3.29	0.01	1.76	-3.53-3.55	1.00	
Other	2	4.04	0.76	1.76	-2.78-4.30	.67	
Screen only	1	5.80	2.52	2.42	-2.36-7.40	.30	
<b>FVIII assay (true)</b>							
BA like	8	3.20					0.4%
NBA like	24	3.17	-0.03	0.63	-1.31-1.24	.96	
Other	12	4.32	0.19	0.71	-1.23-1.62	.79	
<b>Dilution methodology</b>							
Doubling dilutions	41	3.13					0.2%
Other dilution factor	5	5.34	0.19	0.73	-1.27-1.66	.79	
<b>Different reagent</b>							
No	40	3.53					0.4%
Yes	4	2.92	-0.31	0.79	-1.91-1.28	.69	
<b>Centre type</b>							
CCC	22	2.94					8.4%
HTC	20	3.46	0.52	0.40	-0.28-1.32	.20	
Non-UK	22	3.18	-0.31	0.39	-1.09-0.47	.42	
Private	3	3.18	0.36	0.80	-1.23-1.96	.65	
Research	2	4.12	1.18	0.96	-0.73-3.09	.22	
UK other	4	3.28	0.34	0.70	-1.06-1.75	.63	

Abbreviations: BA, Bethesda assay; CCC, comprehensive care centre; HTC, haemophilia treatment centre; NBA, Nijmegen-Bethesda assay; NP, normal pool.



datasets with multiple areas of missing data, as seen in this study through varying denominators for different questions. With the survey being blinded to the investigators, however, this could increase accuracy of responses by allowing omission of responses where information is unknown or differs from consensus. Within this survey, true estimation of the response rate is complex as a proportion of EQA responses ( $n = 24$ ) and survey ( $n = 24$ ) were unpaired. A conservative estimate of response rate was made in reporting, to avoid assumptions that these additional unpaired responses originated from the same participants. For future work, usage of modern survey-based platforms (eg REDCap) could improve response rates and facilitate data-capture.<sup>31</sup> EQA exercises allow provision of standard samples for analysis in laboratories in different geographic regions. There is assumption that laboratories will treat EQA samples exactly the same as a clinical samples, including usage of modifications such as usage of PHT. As a result, this process does not allow evaluation of other pre-analytical factors (eg sampling/transportation) that could affect inhibitor testing results in clinical studies. The current study design is also unable to detect intra-laboratory variability as the analysis was based on a single sample testing point, an area where more study is required. Finally, although some information was collected on FIX inhibitor methodology, the main focus of this exercise was to look at FVIII inhibitor methodology. As a result, these findings may not represent practices that laboratories use for testing for non-FVIII inhibitors.

## 5 | CONCLUSIONS

Standardized, validated approaches to inhibitor testing are an important part of haemophilia care. Improvements in the reporting of inhibitor titres and harmonization of assay cut-offs are required. Variations in testing practice should be locally validated and traceable to facilitate standardization. Understanding of assay limitations may improve diagnostic practice which will become increasingly important as new haemostatic agents emerge.

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## DISCLOSURES

None declared.

## AUTHOR CONTRIBUTION

AR, IJ and DPH designed the research study. DPH, AR, PB and IJ performed the research. PB, AR, SSI and DPH analysed the data. PB, AR,

IJ and DPH wrote the first draft of the paper. All authors reviewed and critically edited the manuscript.

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### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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