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Evaluation of the monocyte activation test for the safety testing of meningococcal B vaccine Bexsero: A collaborative study

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ABSTRACT

The aim of this collaborative study was to evaluate the robustness of the monocyte activation test (MAT) for quantifying the pyrogenic content in the outer membrane vesicle (OMV)-containing vaccine Bexsero: the first meningococcal B vaccine to be licenced. We analysed datasets from 9 laboratories covering 15 test systems for 3 batches of Bexsero with higher, equivalent and lower activity relative to a reference lot in the MAT. Activity was measured in terms of relative pyrogen units (RPU) based on European Pharmacopoeia (Ph. Eur.) MAT Chapter 2.6.30 Method C: Reference Lot Comparison Test. We report that all 15 test systems were consistent in that they showed sample A to be the most active in the MAT; that 13 of 15 test systems had an accuracy of more than 80% and an overall geometric mean RPU of 1.03 with lower and upper 95% confidence limits of 0.97 and 1.09 respectively for a sample with an expected value of 1.00 RPU. We also report larger variability in the results for test systems involving cells from individual blood donations for sample A suggesting that there could be donor to donor differences in sensitivity to the vaccine constituents responsible for the higher activity of this batch. Overall, the consistency and accuracy of the MAT was remarkable given the range of test systems used by participants, all of which are permitted by the Ph. Eur. General MAT Chapter. This is important given the limitations of the rabbit pyrogen test for the control of pyrogenicity in general and particularly with products with intrinsic pyrogenicity such as Bexsero.

1. Introduction

Pyrogens are fever-causing agents derived from bacteria, viruses and fungi and can be found in medicines and vaccines either as contaminants or as an integral part of the product/formulation. When administered parenterally, in addition to fever, pyrogens can cause a rash, febrile convulsions, severe soreness/redness, swelling at the injection site and in extreme cases, life-threatening complications. Consequently, the control of pyrogenic content in parenteral preparations is crucial to assure the safety and quality of medicines and vaccines. There are currently three tests prescribed by the Pharmacopoeias for pyrogen testing. The first developed was the rabbit pyrogen test (RPT), in which the product is administered via intravenous injection into rabbits and the body temperature monitored for fever responses. This test detects all types of pyrogenic material. The second test developed was the bacterial endotoxins test (BET) in which amoebocyte lysate extracted from the Limulus or Tachypleus horseshoe crab clots in the presence of endotoxin. However, its use as an alternative to the RPT is limited because although it detects the most common pyrogen, endotoxin, it fails to detect non-endotoxin pyrogens, for example, peptidoglycan, lipoproteins and bacterial DNA. The third and most recent test developed is the monocyte activation test (MAT).

Monocytic cells play an important role in the innate immune system. They express pattern recognition receptors, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization

Abbreviations: ATCC, American type culture collection; BET, Bacterial endotoxins tests; DSMZ, German collection of microorganisms and cell culture; EE, endotoxin equivalents; ELISA, Enzyme-linked immunosorbent assay; GCV, Geometric coefficient of variation; GM, Geometric mean; GSK, GlaxoSmithKline; IL-1, IL-6 and IL-8, Interleukins -1, -6 and -8; MAT, monocyte activation test; MM6, Mono Mac 6; NIBSC, National Institute of Biological Standards and Control; OCABR, Official control authority batch release; OMCL, Official medicines control laboratory; OMVs, Outer membrane vesicles; PBMC, Peripheral blood mononuclear cells; Ph. Eur., European pharmacopoeia; RPT, Rabbit pyrogen test; RPU, Relative Pyrogen Unit; SNPs, single nucleotide polymorphisms; TLRs, Toll-like receptors; TNFα, Tumour necrosis factor alpha; WB, whole blood; OD, Optical density.

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domain (NOD) -like receptors [1,2] which bind to specific (exogenous) pyrogens, resulting in monocyte activation and the release of endogenous pyrogens (e.g. the cytokines tumour necrosis factor alpha [TNFα] and interleukins [IL] -1β, -6 and -8). These cytokines are mediators of the inflammatory response which includes fever [3]. Since the 1980s, a number of scientists have focused on the development and validation of *in vitro* tests for pro-inflammatory/pyrogenic contaminants based on this concept [4–15] and in 2010, the MAT was included in the Ph. Eur. (Chapter 2.6.30). Three MAT methods are described in the Ph. Eur. (Methods A, B and C), all of which involve the incubation of the test preparation with human monocytes followed by the quantification (e.g. by ELISA) of a cytokine (e.g. IL-6) released by the cells in response to external pyrogens.

The MAT is an animal-free test and thus in accordance with the '3Rs' principle of Replacement, Reduction and Refinement [16] and EU Directive 2010/63/EU concerning the protection of animals. It can, depending on the method used, be applied as a quantitative test (unlike the RPT) and detects both endotoxin and non-endotoxin pyrogens. In addition, it is more physiologically relevant to the human pro-inflammatory response than the RPT since it employs the use of human cells involved in the innate immune system. However, there is scepticism in the use of the MAT for the control of medicines and vaccines among some regulatory authorities and scientists in industry due to the difficulties associated with validating the test, setting appropriate pass/fail specifications and the limited availability/accessibility of monocytic cells which are fit for purpose. The latter has recently been addressed by the validation of the use of cryogenically preserved peripheral blood mononuclear cells (PBMC) isolated from blood sourced from donation facilities for use in the MAT [17,18]. Additionally, access to commercially available cell sources has improved significantly in the last year [19].

In 2014, the National Institute for Biological Standards and Control (NIBSC), UK, validated and implemented the MAT as a replacement for the RPT for the Official Control Authority Batch Release (OCABR) of Bexsero. Bexsero is a vaccine developed by GlaxoSmithKline (GSK) for the control of group B meningococcal disease caused by the Gram negative bacteria *Neisseria meningitidis.* Bexsero was the first meningococcal group B vaccine to be licenced for use in humans, obtaining an EU licence in January 2013 and subsequently in a number of other territories including Canada, Australia and the USA [20]. Since its licensure it has been introduced into the UK paediatric vaccine schedule, used to control outbreaks in two US universities [21,22] and has a growing private market worldwide. The number of doses of this vaccine administered to patients is thus considerably greater than any other group B vaccine to date.

It is a multivalent vaccine containing three recombinant proteins and outer membrane vesicles (OMVs). Commercially produced OMVs are detergent extracted and in addition to surface structures such as endotoxin, porins, peptidoglycan, muramylpeptides and lipoproteins, can contain bacterial DNA and cytosolic proteins. Bexsero has been commonly associated with soreness at the site of injection, fever and in some cases febrile convulsions following its administration [23,24]. These responses are consistent with administering endotoxin to patients and are not unexpected with vaccines containing OMVs, however the role of other pyrogenic components remains unclear. The necessity to develop and implement the MAT at NIBSC was prompted by the problems encountered in the use of the RPT for the batch release of this intrinsically pyrogenic vaccine [25]. As the vaccine contains high levels of endotoxin, prior to testing it had to be diluted to a pre-established sub-pyrogenic dose before it was injected into rabbits. All batches were required to pass the RPT at this dilution. However, the rationale for this was flawed; the RPT was originally designed to confirm the absence of pyrogens. The Pharmacopoeial methods prescribe the use of algorithms established for this purpose and the numbers of rabbits used in the test were designed to rule out false positive and negative results and provide a qualitative result of pass or fail. Switching to use this test as a consistency rather than safety test requires recalculation of the number of animals used to provide the power to discriminate between batches containing different levels of pyrogens. In addition, since the RPT was originally developed to confirm the absence of pyrogens in large volume parenterals given intravenously to patients, the pharmacopoeias prescribe that test samples are injected intravenously in rabbits. Therefore, the RPT is not appropriate for the control of vaccines given intramuscularly or subcutaneously as these routes of administration are likely to result in smaller responses to pyrogens.

The MAT originally developed and implemented by NIBSC for the OCABR testing of Bexsero involved the use of PBMC separated by density gradient centrifugation from fresh blood donations (without pooling) and IL-6 as the measured response, quantified by ELISA as previously described [26]. Not surprisingly, it was shown during the development phase that the IL-6 dose-response curve for Bexsero was not parallel to the dose-response curve for the 3rd WHO International Standard for Endotoxin in the majority of donors. The lack of parallelism most likely resulted from dissimilarities in the pyrogenic content. Consequently, it was not appropriate to quantify the pyrogenic content of Bexsero in terms of endotoxin equivalents (EE) as described in Method A of the Ph. Eur. MAT chapter. Parallelism is indeed important to ensure the accurate assignment of potency. Instead, a reference lot of Bexsero vaccine was identified and pyrogenic activity assigned an arbitrary value of 1. The pyrogenic content of test batches was quantified in terms of activity compared with this reference lot. Results were reported as Relative Pyrogen Units (RPU) and were calculated using parallel-line analysis of the linear part of the dose-response curves for the reference and test batch. This approach is based on the same principle as Ph. Eur. Method C: Reference Lot Comparison Test.

All three methods described in the Ph. Eur. MAT chapter permit the use of whole blood (WB), PBMC or a suitable monocytic cell-line, of which the former two can be fresh or cryogenically preserved and may be pooled from at least four different donors or used as individual donations. In addition, a variety of readouts are permitted (e.g. TNFα, IL-1β, IL-6, IL-8) provided that it is validated. It is conceivable that the flexibility afforded by the Ph. Eur. chapter could lead to inconsistent outcomes for the same products in laboratories using the MAT. We report here the results of a collaborative study carried-out to evaluate the robustness of the MAT. We assessed the consistency of results generated by 9 laboratories in 9 countries using a range of MAT systems with regards to the cell source and readout for 3 batches of Bexsero associated with higher, equivalent and lower activity relative to a reference lot in the MAT. The purpose of this study was to promote the acceptance and use of this 'animal-free' test as a replacement for the RPT both in industry and in the regulatory network for the control of intrinsically pyrogenic vaccines such as those containing OMV. This is important given the limitations of the RPT for testing such vaccines and that OMV technology is not exclusive to Bexsero and its use is rapidly increasing [27,28].

2. Methods

All 9 of the participating laboratories had some experience of testing for pyrogens in parenteral preparations and in performing cell-based tests. Ten syringes each of Bexsero samples A, B and C, and the reference lot of Bexsero were provided and participants were required to generate results for each of the samples using

Ph. Eur. MAT Method C: Reference Lot Comparison Test (2.6.30). This was to allow all results to be calculated in terms of Relative (to the reference lot) Pyrogen Units (RPU), i.e. harmonised units which could be compared across laboratories. Previous experiments had shown that Method A was inappropriate (see above) at least under the test conditions routinely used at NIBSC. Participants were asked to use their own 'in house' procedures in terms of the cells used, the response measured, materials and reagents used and incubation periods. Participants were required to incubate monocytic cells with 5 x 2-fold dilutions of the test and reference materials which covered the linear part of the dose-response curve for their system (typically 1:1,500–1:24,000 final dilution within the well) and each dilution was to be tested in quadruplicate as shown in the plate layout in Fig. 1. In addition, all samples, the reference lot and controls were to be tested in the same 96-well, microtiter plate for each donation/pool/passage of cells. Test controls included the 3rd WHO International Standard for Endotoxin [29] or an in house reference calibrated in IU at a concentration known to stimulate a response in the test system (positive control) and untreated cells in culture medium (negative control). Following the incubation period, participants were asked to use their 'in house' ELISAs to quantify the chosen cellular response to test material (e.g. IL-6) in the cell-conditioned medium.

2.1. Analysis

Data analyses were carried-out at NIBSC. Participants were required to copy their raw data (optical density; OD values) for each test plate into Excel templates (provided by NIBSC) that were of the same format as the plate layout in Fig. 1. Dixon's Test for outliers ($\alpha = 0.02$) was used to exclude up to one anomalous OD value within the 4 replicates for each treatment. Only data from test plates where the endotoxin positive control mean OD was above the mean OD of the negative control were included in the analysis. Sample dilutions giving a mean OD below the mean OD of the negative control tested on the same test plate were excluded from the analysis.

Parallel-line analysis of the dose-response curves (dilution-OD) for the test and reference samples (on the same test plate) using a linear fit (taking the 3 dilutions which generated the steepest slope) was used to calculate a Relative Pyrogen Unit (RPU) value for each sample tested by each laboratory. Analysis was performed using CombiStats™ [30]. Validity criteria set for OCABR testing of Bexsero at NIBSC for the parallel-line analysis were applied as follows: regression; $p < 0.05$, non-linearity; $p > 0.01$, non-parallelism; $p > 0.01$. If the validity criteria were not met using the linear fit, a sigmoidal fit was applied. Log transformation was performed if necessary.

For datasets where the non-linearity and/or non-parallelism validity criteria could not be met using a linear or sigmoidal fit, RPU values were estimated regardless using linear parallel-line analysis to allow for an approximate evaluation. These 'invalid' RPU values are highlighted in all relevant tables and graphs and were excluded from calculations of overall geometric means, geometric coefficients of variation (unless highlighted, as in Table 2) and upper and lower confidence limits.

2.2. Test samples

Bexsero test lots (samples A, B and C) and the reference lot were kindly provided by GSK (product manufacturer) as syringes of final product and were distributed to the participating laboratories under temperature controlled conditions by NIBSC. Preliminary testing at NIBSC using the MAT showed that sample A stimulated higher IL-6 responses and sample B stimulated lower IL-6 responses in comparison with the reference lot giving RPU values above and below 1.00 respectively. Sample C was the same material as the reference lot (i.e. a coded duplicate) and so in theory should have given an RPU value of 1.00. The reference lot was the same reference lot used for the OCABR testing of Bexsero at NIBSC at the time of the study.

3. Results

3.1. Participants and the test systems used

Data were received from 9 laboratories in 9 countries including 8 official medicines control laboratories which were ANSM (France), AGES (Austria), DKMA (Denmark), HC (Canada), NoMA (Norway), PEI (Germany), SUKL (Czech Republic) and NIBSC (UK), and the manufacturer of Bexsero, GSK (Italy). Some laboratories provided results for more than one MAT system giving a total of 15 different formats included in the collaborative study, all of which were permitted by Ph. Eur. MAT Chapter 2.6.30. The MAT systems used (including the types of cell preparation and the cytokine readouts measured) and the number of independent tests carried-out for each test sample are summarised in Table 1.

3.2. Validity of results

It was possible to generate valid RPU using a parallel-line analysis of the dose-response curves for the test and reference preparations according to the NIBSC OCABR validity requirements detailed in Section 2.1 for 75%, 89% and 86% of all the individual RPU values generated for samples A, B and C respectively. The test systems and laboratories for which these validity criteria were not met are indicated in Table 2. For sample A, the use of individual donations of fresh WB gave the highest frequency of non-parallel results from any one test system with 7 and 8 of 8 blood donors' over 2 independent tests giving statistically invalid results for IL-6 and IL-1β readouts respectively. These two test systems were specific to laboratory 5 and also gave the highest frequency of invalid results for

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL	A-D1	A-D2	A-D3	A-D4	A-D5	B-D1	B-D2	B-D3	B-D4	B-D5	-
B	CTRL	A-D1	A-D2	A-D3	A-D4	A-D5	B-D1	B-D2	B-D3	B-D4	B-D5	-
C	CTRL	A-D1	A-D2	A-D3	A-D4	A-D5	B-D1	B-D2	B-D3	B-D4	B-D5	-
D	CTRL	A-D1	A-D2	A-D3	A-D4	A-D5	B-D1	B-D2	B-D3	B-D4	B-D5	-
E	E	C-D1	C-D2	C-D3	C-D4	C-D5	Ref-D1	Ref-D2	Ref-D3	Ref-D4	Ref-D5	-
F	E	C-D1	C-D2	C-D3	C-D4	C-D5	Ref-D1	Ref-D2	Ref-D3	Ref-D4	Ref-D5	-
G	E	C-D1	C-D2	C-D3	C-D4	C-D5	Ref-D1	Ref-D2	Ref-D3	Ref-D4	Ref-D5	-
H	E	C-D1	C-D2	C-D3	C-D4	C-D5	Ref-D1	Ref-D2	Ref-D3	Ref-D4	Ref-D5	-

Fig. 1. 96-well test plate layout. CTRL; negative control, E; endotoxin positive control, A, B or C; Bexsero samples A, B or C, Ref; Bexsero reference lot, D1 – D5; dilutions.

Table 1
MAT systems used by participating laboratories. Cryo: cryopreserved.

Laboratory/ System		Cells		Individual (I) or Pool (P)	Number of tests[a]	Donors per test	Readout	Notes
1		PBMC	Fresh	I	2	4	IL-6	8 different donors
2		PBMC	Cryo	I	2	4	IL-6	8 different donors
3	A	PBMC	Cryo	I	1	4	IL-6	4 different donors; buffy coats
	B	PBMC	Cryo	I	1	4	IL-6	4 different donors; leukocyte bags
4		PBMC	Cryo	I	3	4	IL-6	Same 4 donors of buffy coats in each independent test
5	A	PBMC	Fresh	I	2	4	IL-6	8 different donors
	B	WB	Fresh	I	2	4	1. IL-1β 2. IL-6	8 different donors
	C	WB	Fresh	P	1	4	1. IL-1β 2. IL-6	Same donors as used by Lab 5B, test 2
6		WB	Fresh	P	2	4	IL-1β	8 different donors
7		WB	Cryo	P	3	8	IL-1β	Merck Pyrodetect Cryo-blood kit. Same lot number used for 3 independent tests
8		WB	Cryo	P	2	8	IL-1β	Merck Pyrodetect Cryo-blood kit. Same lot number used for 2 independent tests
9	A	Mono Mac 6	Cell Line	–	2	1	IL-6	Used at passage 13. Source: DSMZ
	B	THP-1	Cell Line	–	2	1	TNFα	Used at passage 12. Source: ATCC

[a] Independent repetitions.

samples B and C, although the frequency was much lower for these samples with a maximum of 3 out of 8 donors' in two independent tests not meeting the criteria for any one sample. As illustrated in Fig. 2, the dose response curve generated for sample A with fresh WB resulted in a higher maxima than that of the reference lot. Furthermore, a statistical analysis showed significant ($p < 0.01$) non-parallelism between the dose-response curves of the reference and sample A using WB ($p = 0.002$) which was not the case for PBMC ($p = 0.917$). Whilst calculating an RPU value from parallel-line analysis using these curves may not be statistically valid, it does enable an approximate value to be generated. Data not meeting the validity criteria has been highlighted in all tables and graphs and are included only to allow for the identification of trends and differences between the systems.

3.3. Consistency and accuracy of results

Overall, RPU results for sample A were much less consistent than for samples B and C. This is highlighted by the larger spread of RPU values around the geometric mean for sample A in Fig. 3 compared with that of samples B and C, and by the notably higher geometric coefficient of variation (GCV) for sample A (GCVs of 38%, 27% and 25% for samples A, B and C respectively; Table 3). In addition, donor to donor variability in RPU results was larger for sample A than for sample C in 7 of the 8 test systems in which individual donations of whole blood/PBMC were cultured. For these 7 test systems, GCVs were in the range of 8–49% for sample A compared with 4–29% for sample C. One laboratory test system, LAB 5 A, showed notably low donor to donor variability in RPU for all 3 samples (GCV < 10% for samples A, B and C).

An accurate assessment of within–test system variability (by way of GCV calculation) for 6 of the 15 test systems was not possible due to a lack of independent test repeats (which also prevented an accurate assessment of the between-test system variability). LAB 7 and LAB 8 both used a commercial MAT kit containing a pool (from 8 different donors) of cryogenically preserved whole blood and performed 3 and 2 independent tests, respectively per sample. The within-test system variability for LAB 7 and LAB 8 was considerable for sample C given that this sample was the same material as the reference lot and that both these laboratories used the same kit batch number (hence the same blood pool) for each independent test carried-out.

The inaccuracy of each test system shown in Table 2 was based on the test system GM RPU for sample C compared with the expected RPU of 1.00. Nine of 15 laboratory test systems returned an inaccuracy value $\leq$10%, 4 of 15 laboratory test systems returned an inaccuracy value between 11% and 20% and only 2 of 15 laboratory tests systems returned an inaccuracy value >20%. LAB 4, who used PBMC from individual donations, generated relatively high RPU for all samples compared with the overall GM RPUs as shown in Fig. 4. Not surprisingly, the inaccuracy value for this laboratory test system was 35%.

The GM of all RPU generated for each sample, as shown in Table 3, were as anticipated with sample A showing the most relative activity (GM RPU 2.13), sample B showing the least relative activity (GM RPU 0.83) and with sample C, i.e. the same material as the reference lot, showing equivalent activity to the reference with a GM RPU 1.03 with the lower and upper 95% confidence limits for sample C of 0.97 and 1.09 RPU respectively.

4. Discussion

This collaborative study was carried-out to evaluate the inter-laboratory robustness of the MAT for measuring pyrogen levels in the intrinsically pyrogenic meningococcal B vaccine Bexsero. We analysed the datasets generated by 9 different laboratories covering 15 test systems for 3 batches of Bexsero associated with higher (sample A), moderate (sample C) and lower (sample B) relative activity in the MAT. Activity was measured in terms of relative pyrogen units by way of comparison of responses stimulated by the test sample to responses stimulated by the reference lot using parallel-line analysis with validity criteria as implemented for the OCABR testing of Bexsero at NIBSC.

The aim of this study was to evaluate how consistent the results would be using the range of test systems permitted in the Ph. Eur. The overall consistency of results generated was remarkable given the range of test formats employed by the participants and the limited method development undertaken. Based on GM RPU, all 15 laboratory test systems were capable of showing that sample A had the highest relative activity in the MAT. Samples B and C differed in their overall GM RPU of only 0.2 RPU (0.83 and 1.03 RPU respectively, see Table 3) suggesting that the activity of sample B is very similar to that of the reference, and that it is particularly difficult for test systems to distinguish between the two. Samples A, B and C were ranked as expected based on the GM of all RPU

Table 2
MAT results and geometric means (GM) in terms of activity relative to the reference lot of Bexsero (Relative Pyrogen Units; RPU) for samples A, B and C generated by 9 laboratories using the test systems listed in Table 1. Inaccuracy (%) = percentage inaccuracy for sample C, assuming an RPU of 1. Data not conforming to the validity criteria are denoted in grey. *Calculated value includes data not conforming to validity criteria.

Sample A RPU																	
Lab / System	1	2	3	4	Assay GM	5	6	7	8	Assay GM	9	10	11	12	Assay GM	Overall GM	GCV (%)
1	1.95	2.82	2.83	1.50	2.20	1.31	1.45	1.57	1.42	1.43	-	-	-	-		1.78	36
2	2.84	1.87	1.64	1.82	2.00	1.99	2.01	2.31	1.65	1.98	-	-	-	-		1.99	20
3 A	1.34	1.50	1.53	1.12	1.36	-	-	-	-		-	-	-	-		1.36	15
3 B	1.36	1.79	2.17	2.95	1.99	-	-	-	-		-	-	-	-		1.99	39
4	5.36	2.34	3.01	2.65	3.16	3.66	2.34	2.92	2.76	2.88	3.11	4.10	3.79	2.66	3.37*	3.06	27
5 A	1.92	1.92	1.69	1.64	1.79	1.97	1.85	1.69	1.84	1.83*	-	-	-	-		1.81	8
5 B1	7.25	3.23	3.10	4.00	4.13*	2.70	1.97	2.33	3.57	2.58*	-	-	-	-		3.26*	49*
5 B2	6.87	4.26	4.19	4.09	4.73*	2.97	1.90	3.41	5.09	3.15*	-	-	-	-		3.86*	46*
5 C1	2.61	-	-	-		-	-	-	-		-	-	-	-		2.61	n/a
5 C2	2.46	-	-	-		-	-	-	-		-	-	-	-		2.46	n/a
6	1.89	-	-	-		2.31	-	-	-		-	-	-	-		2.09	n/a
7	1.44	-	-	-		2.50	-	-	-		2.26	-	-	-		2.01	34
8	2.25	-	-	-		2.84	-	-	-		-	-	-	-		2.53	n/a
9 A	2.37	-	-	-		2.04	-	-	-		-	-	-	-		2.37	n/a
9 B	2.05	-	-	-		2.06	-	-	-		-	-	-	-		2.05	n/a

Sample B RPU																	
Lab / System	1	2	3	4	Assay GM	5	6	7	8	Assay GM	9	10	11	12	Assay GM	Overall GM	GCV (%)
1	0.95	0.95	0.76	0.70	0.83	0.71	0.84	1.12	0.81	0.86	-	-	-	-		0.84	18
2	1.03	0.73	0.80	0.75	0.82	0.73	0.80	0.55	0.70	0.69	-	-	-	-		0.75	19
3 A	0.63	0.70	0.63	0.55	0.63	-	-	-	-		-	-	-	-		0.63	10
3 B	0.63	0.69	0.85	0.87	0.75	-	-	-	-		-	-	-	-		0.75	17
4	1.31	0.93	0.66	0.65	0.85	0.99	0.69	1.22	0.89	0.93	0.85	1.24	1.01	0.76	0.95	0.91	28
5 A	0.83	0.90	0.84	0.85	0.85*	0.95	0.93	0.91	0.81	0.90	-	-	-	-		0.88	6
5 B1	1.71	0.68	1.27	0.73	1.02*	0.72	0.92	1.09	0.93	0.90*	-	-	-	-		0.82	22
5 B2	1.29	0.81	0.73	0.36	0.72*	0.62	0.82	1.13	0.75	0.81	-	-	-	-		0.71	47
5 C1	1.36	-	-	-		-	-	-	-		-	-	-	-		1.36	n/a
5 C2	0.87	-	-	-		-	-	-	-		-	-	-	-		0.87	n/a
6	0.85	-	-	-		1.07	-	-	-		-	-	-	-		1.07	n/a
7	0.79	-	-	-		1.10	-	-	-		1.41	-	-	-		1.07	34
8	1.07	-	-	-		0.56	-	-	-		-	-	-	-		0.77	n/a
9 A	1.25	-	-	-		0.99	-	-	-		-	-	-	-		0.99	n/a
9 B	0.86	-	-	-		0.72	-	-	-		-	-	-	-		0.79	n/a

Sample C RPU																		
Lab / System	1	2	3	4	Assay GM	5	6	7	8	Assay GM	9	10	11	12	Assay GM	Overall GM	GCV (%)	Inaccuracy (%)
1	0.97	1.27	1.06	0.98	1.06	0.79	0.87	1.02	0.98	0.91	-	-	-	-		0.98	15	2
2	1.08	0.89	1.01	1.04	1.00	0.91	1.33	1.11	1.22	1.13	-	-	-	-		1.06	15	6
3 A	0.86	0.92	1.02	0.71	0.87	-	-	-	-		-	-	-	-		0.87	16	13
3 B	0.94	0.93	0.70	1.21	0.93	-	-	-	-		-	-	-	-		0.93	25	7
4	1.79	1.18	1.29	1.28	1.37	1.27	1.07	1.21	1.24	1.19	1.91	1.43	1.44	1.35	1.52*	1.35	19	35
5 A	1.05	1.06	1.01	0.97	1.02*	1.05	1.09	1.02	1.01	1.04	-	-	-	-		1.04	4	4
5 B1	1.65	0.70	0.97	0.90	1.00*	1.09	1.00	0.91	0.94	0.98	-	-	-	-		0.92	27	8
5 B2	1.30	0.73	0.58	0.71	0.79*	0.89	0.99	1.00	1.01	0.97	-	-	-	-		0.85	29	15
5 C1	0.99	-	-	-		-	-	-	-		-	-	-	-		0.99	n/a	1
5 C2	0.89	-	-	-		-	-	-	-		-	-	-	-		0.89	n/a	11
6	0.95	-	-	-		0.98	-	-	-		-	-	-	-		0.96	n/a	4
7	0.91	-	-	-		1.38	-	-	-		1.80	-	-	-		1.31	41	31
8	0.56	-	-	-		1.22	-	-	-		-	-	-	-		0.83	n/a	17
9 A	1.10	-	-	-		0.65	-	-	-		-	-	-	-		1.10	n/a	10
9 B	0.99	-	-	-		0.89	-	-	-		-	-	-	-		0.94	n/a	6

generated for each sample and the overall GM RPU of 1.03 with lower and upper 95% confidence limits of 0.97 and 1.09 respectively for sample C were remarkably close to the expected value of 1.00.

The results of this collaborative study also demonstrated a good level of accuracy for most test systems, with 13 of 15 showing greater than 80% accuracy in their GM RPU for sample C. The highest inaccuracy value was for LAB 4 who used cryopreserved PBMC from individual donations. Interestingly, nearly all donor RPU for LAB 4 were higher than the GM of all RPU for each sample. It is possible this was due to the differential treatment of the samples and the reference lot. A procedure which resulted in lower responses than expected to the reference lot would give higher RPU values for the samples. This may have been resolved with additional method development.

The parallel-line method of analysis and associated validity criteria to estimate RPU in this study was developed for use with data generated using PBMC/IL-6 at NIBSC therefore the inability to fit all the data in the study to this same model with the breadth of test systems used was not unexpected. The highest frequency of statistically invalid RPU was for sample A using a test system which involved fresh WB from individual donations (i.e. not pooled) with IL-1β and IL-6 readouts (LAB 5 B1 and 2). As both IL-1β and IL-6 were quantified from the same cell-conditioned medium it is not surprising that they were equally affected. Although there were cases of invalidity also for sample C for fresh WB from individual donors, these cases were much less frequent than for sample A. It is clear that sample A stimulated more activity in the MAT compared with the reference lot in all systems used, resulting in RPUs all greater than 1.00. With fresh WB (not pooled), the enhanced

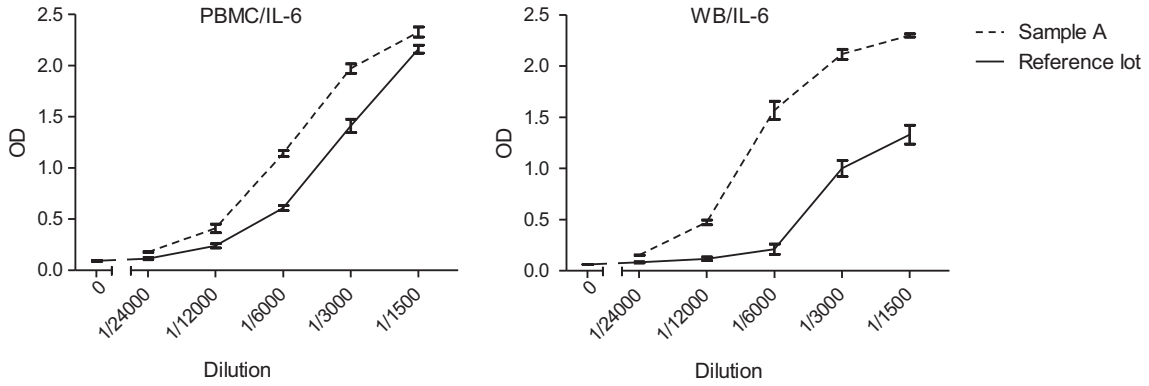

Fig. 2. Typical dose response curves generated from two individual donors for the reference lot (continuous line) and sample A (dashed line) by Lab 5 using PBMC and WB with IL-6 as the readout. Data presented as means ± SEM (n = 4), outliers excluded by Dixon's test as described in Section 2.1.

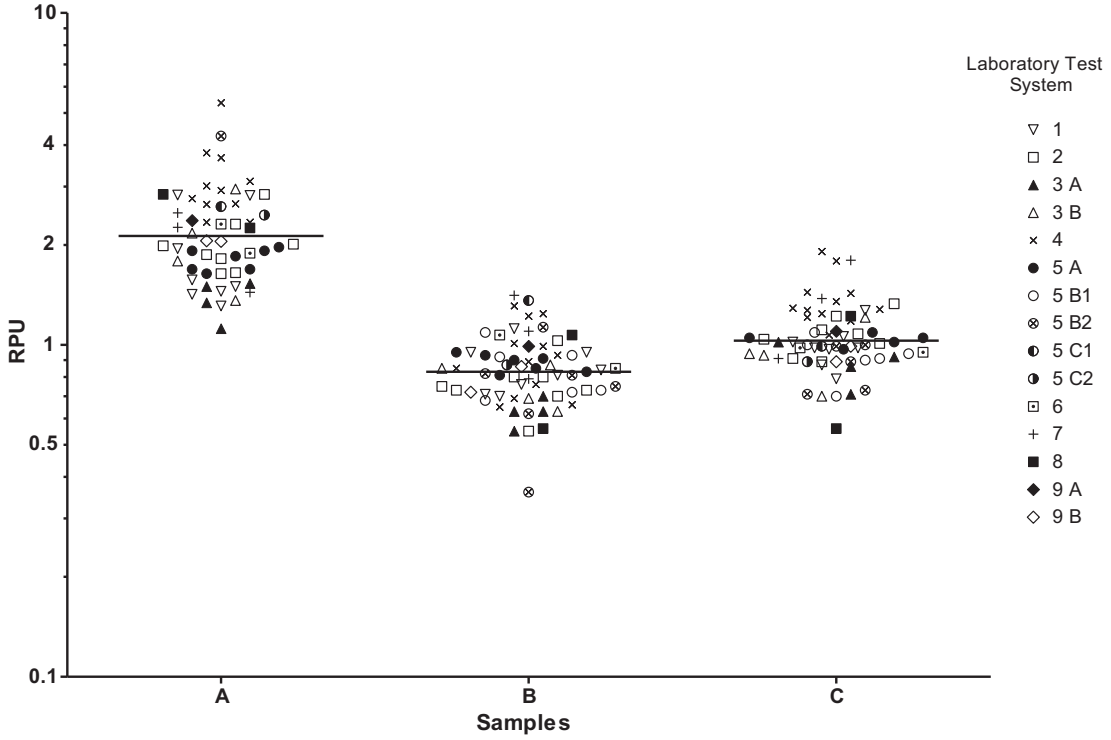

Fig. 3. MAT results in terms of activity relative to the reference lot of Bexsero (Relative Pyrogen Units; RPU) for Bexsero samples A, B and C generated by 9 laboratories using the test systems listed in Table 1. Each symbol represents a valid RPU value generated from responses of an individual donation of blood cells, one pool (from multiple donors) of cells or one passage of a cell-line. Continuous horizontal lines represent the geometric mean of all valid RPU values generated by the participating laboratories for a given sample.

Table 3
Geometric mean relative pyrogen units (GM RPU), geometric coefficient of variation (GCV) and lower (LCL) and upper (UCL) confidence limits based on all valid RPU generated for Bexsero samples A, B and C.

	Sample		
	A	B	C
GM RPU	2.13	0.83	1.03
GCV (%)	38	27	25
95% LCL	1.95	0.78	0.97
95% UCL	2.32	0.88	1.09

activity of sample A resulted in a steeper dose-response curve compared with that of the reference lot, culminating in statistically significant non-parallelism for a majority of blood donors as illustrated in Fig. 2. Differences in the response of PBMC and WB to meningococcal OMVs have been observed by others [31–33]. The reason(s) for the enhanced activity with fresh WB (not pooled) in this study are not clear and the possibilities are extensive. What is clear is that in order to implement LAB test systems 5 B1 and 2 for batch release, a different statistical model and validity criteria would need to be validated to accommodate the non-parallelism.

For test systems which employed the use of individual blood donations, the greater donor to donor variability in RPU for sample A compared with that of sample C is surprising as it could be expected that donor responses are normalised by the use of a reference lot. The pyrogenic component or components responsible for the extra MAT activity stimulated by sample A are not known, but it is possible that there is some synergistic effect caused by additional ligands of innate receptors (such as TLRs and NODs

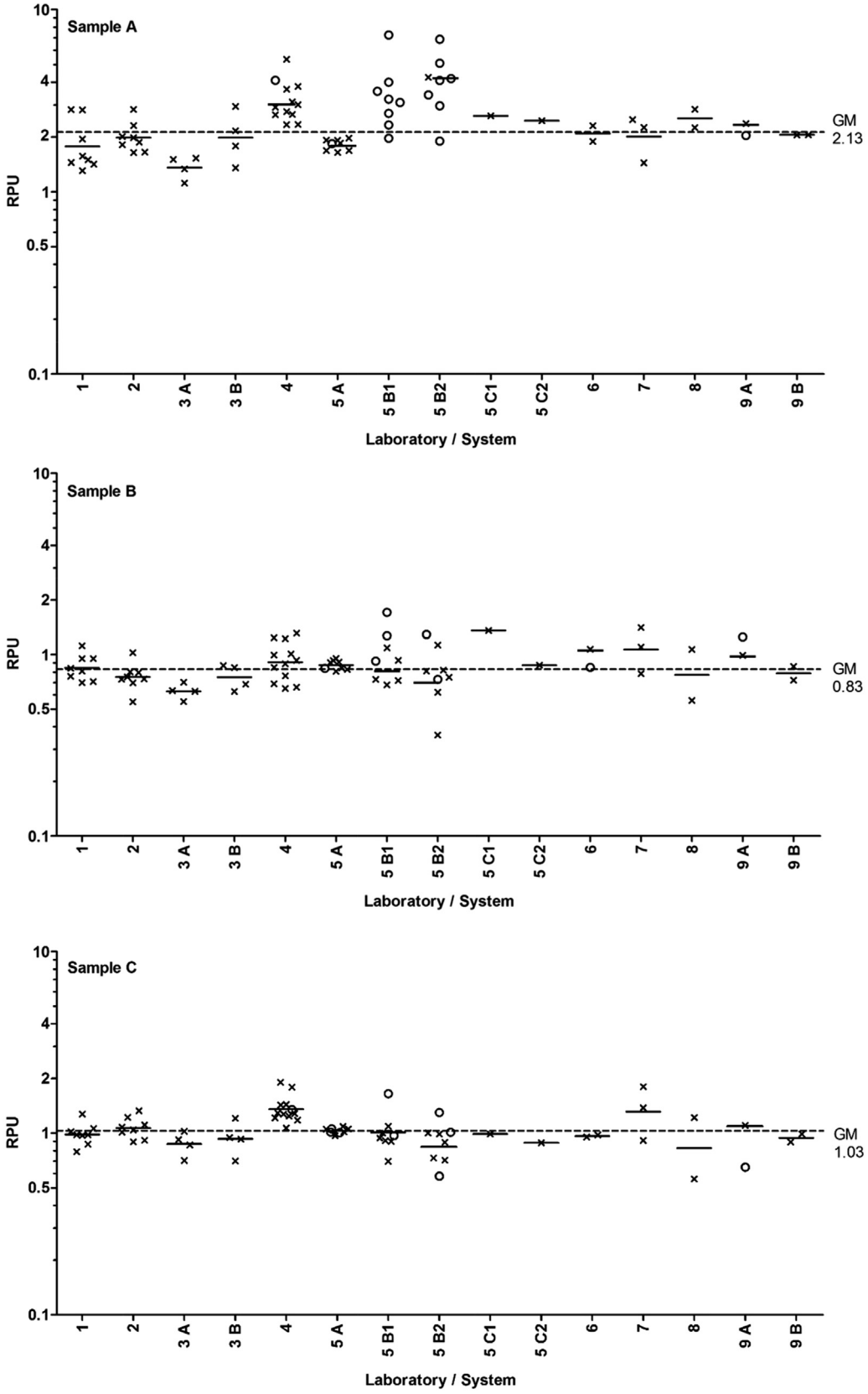

Fig. 4. MAT results in terms of activity relative to the reference lot of Bexsero (Relative Pyrogen Units; RPU) for Bexsero samples A, B and C generated by 9 laboratories using the systems listed in Table 1. Each symbol is an RPU value generated from responses of an individual donation of cells, one pool (from multiple donors) of cells or one passage of a cell-line. Crosses are values conforming to assay validity criteria, open circles are approximate values using statistically invalid data described in Section 2.1. Continuous horizontal lines represent the geometric mean (GM) of all RPU values generated for each system/laboratory, excluding statistically invalid data. Broken horizontal lines represent the overall GM i.e. of all valid RPU values generated for a given sample.

[1,34]) in this vaccine sample, presumably from the OMV component. The increase in RPU variability for sample A suggests a difference in the sensitivity of some donors to the components responsible for the extra activity associated with this sample. This could be attributed to polymorphisms in the TLR genes responsible for mediating responses to different pyrogens [18]. TLR single nucleotide polymorphisms (SNPs) have been associated with higher susceptibility to disease and disease severity (reviewed by Gianchecchi et al. [35]), and indeed may represent the variability in fever responses in vaccine recipients. The donor to donor variability in RPU for sample A raises concern about the appropriateness of cell-lines for testing this vaccine given that each cell-line is derived from only a single individual. Additionally, whilst the majority of systems covered the dose response in the dilution range 1:1,500–1:24,000, the cell lines required much higher concentrations (1:20-1:1000 for MM6 and 1:90-1:7290 for THP-1), indicating a reduced sensitivity. This is an example of where careful consideration would be required in setting appropriate batch pass/fail specifications. It would be inappropriate to apply the same specification to a test system using MM6 cells and one in which fresh WB from individual donors is used, especially where all donor RPU are required to comply with the specification for the batch to pass. That said, test systems in this study employing the use of cell-lines successfully ranked all three samples in the expected order in terms of their activity in the MAT. However, for the cell-line MM6 (LAB 9A) a valid RPU could only be calculated for each sample using data from one of two independent tests. This issue with invalidity may or may not be resolved by improvement of test consistency and operator performance.

In summary, this collaborative study shows that for the OMV-containing vaccine Bexsero, the MAT is generally a robust method for measuring the pyrogenic content of this vaccine when carried out as a reference lot comparison test. However, multiple factors can influence the magnitude of the RPU measured; the cell source and donor sensitivity seem to be particularly important. The data generated suggests careful consideration should be taken when choosing the statistical model, validity criteria and pass/fail specification for each given test system, and the need for each laboratory to validate their methods in-house. In the future the MAT should be considered as an alternative to the RPT for the routine testing of intrinsically pyrogenic vaccines.

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Conflict of Interest

The authors have no conflict of interest concerning this collaborative study.

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