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Monocyte-activation test to reliably measure the pyrogenic content of a vaccine: An *in vitro* pyrogen test to overcome *in vivo* limitations

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ABSTRACT

Pyrogen content is one of the critical quality attributes impacting the safety of a product, and there is an increasing need for assays that can reliably measure this attribute in vaccines. The Limulus amebocyte lysate (LAL) assay and the rabbit pyrogen test (RPT) are the canonical animal-based pyrogen tests currently used to release vaccines; however, there are several drawbacks associated with these tests when applied to Bexsero, intrinsically pyrogenic product, containing a meningococcal Outer Membrane Vesicle component. While the RPT, as applied to Bexsero at its given dilution, ensures safe vaccine, it is highly variable and prone to false positive results. On the other hand, the LAL assay although quantitative, can detect only endotoxin pyrogens and is not sufficient for monitoring the safety of Bexsero, which contains both LPS and non-endotoxin pyrogens. Being aware of these limitations of the RPT and LAL when applied to Bexsero, the Monocyte Activation Test (MAT) which is sensitive to both endotoxin and non-endotoxin based pyrogens has been developed as an alternative pyrogen test. Here, the development and the validation of a MAT assay adapted from the European pharmacopoeia for Bexsero, is described. The MAT assay is then used for monitoring the safety and consistency of Bexsero vaccines at release, providing great advantages in terms of reduced variability with respect to RPT, reduction of animal use, in line with the 3Rs principle concerning the protection of animals and faster time to market. In addition the correlation of the MAT to the RPT has been demonstrated supporting the replacement of the *in vivo* method and the potential application of the assay to other intrinsically pyrogenic vaccines.

1. Introduction

Various *in vivo* methods, such as the Rabbit Pyrogen Test and the diphtheria and tetanus potency tests, have played a central role in controlling the safety and potency of vaccines, and those methods are described within monographs in the Pharmacopoeias [1,2]. However, the *in vivo* methods have inherent limitations, such as high variability and complexity due to the use of animals, making them not always fully suitable for appropriately monitoring consistency of production or for accurately measuring critical quality attributes [3].

As a consequence, in the recent years, several efforts have been directed towards the development of *in vitro* methods to replace the *in vivo* ones for quality control of vaccines [4–6].

The *in vitro* tests represent a superior alternative to *in vivo* methods. In general, the *in vitro* methods exhibit a much lower variability with higher sensitivity, allowing a better evaluation of the critical quality attributes for the product of interest, with reduction of time and costs for lot release testing. Additionally, for ethical reasons, there is a global commitment to the reduction of animal usage in accordance to the 3Rs principle (Refinement, Reduction, Replacement) [3,7]. In Europe the implementation of the Directive 2010/63/EU paved the way for the replacement of *in vivo* test with reliable and validated *in vitro* test, wherever possible.

Bexsero [8] has been developed to prevent invasive disease caused by serogroup B Neisseria meningitidis; the vaccine is approved for use in different age groups in many countries worldwide. Bexsero is a multicomponent vaccine consisting of three recombinant protein antigens (rP), i.e. the factor H binding protein (fHbp) [9,10], the Neisserial adhesin A (NadA) [11] and the Neisserial heparin-binding antigen (NHBA) [12], and the Outer Membrane Vesicles (OMV) [13–15] from Gram-negative Neisseria

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meningitidis serogroup B; all components are adsorbed onto aluminum hydroxide [16]. The OMV are purified from the bacterial membranes and are inherently pyrogenic, containing both endotoxin (meningococcal lipopolysaccharide) and non-endotoxin (e.g. lipoproteins) fever-inducing components [17].

The *in vivo* Rabbit Pyrogen Test (RPT) [18–20], originally developed for controlling products that should not contain any amounts of pyrogens, has undergone several adaptations from the procedures described in the pharmacopoeia, for the application to a product that is inherently pyrogenic, such as Bexsero [17]. Since batches released with the adapted RPT have been demonstrated to be safe both in clinical studies and commercial experience, the test has provided an ongoing assurance of the safety of manufactured materials.

While the RPT, as applied to Bexsero at its given dilution, ensures safe vaccine, it is highly variable and prone to false positive results. Given this intrinsic propensity to false positives, the RPT failures may exclude safe vaccine batches from availability to target populations. On the other hand, the Limulus amebocyte lysate (LAL) [21,22] is a very sensitive method originally designed for detecting potential endotoxin contaminants in pyrogenic-free products which required extensive modifications when applied to Bexsero. In addition, the assay although quantitative, can detect only endotoxin pyrogens and is not sufficient for monitoring the overall safety of Bexsero.

Due to their original nature of control testing for non-intrinsic pyrogenic products and the need of adaptation when applied to Bexsero, both RPT and LAL methods have continuously shown challenges, paving the way for the Monocyte Activation Test (MAT) as an alternative pyrogen test for Bexsero release. The MAT is a cell-based *in vitro* test for assessing pyrogenicity [23], which has been recently included into the European Pharmacopoeia [24] as compendial method and mentioned by USP [25]. The MAT is a sensitive quantitative test able to detect both endotoxin and non-endotoxin pyrogens in a product, at very low concentrations, rather than simply screening for the presence or absence of pyrogenic contaminants like done by RPT [26]. The MAT combines the *in vitro* performance of the LAL test with the wide range of pyrogens detectable by the RPT and can potentially replace both methods [27].

The MAT applied to Bexsero was developed in strict collaboration with European Official Medicine Control Laboratories [28] which were experiencing similar limitations when releasing Bexsero using RPT [17]. MAT was approved by European Medicines Agency in March 2017 and rolled out to other Jurisdictions accordingly.

This study describes the development and validation of a MAT, adapted on the basis of the Bexsero specific properties, as *in vitro* method in alternative to RPT for assessing consistency at release with Bexsero batches proven to be safe in humans, providing great advantages in terms of reduction of animal use, faster time to market and reduced variability with respect to RPT.

2. Materials and methods

2.1. Materials

Consumables, reagents and test samples for validation were described in Supplementary Materials and Methods.

2.2. Methods

All the methods were performed as described in Supplementary Materials and Methods.

3. Results

3.1. Choice of IL-6 as the most suitable read-out for MAT applied to Bexsero

The main fever-inducing cytokines used as read-out for pyrogen detection in MAT are interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα). The preferred MAT read-out is usually IL-6 because IL-6, unlike IL-1β and TNFα, is secreted entirely into the PBMC supernatant in large quantities, thereby permitting its complete estimation [30].

In order to confirm the superiority of IL-6 also as readout for the MAT applied to Bexsero, a multiplex cytokine analysis was performed on PBMC supernatants after stimulation with Bexsero lots. The simultaneous measurement of IL-1β, IL-6 and TNFα showed the ability of the Bexsero vaccine to induce titration-dependent production of proinflammatory cytokines in human monocytoid cells. Among these, the IL-6 resulted to be the most abundant cytokine released by PBMCs of all the four tested donors, following stimulation with the Bexsero vaccine (almost 3-fold difference, Supplementary Fig. 1). IL-6 was therefore confirmed as the most suitable readout for MAT applied to Bexsero.

3.2. OMV component stimulates PBMC to produce IL-6, in a concentration-dependent manner

The Bexsero vaccine has intrinsic endotoxin and non-endotoxin pyrogenic components which are present in the OMV and are able to induce IL-6 production in PBMC [17]. In order to confirm the role of OMV component in the stimulation of PBMC, the MAT assay was performed on a trivalent formulation containing only the three recombinant protein antigens (i.e. without OMV), adsorbed to aluminum hydroxide, and the trivalent formulation spiked with increasing concentrations of OMV up to the full dose present in the Bexsero vaccine (Fig. 1, Supplementary Fig. 2).

Data demonstrated that the three recombinant protein antigens in the absence of the OMV did not induce IL-6 production, while PBMCs exhibited a dose-dependent response to the OMV with IL-6 levels directly proportional to the OMV content in the tested sample.

3.3. MAT applied to Bexsero is designed as a "reference lot comparison test" using a Bexsero lot as reference

Method A (Quantitative test) [24], which involves a comparison of the tested sample with a standard endotoxin dose-response curve, and Method C (Reference lot comparison test) [24], which involves a comparison of the tested sample with a reference lot of the same preparation, were compared to each other for their suitability applied to MAT for Bexsero vaccine.

Graphical evaluation of the results showed that the Endotoxin and Bexsero curves did not exhibit the same behaviour, with an evident lack of parallelism, making questionable the use of Method A for the MAT applied to Bexsero (Fig. 2A).

When a 4-Parameter Logistic Model (4-PL) was fit to the log transformed data, gaining a whole evaluation of the dose-response relationships, the statistical analysis confirmed the observed behaviours. When a Bexsero vaccine curve was compared to the Endotoxin standard, the majority of the assays (93.3%) were invalid based on a lack of parallelism (non-parallelism p-value < 0.05). In addition it was observed that the linear part of the dose-response curve of the Endotoxin standard was more constant among donors with respect to the dose-response curve of a Bexsero batch. As consequence the calibration of the Bexsero sample versus the Endotoxin standard led to a greater variability of the response among different donors. The distance between the linear

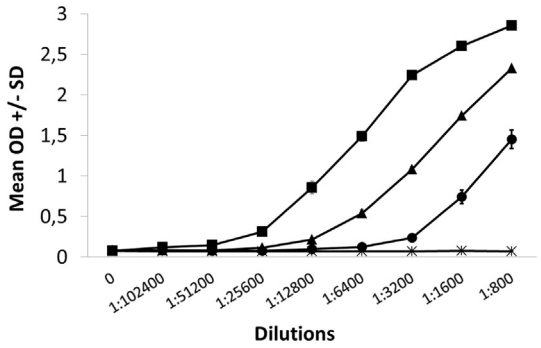

Fig. 1. OMV component of Bexsero stimulates PBMC to produce IL-6, in a concentration-dependent manner. Cryo-preserved PBMCs from three individual donors were stimulated with different MenB formulations containing increasing concentration of the OMV component. Mean ± SD (Standard Deviation) of OD expressing IL-6 levels secreted by quadruplicate cell cultures of one representative donor upon stimulation with serial dilutions of: trivalent MenB formulation containing the three MenB recombinant antigens NHBA-953, 936-fHbp and NadA (✱); the trivalent formulation plus 12.5 μg/ml of OMV (●); the trivalent formulation plus 25 μg/ml of OMV (▲); the trivalent formulation plus 50 μg/ml of OMV (■).

part of the different curves (Bexsero *vs* Bexsero and Bexsero *vs* Endotoxin) were calculated and expressed as Relative Response (RR). When a Bexsero test batch was calibrated against a second Bexsero batch, variability of results among donors showed a Coefficient of Variation (CV) of 9%; on the contrary, the CV increased to 47% when a Bexsero batch was calibrated against the endotoxin standard (Fig. 2B).

In conclusion, the Method C was identified as the only method applicable to Bexsero due to a lack of parallelism with the Endotoxin standard curve.

3.4. The mathematical model for the MAT applied to Bexsero is a parallel line assay shifted

A mathematical model for the Reference lot comparison test format can be linear (Parallel Line Assay, PLA) or non-linear (4-PL). A comparison between the models was performed on data obtained from an experimental study designed to achieve an overview on the major sources of variability for the MAT assay (e.g. operators, donors, analytical sessions and plates). The selection was then driven by an evaluation of the assay precision, the variability among assays and the number of invalid assays.

An improved version of the PLA (referred to as PLA shifted, PLAS) resulted to be the most promising one allowing a RR estimation with a precision and %CV comparable to the ones obtained with the 4-PL model but with a lower percentage of invalid assays (Table 1).

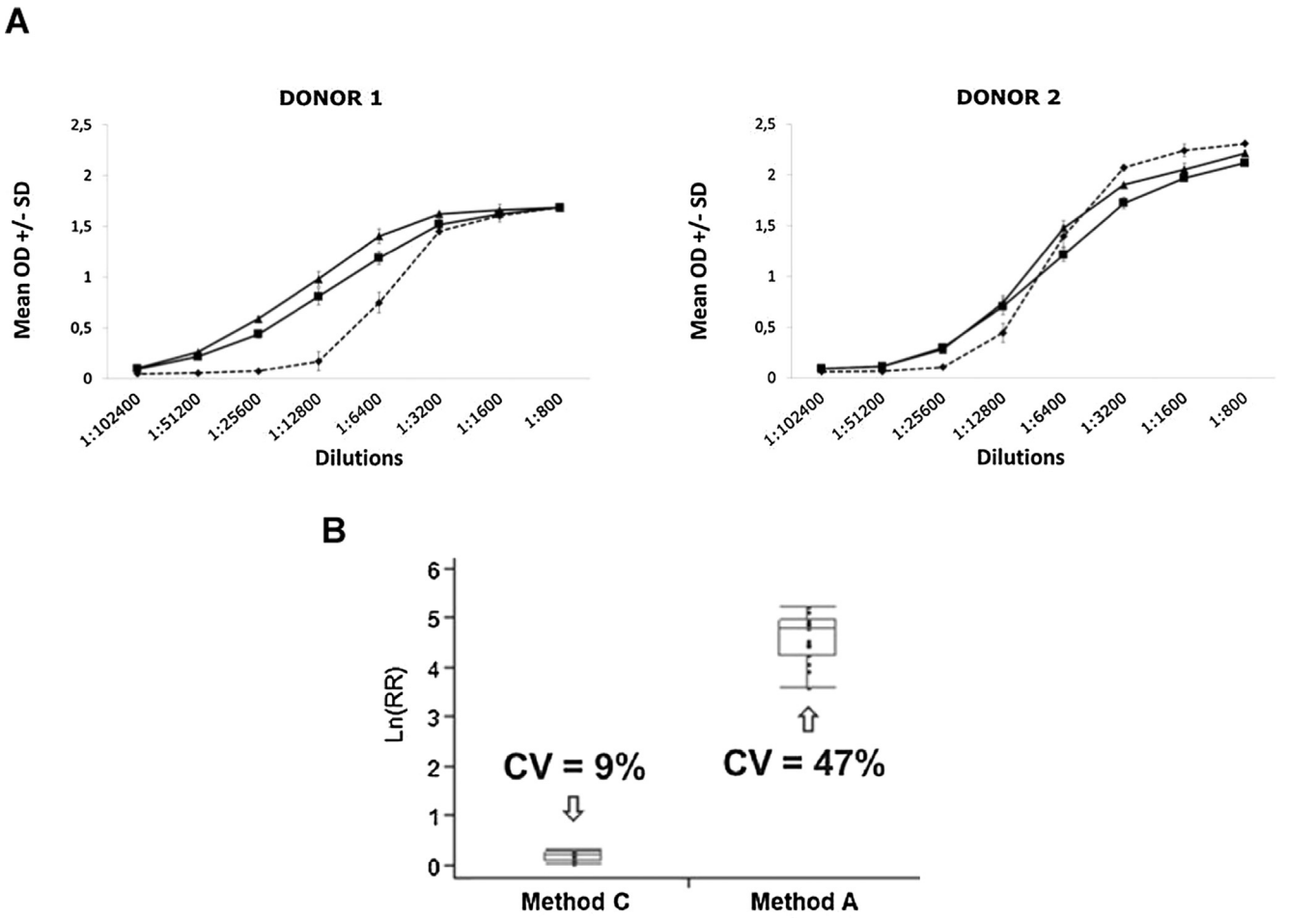

Fig. 2. Comparative analysis of method A and C for their suitability in MAT applied to Bexsero vaccine. (A) Cryo-preserved PBMCs from 15 donors were stimulated with CSE (control standard endotoxin) from *E. coli* strain O55:B5 (calibrated against the 3rd WHO International Standard (IS) for endotoxin (NIBSC code 10/178)) [29] and two Bexsero batches, one used as reference (selected as representative of the Bexsero production) and the other as test lot. Mean ± SD (Standard Deviation) of OD values expressing IL-6 levels secreted by quadruplicate cell cultures of two representative donors upon stimulation with serial dilutions of: Endotoxin standard, starting from 2 EU/ml (◆); Bexsero Test batch (▲); Bexsero Reference batch (■). (B) Graphical representation of the Ln(RR) (Relative Response expressed as natural logarithms) values of the Bexsero Test batch, calculated against the Endotoxin (Method A) and a second Bexsero batch (Method C) as reference, testing 15 different donors. Rectangular boxes indicate the average ± SD (Standard Deviation) of Ln(RR) values. % Coefficient of Variation (%CV) is indicated.

Table 1
Statistical output of the mathematical model comparison for the MAT applied to Bexsero. The comparison among 4-PL, PLA and PLAS model was performed by the evaluation of the assays precision (measured with the percentiles of the % width of 95% Confidence Interval (CI)), the variability among assays (measured with Intermediate Precision (IP) %CV) and the number of invalid assays. The statistical output of the model comparison showed that the PLAS model allowed a RR estimation with a precision and %CV comparable to the ones obtained with the 4-PL model but with a lower percentage of invalid assays. The low rate of invalid assays was probably due to the independent selection of dilution ranges for reference and test batch.

	Precision	IP CV%	Invalid assays
4-PL model	19–20%	6%	17–44%
PLA model	22–39%	7%	11–39%
PLAS model	22–27%	7%	11%

The PLAS model consisted in a PLA performed by considering 3 consecutive dilution points in the linear range of the vaccine response that can be shifted between the Reference and Test vaccine. A schematic view of the selected mathematical model for MAT is shown in Supplementary Fig. 3.

Finally, to define the most appropriate number of donors to be tested for an assay the Geometric Mean of RR (GM(RR)) %CV varying number of sessions and number of donors per session was computed. Results, shown in Table 2, indicated that there was a decrease in variability (as GM(RR) %CV) by executing the assay with multiple donors, within the same session, or among multiple sessions. However, it should be noticed that multiple sessions did not markedly improve the assay precision; therefore, balancing this finding with operational aspects, the final assay was designed considering four donors tested in a single analytical session and a reportable result based on the GM of the 4 RR values.

3.5. MAT applied to Bexsero is a precise and accurate analytical method for its intended purpose

MAT assay applied to Bexsero underwent a product specific validation for a quantitative test, using three Bexsero batches in two different laboratories. Lack of interference with the detection system was confirmed by measuring the mean % activity of human IL-6 in the presence of a wide range of Bexsero dilutions (from 1/50 to 1/6400). Results ranged from 84% to 94% (Supplementary Table 1), confirming that the 1/50 dilution can be considered as the minimum Bexsero dilution at which the sample does not interfere with MAT detection system. A summary of the parameters investigated during MAT validation and the obtained results is reported in Supplementary Table 2. Briefly, results confirmed that the MAT was precise with an overall geometric CV% < 15% and accurate in the explored range of response levels from 0.50 to 2.00.

3.6. MAT correlates with RPT and LAL

The potential correlation between MAT and RPT or LAL was evaluated. Towards this goal, 87 Bexsero batches were tested with MAT, RPT and LAL and results were collected for the comparison analysis.

In order to perform a correlation analysis with RPT results, since pass/fail was not informative, the average values of the temperature rises observed for each lot were used.

Correlation analyses with MAT results versus RPT results and LAL results were performed. A significant positive correlation ($R = 0.41$, p-value < 0.0001) was observed between MAT and RPT results, confirming that the MAT was correctly measuring the pyrogenic content present in the Bexsero and supporting its implementation instead of RPT (Fig. 3A). A significant positive correlation ($R = 0.22$, p-values = 0.04) was also obtained between MAT and LAL results when an anomalous single observation (represented as empty circle in Fig. 3B) was excluded from the analysis.

3.7. MAT assay is applicable to different vaccines having intrinsic pyrogenic compounds

In order to evaluate the applicability of the MAT assay to other vaccines known to contain components derived from N. Meningitidis serogroup B, the assay has been applied to Trumenba (Pfizer's vaccine) [31,32] and Comvax (Merck's vaccine) [33] in parallel with Bexsero.

Result showed that the IL-6 production upon stimulation with Trumenba was at least 100-fold higher than the corresponding cytokine production induced by Bexsero, as demonstrated by evaluating the distance between the corresponding response curves (Fig. 4). Conversely, PBMC response to Comvax was closest to the response induced by Bexsero, as demonstrated by the almost

Table 2
Identification of the most appropriate number of donors to be tested for a single MAT session. The most appropriate number of donors to be tested for an assay was evaluated by applying the format variability formula suggested in USP <1033> (Validation of Bioassays). Results were expressed as GM(RR) %CV values varying number of sessions and number of donors per session used for one test. Highlighted boxes indicated the combination of Donor/Analytical session requiring 4 donors. The final assay format was designed considering four donors tested in a single analytical session.

	Number of analytical sessions				
Number of donors per session	**1**	**2**	**3**	**4**	**5**
1	12%	9%	8%	8%	7%
2	10%	8%	7%	7%	7%
3	9%	8%	7%	7%	6%
4	9%	7%	7%	6%	6%
5	9%	7%	7%	6%	6%

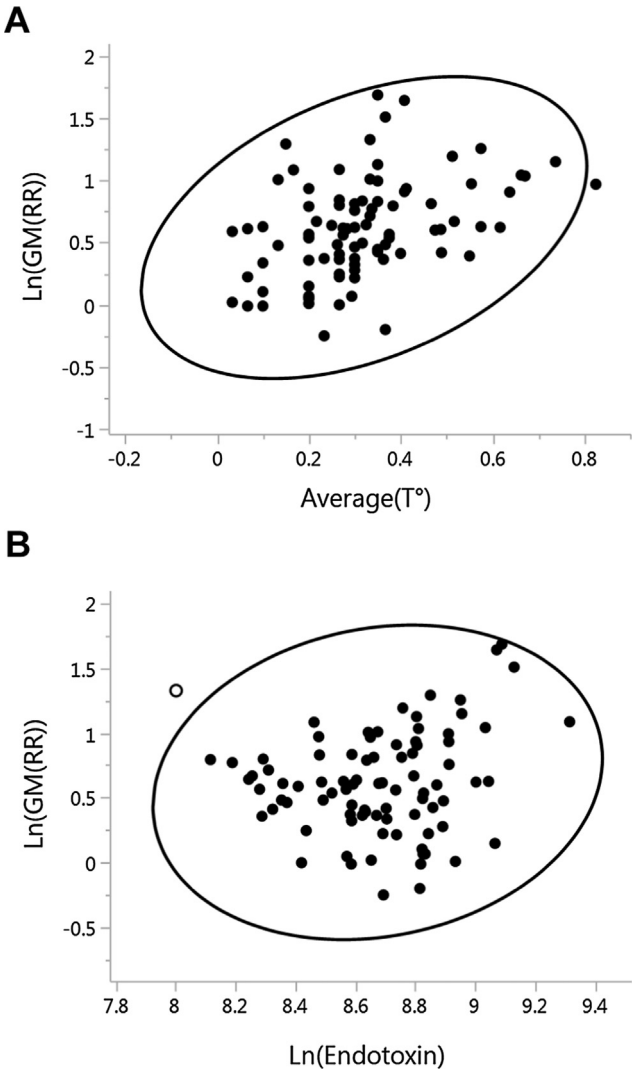

Fig. 3. Parallel comparison of MAT, RPT and LAL for Bexsero batches. A correlation analysis was performed on the results of 87 Bexsero batches tested with both MAT, RPT and LAL test. MAT results were expressed as the GM of four RR values obtained for each lot (Ln(GM(RR))). LAL test results were provided as International Unit (IU) values/ml, expressing the endotoxin content of each Bexsero batch tested (Ln (Endotoxin)). For MAT and LAL results are transformed in log-natural scale. In the case of RPT the average values of the temperature rises (Average(T°)) observed for each lot were used. Scatter plots between (A) MAT Ln(GM(RR)) and RPT Average(T°) and between (B) MAT Ln(GM(RR)) and LAL Ln(Endotoxin) were shown. A bivariate normal ellipse (p = 0.990) was represented in dark grey. In the scatter plot between MAT values and LAL values an anomalous single observation excluded from the correlation analysis was represented as empty circle.

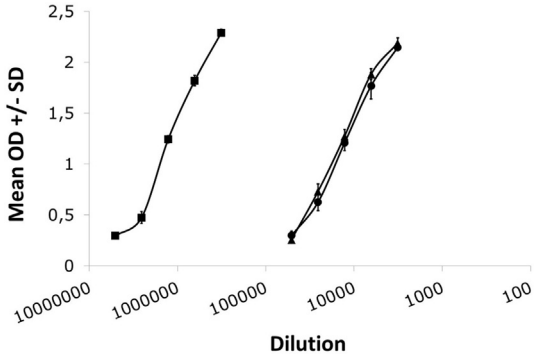

Fig. 4. MAT assay applied to different vaccines having intrinsic pyrogenic compounds. Cryo-preserved PBMC of 6 different donors were stimulated with three different vaccines containing components derived from *N. meningitidis* serogroup B. Since the assay format for the MAT applied to Bexsero was designed as a relative comparison based on the measurement of the distance (expressed as RR value) between the linear part of the test and reference curves of the same vaccine, the RR estimation was not suitable to compare Bexsero with other vaccines. For this reason, the comparative analysis was made by considering the IL-6 production (expressed as OD values) upon PBMC stimulation with the vaccines and by graphically evaluating the corresponding linear part of the response curves. Mean ± SD (Standard Deviation) of OD expressing IL-6 levels secreted by quadruplicate cell cultures of one representative donor upon stimulation with serial dilutions of: Bexsero vaccine (●); Trumenba (■) (Pfizer's MenB vaccine) composed of two recombinant lipidated factor H binding protein (fHBP) variants from *Neisseria meningitidis* serogroup B. The recombinant proteins were adsorbed to aluminum phosphate; Comvax (▲) (Merck's Hib-HB vaccine) composed of an Haemophilus influenzae type b capsular polysaccharide covalently bound to an outer membrane protein complex (OMPC) of *Neisseria meningitidis* serogroup B, and Hepatitis B surface antigen (HBsAg) from recombinant yeast cultures. Comvax was adjuvanted with amorphous aluminum hydroxyphosphate sulphate. Accordingly, the multi-vaccine comparative analysis was performed adjusting the starting dilution of each vaccine to get the full titration of response curve for all the tested samples. Results were represented by plotting the OD values against the vaccine dilutions (expressed in Logn scale).

perfect overlapping of the corresponding response curves. Lack of IL-6 production in the presence of all the Placebo buffers alone demonstrated the absence of any matrix effect (data not shown).

4. Discussion

RPT and LAL, originally developed for products usually free from pyrogenic contamination, present challenges and limitation when applied to a complex product with intrinsic pyrogenicity, such as Bexsero vaccine. Instead, the MAT has been demonstrated to be a valuable and fit-for-purpose alternative method allowing a more consistent, reproducible, robust, and quantitative approach to pyrogen testing.

So far, the MAT assay has been primarily used to detect contaminants in products which are not intrinsically pyrogenic [34,35]; therefore, the application to Bexsero vaccine has required a product-specific development and validation. The specificity of MAT for the detection of the known pyrogenic component present in the Bexsero vaccine (OMV) was confirmed since there was no response to the three recombinant protein antigens without OMV.

The MAT format for Bexsero followed the European Pharmacopoeia guidelines since it used cryopreserved human PBMCs as cell source and the released pro-inflammatory cytokine IL-6 as readout. The choice of IL-6 was based on experimental evidences: the concentrations of IL-6 detected in PBMC supernatant following administration of the Bexsero vaccine were higher than the concentrations of the other classical fever-inducing cytokines (IL-1β and TNFα). This finding was in agreement with the observation that IL-6, unlike IL-1β and TNFα, was secreted entirely into the cell supernatant in large quantities, thereby permitting its complete estimation [30,35,36]. Moreover, the IL-6 was the analyte in blood that better correlated with fever in humans [37].

The reference lot comparison test (Method C) using a Bexsero batch as internal Reference was determined to be the most suitable for Bexsero, since a lack of parallelism between endotoxin and vaccine response curves was observed making the use of Standard Endotoxin as reference, inapplicable. The lack of parallelism is ascribed to the presence of OMV in the Bexsero vaccine, a much more complex and heterogeneous pro-inflammatory matrix as compared to the single component solution of *E. coli* endotoxin. OMVs contain not only LPS from *Neisseria* (which in itself is structurally different from *E. coli* LPS) but also many others non-endotoxin IL-6 inducing factors (e.g. lipoproteins, periplasmic components) [38]. More dynamic interactions and synergistic rather than simply additive effects between the OMV endotoxin and non-endotoxin pyrogen components are therefore expected, with consequent lack of parallelism with the *E. coli* endotoxin standard.

Moreover, Method C is recommended when the pyrogenic content of a product was inherently high or to address a high donor variability [24]. In this respect, it should be noted that, while the response against endotoxin was generally constant among different donors, the response to non-endotoxin compounds could differ significantly [39–41]. Indeed, we observed a significant reduction in the variability of results among donors by using a Bexsero lot as reference respect to Endotoxin standard.

The reportable result of the MAT applied to Bexsero is a GM of 4 individual results (RR) from four different donors. The choice of the GM(RR) as reportable result is preferred in order to have a single numeric/quantitative result for each test batch.

Although the calculation of the GM is not mentioned in the Pharmacopoeia (that however allows pooling donors), its use ultimately allows assessing consistency at release with Bexsero batches proven to be safe in humans, without the application of a pass/fail criterion, considered not properly suitable and too stringent for a product with intrinsic endotoxin and non-endotoxin pyrogens.

Assay validation confirmed an acceptable precision for a biological assay, with an overall GCV% below 15%. Reproducibility of the results was also demonstrated across laboratories in a collaborative study [42].

A full correlation between *in vivo* and *in vitro* tests may not always be possible. In fact, many of the *in vivo* assays for vaccines have historically proven their value in ensuring the efficacy and safety of vaccines, without the requirement of formal product specific validations (such as for RPT), making a formal one-to-one comparison challenging or even impossible. Another aspect is that the quality attributes could be assessed differently between two methods.

The dilution needed to perform the RPT on Bexsero, in combination with the variability of the temperature responses, make the RPT prone to false positive results [17]. For this reason, a direct evaluation of lack of differences in the ability of the two methods to detect pyrogenic samples has been considered not suitable. Nevertheless, a statistically significant correlation of MAT values with rabbit temperature increase was shown, supporting the replacement of the RPT with MAT. Regarding the correlation with LAL, the significance was lower with respect to the one observed with RPT and obtained only by removing an outlier value. This was in general expected due to the fact that the two methods did not measure exactly the same attributes. Moreover, previous works have already reported that the MAT response to endotoxins did not always correlate with LAL assay results [43–45].

MAT can be applied to many other vaccines to quantify the amount of pyrogenic compounds, in case of intrinsically pyrogenic vaccines. In this study the IL-6 production of PBMC stimulated with Bexsero and other two vaccines containing meningococcal-derived components (Trumenba and Comvax) was compared. In particular, Trumenba showed a much greater fold induction in IL-6 release than Bexsero and Comvax, which conversely exhibited the same reactogenicity profile. The higher activation of IL-6 by the Trumenba could be due to the presence of lipoproteins [46], which are known to be able to activate the Toll-like receptor 2 [47]. Interestingly, this vaccine was indicated only for a target population aged 10 through 25 years while both Bexsero and Comvax were given to infants.

In conclusion, we have demonstrated the development and validation of a MAT, adapted on the basis of the Bexsero specific properties, as *in vitro* method in alternative to RPT. The assay is suitable for assessing consistency at release with Bexsero batches proven to be safe in humans. Its application allows a markedly simplification of the release testing panel together with a drastic reduction of animal use.

The successful outcome of the product-specific validation of MAT applied to a complex, adjuvanted and pyrogenic vaccine like Bexsero together with the ever-increasing interest of international regulations to such assay to reduce and replace animal testing [48] led to MAT submission and approval by different jurisdictions around the world.

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Author contributions

All authors attest they meet the ICMJE criteria for authorship. SV, GS, FB, SF, DS, BC were involved in the conception and design of the study. SV, GS performed the experiments. FB, SF and MP analyzed and interpreted the results. SV and BC were involved in drafting the paper. All authors were involved in revising the paper critically for important intellectual content and approved the final version.

Trademark statement

- Bexsero is a trade mark of the GSK group of companies.
- Trumenba is a trade mark of the Pfizer group of companies.
- Comvax is a trade mark of the Merck group of companies.

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This study was sponsored by Novartis Vaccines, now acquired by the GSK Vaccines srl. GSK took responsibility for all costs incurred in publishing and was involved in all stages of the study conduct and analysis.

Conflict of interest (COI) statement

All authors have declared the following interests: SV, GS, FB, MP, EB, LG, DS, BC were employees of Novartis Vaccines when the study initiated. Following the acquisition of Novartis Vaccines by the GSK group of companies in March 2015, SV, GS, FB, MP, EB, LG, DS, BC are now permanent employees of the GSK group of companies. SF was permanent employee of GSK group of companies at the time of the study (until November 2016). SF is now a researcher of University of Calabria.

Policy and ethics

Informed consent was obtained for use of human samples. Experiments using animals have been conducted in accordance with institutional guidelines.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.10.082.

References

[1] Martin WJ, Marcus S. Studies on bacterial pyrogenicity. 3. Specificity of the United States pharmacopeia rabbit pyrogen test. Appl Microbiol 1964;12:483–6.
[2] Robinson A, Funnell SGP. Potency testing of acellular pertussis vaccines. Vaccine 1992;10(3):139–41.
[3] De Mattia F, Chapsal JM, Descamps J, Halder M, Jarrett N, Kross I, et al. The consistency approach for quality control of vaccines - A strategy to improve quality control and implement 3Rs. Biologicals 2011;39(1):59–65.

[4] Hartung T, Aaberge I, Berthold S, et al. ECVAM workshop on novel pyrogen tests based on the human fever reaction. Altern Lab Anim 2001;29(2):99–123.
[5] Hendriksen CF. Refinement reduction and replacement of animal use for regulatory testing: current best scientific practices for the evaluation of safety and potency of biologicals. ILAR J 2002;43(Suppl.):S43–8.
[6] Kulpa-Eddy J, Dusek D. Application of the consistency approach to reduce animal use in vaccine potency testing. Proc Vaccinol 2011;5:232–5.
[7] Russell WMS and Burch RL. The principles of humane experimental technique. Methuen, London; 1959 (Reprinted by UFAW, 1992).
[8] Pizza M, Scarlato V, Masignani V, Giuliani MM, Arico B, Comanducci M, et al. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science 2000;287(5459):1816–20.
[9] Schneider MC, Prosser BE, Caesar JJ, Kugelberg E, Li S, Zhang Q, et al. Neisseria meningitidis recruits factor H using protein mimicry of host carbohydrates. Nature 2009;458(7240):890–3.
[10] Madico G, Welsch JA, Lewis LA, McNaughton A, Perlman DH, Costello CE, et al. The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. J Immunol 2006;177(1):501–10.
[11] Capecchi B, Adu-Bobie J, Di Marcello F, Ciucchi L, Masignani V, Taddei A, et al. Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells. Mol Microbiol 2005;55(3):687–98.
[12] Serruto D, Spadafina T, Ciucchi L, Lewis LA, Ram S, Tontini M, et al. Neisseria meningitidis GNA2132, a heparin-binding protein that induces protective immunity in humans. Proc Natl Acad Sci USA 2010;107(8):3770–5.
[13] Snape MD, Dawson T, Oster P, Evans A, John TM, Ohene-Kena B, et al. Immunogenicity of two investigational serogroup B meningococcal vaccines in the first year of life: A randomized comparative trial. Pediatr Infect Dis J 2010;29(11):e71–9.
[14] Parikh SR, Andrews NJ, Beebeejaun K, Campbell H, Ribeiro S, Ward C, et al. Effectiveness and impact of a reduced infant schedule of 4CMenB vaccine against group B meningococcal disease in England: A national observational cohort study. Lancet 2016;388(10061):2775–82.
[15] Acevedo R, Fernández S, Zayas C, et al. Bacterial outer membrane vesicles and vaccine applications. Front Immunol 2014;5(121):1–6.
[16] Giuliani MM, Adu-Bobie J, Comanducci M, Arico B, Savino S, Santini L, et al. A universal vaccine for serogroup B meningococcus. Proc Natl Acad Sci USA 2006;103(29):10834–9.
[17] Vipond C, Findlay L, Feavers I, Care R. Limitations of the rabbit pyrogen test for assessing meningococcal OMV based vaccines. ALTEX 2016;33:47–53.
[18] Hort EC, Penfold WJ. Microorganisms and their relation to fever: preliminary communication. J Hyg (Lond) 1912;12(3):361–90.
[19] Greisman SE, Hornick RB. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc Soc Exp Biol Med 1969;131(4):1154–8.
[20] Roberts KJ. The pyrogen test. In: Endotoxins - pyrogens, LAL testing and depyrogenation. CRC Press; 2007. p. 261–71.
[21] Bang FB. A bacterial disease of Limulus polyphemus. Bull Johns Hopkins Hosp 1956;98:325–51.
[22] Williams KL. Endotoxin relevance and control overview. In: Endotoxins - pyrogens, LAL testing and depyrogenation. CRC Press; 2007. p. 27–45.
[23] Hoffmann S et al. International validation of novel pyrogen tests based on human monocytoid cells. J Immunol Methods 2005;298(1–2):161–73.
[24] European Directorate for the Quality of Medicines. Monocyte activation test, Chapter 2.6.30. European Pharmacopoeia 9th ed., Version 9.2; 2017.
[25] United States Pharmacopeial Convention. Pyrogen Test, USP <151>. United States Pharmacopeia (USP) 39-NF34; 2016.
[26] Hartung T. The human whole blood pyrogen test - lessons learned in twenty years. ALTEX 2015;32(2):79–100.
[27] Fennrich S, Hennig U, Toliashvili L, Schlensak C, Wendel HP, et al. More than 70 years of pyrogen detection: current state and future perspectives. Altern Lab Anim 2016;44(3):239–53.
[28] Vipond C, Sutherland J, Nordgren K, Kemp G, Rory Care AH, Findlay L. Replacement of the rabbit pyrogen test with the monocyte activation test for the control/safety testing of an OMV-based Meningococcal B vaccine. Vaccine 2018. in press.
[29] Findlay L, Desai T, Heath A, Poole S, Crivellone M, Hauck W, et al. Collaborative study for the establishment of the WHO 3(rd) international standard for endotoxin, the Ph. Eur. endotoxin biological reference preparation batch 5 and the USP reference standard for endotoxin lot H0K354. Pharmeur Bio Sci Notes 2015;2015:73–98.
[30] Poole S et al. Proceedings of the European Workshop on detection and quantification of pyrogen. Pharmeuropa special 1989;1:17–8.
[31] Gohil K. Pharmaceutical approval update. Pharm Therapeut PT 2015;40(6):362–3.
[32] TRUMENBA® (Meningococcal Group B Vaccine) Prescribing Information. Philadelphia, PA: Pfizer, Inc; 2015.
[33] Davis RL, Black S, Shinefield H, Mahoney L, Zavitkovsky A, Lewis E, et al. Post-marketing evaluation of the short term safety of COMVAX. Vaccine 2004;22(3–4):536–43.
[34] Fennrich S, Fischer M, Hartung T, et al. Detection of endotoxins and other pyrogens using human whole blood. Dev Biol Stand 1999;101:131–9.
[35] Poole S. 'Monocyte test' for pyrogen. Pharmeuropa 1989;1(5):238.
[36] Taktak YS, Selkirk S, Bristow AF, Carpenter A, Ball C, Rafferty B, et al. Assay of pyrogens by interleukin-6 release from monocytic cell lines. J Pharm Pharmacol 1991;43(8):578–82.
[37] Gaines Das RE, Brügger P, Patel M, Mistry Y, Poole S. Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs: test design and data analysis. J Immunol Methods 2004;288(1–2):165–77.
[38] Vipond C, Suker J, Jones C, Tang C, Feavers IM, Wheeler JX. Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. Proteomics 2006;6(11):3400–13.
[39] Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, et al. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci USA 2005;102(27):9577–82.
[40] Kanegasaki S, Tanamoto K, Yasuda T, Homma JY, Matsuura M, Nakatsuka M, et al. Structure-activity relationship of Lipid A: comparison of biological activities of national and synthetic Lipid A's with different fatty acid compositions. J Biochem 1986;99(4):1203–10.
[41] Munford RS, Varley AW. Shield as signal: Lipopolysaccharides and the evolution of immunity to Gram-negative bacteria. PLoS Pathog 2006;2(6):e67.
[42] Findlay L, Sutherland J, Desai T, Hockley J, Care R, Nordgren K, et al. Collaborative Study Group. Evaluation of the monocyte activation test for the safety testing of meningococcal B vaccine Bexsero: A collaborative study. Vaccine 2019;37(29):3761–9.
[43] Dehus O, Hartung T, Hermann C. Endotoxin evaluation of eleven lipopolysaccharides by whole blood assay does not always correlate with Limulus amebocyte lysate assay. J Endotoxin Res 2006;12(3):171–80.
[44] Brandenburg K, Howe J, Gutsman T, Garidel P. The expression of endotoxic activity in the Limulus test as compared to cytokine production in immune cells. Curr Med Chem 2009;16(21):2653–60.
[45] Gutsmann T, Howe J, Zähringer U, et al. Structural prerequisites for endotoxic activity in the Limulus test as compared to cytokine production in mononuclear cells. Innate Immun 2010;16(1):39–47.
[46] Luo Y, Friese OV, Runnels HA, et al. The dual role of lipids of the lipoproteins in Trumenba, a self-adjuvanting vaccine against meningococcal meningitis B disease. AAPS J 2016;18(6):1562–75.
[47] Massari P et al. Cutting edge: Immune stimulation by Neisserial porins is Toll-like receptor 2 and MyD88 dependent. J Immunol 2002;168:1533–7.
[48] Beken S, Kasper P, van der Laan JW. Regulatory acceptance of alternative methods in the development and approval of pharmaceuticals. Adv Exp Med Biol 2016;856:33–64.