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A Comparison of Pyrogen Detection Tests in the Quality Control of Meningococcal Conjugate Vaccines: The Applicability of the Monocyte Activation Test

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Summary — The meningococcal C conjugate vaccine (MenCC) is an interesting model with which to test the efficacy of the Monocyte Activation Test (MAT) as an alternative method of pyrogen testing in the quality control of vaccines. The MenCC that has been produced by Bio-Manguinhos in Brazil is in the final development stage, and, as recommended in the guidelines for MenCC production, its pyrogen content must be determined by using the *Limulus* Amoebocyte Lysate (LAL) assay and the Rabbit Pyrogen Test (RPT). This represents an ideal opportunity to compare LAL and RPT data with data obtained by using a MAT system with cryopreserved whole blood and IL-6/IL-1β as marker readouts. In order to assess the compatibility of the MAT with MenCC, endotoxin and non-endotoxin pyrogen content was quantified by using MenCC samples spiked with lipopolysaccharide (LPS), lipoteichoic acid or zymosan standards. The presence of the aluminium-based adjuvant interfered with the MAT, increasing the readout of IL-1β in LPS-spiked MenCC batches. This infringed the product-specific validation criteria of the test, and led to IL-6 being chosen as the more suitable marker readout. No pyrogenic contaminants were identified in the MenCC batches tested, demonstrating consistency among the different systems (MAT, RPT and the LAL assay). In conclusion, the introduction of the MAT during MenCC development could contribute to the elimination of animal tests post-licensing, ensuring human protection based on an effective non-animal based method of quality control.

Key words: *alternative methods, health surveillance, meningococcal C conjugate vaccine, monocyte activation test, pyrogen.*

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Introduction

Meningococcal meningitis is a bacterial form of meningitis, which is a serious infection of the meninges that can cause severe brain damage and is fatal in 50% of cases if left untreated. Routine vaccination with meningococcal conjugate vaccines is the most effective public health strategy to control meningitis caused by most serogroups of *Neisseria meningitides*. The use of conjugate vaccines is necessary, because standard polysaccharide vaccines are not able to protect individuals below 2 years of age or successfully establish long-term protection (1–3).

In Brazil during 2016, there were 1105 confirmed cases of meningoccal disease and 243 reported deaths. Serogroup C was responsible for 303 and 73 of the confirmed cases and deaths, respectively (4). In 2002, a significant shift from serogroup B to serogroup C capsular type was observed. Serogroup C has since become the prevalent serogroup, accounting for 67% of the cases of meningoccal disease in Brazil. This confirmed the need for a meningococcal C conjugate vaccine (MenCC) in the Brazilian National Immunisation Programme (NIP; 5). After four years of infant and toddler immunisation in Brazil, serogroup C infection rates in the vaccine target population were reduced by 65, to 92% (5). However, since MenCC was not administered to older age groups such as adolescents, the asymptomatic meningococcal transmission was not affected. Thus, this current vaccination schedule is not capable of conferring herd immunity, which reinforces the need for a broader age-based coverage for

a reduction in microorganism transmission. Proof of the establishment of herd immunity will be possible only after large-scale vaccine administration is extended to older individuals, and an effective reduction in the cases of meningococcal meningitis is observed (6, 7). These observations, in addition to the epidemiological studies that have taken place in Brazil involving vaccinated groups, will provide a baseline to ascertain the impact of the upcoming catch-up dose of the MenCC in adolescents (12–13 years-old), which has been approved as part of the national 2017–2018 immunisation schedule (5).

The Immunobiological Technology Institute unit (Bio-Manguinhos) at Fiocruz, which produces immunobiological products and is the main producer of yellow fever vaccine worldwide, has developed a monovalent MenCC, by using the modified reductive amination method and tetanus toxoid (TT) as the carrier protein, according to World Health Organisation (WHO) requirements (8–11). All steps for the production, purification and quality control of the MenCC were standardised, and several batches of the conjugate were produced, at different scales, under reproducible conditions (9). The vaccine was submitted to Phase I and Phase II clinical trials in adults and in children aged one to nine years, respectively, and demonstrated satisfactory safety and immunogenicity (12, 13). MenCC production was scaled up to obtain industrial batches, with satisfactory results (11). Three batches of vaccine were produced under Good Manufacturing Practice (GMP) conditions in an industrial setting, and submitted to the third Phase I field study, in which the batches were compared to a commercially available vaccine (NeisVac-C®). The data from this Phase I field study revealed that the MenCC caused no significant adverse events, and both vaccines tested had similar immunogenicity. A Phase II/Phase III clinical trial of MenCC, involving volunteers aged 11 to 19 years, will take place soon.

The production process and quality control of conjugate vaccines are more complex than those for their unconjugated capsular polysaccharide equivalents (8). The standard GMP recommendations for pharmaceuticals (14) and for biological products (8) should be applied to the production of MenCCs. Among the tests required to ensure the quality of the production systems and control processes for biotechnological products, the pyrogen test is mandatory to guarantee the absence of contamination with microorganisms with potential pyrogenic activity (15).

From a regulatory point of view, the *Limulus* Amoebocyte Lysate (LAL) assay was accepted as an official test for endotoxin detection, because Gram-negative bacteria are recognised as the greatest source of pyrogenic contamination (16). As recommended by the US Pharmacopoeia, the LAL assay should substitute, whenever possible, for the *in vivo* Rabbit Pyrogen Test (RPT): *The pyrogen test on rabbits should be performed only if the product is incompatible with the LAL test* (17). However, for some vaccines — such as tick-borne encephalitis vaccines, polysaccharide vaccines (18) and outer membrane vesicle (OMV) vaccines against meningococcal disease (19) — there are limitations to the use of the LAL assay that justify the use of the animal test to detect non-endotoxin pyrogen (NEP) molecules.

The current study was based on the premise that the pyrogen testing required for the quality control of MenCC (i.e. the RPT and the LAL assay) could serve as an opportunity to compare RPT and LAL data with the results obtained with another non-animal method, the MAT. The European Pharmacopoeia recommends the use of the LAL assay, to ensure an acceptable level of pyrogenicity of the final product (20), and the WHO recommends that final batches of the vaccine should be tested for pyrogenic activity with the RPT or the LAL assay (8). In addition, some agencies, such as the US Center for Veterinary Medicine, require that the first three batches manufactured be tested by using both the LAL assay and the RPT, to determine whether other types of pyrogenic substances are present.

The replacement of animal experiments by validated non-animal alternatives is statutory in Europe, a trend guided by the Three Rs principles (22) and one which is emerging wordwide. The revision of the chapter on the Monocyte Activation Test (MAT; 23) by the European Pharmacopoeia Commission (24), thereby replacing the RPT by the MAT (on a case-by-case basis, subject to product-specific validation), reinforces the move to reduce the use of laboratory animals in pyrogenic tests. The MAT is a validated *in vitro* alternative to the RPT that has been recognised by the European Pharmacopoeia since 2010. It combines the benefits of an *in vitro* method (non-animal and high-throughput) with the advantage of being able to recognise a broad spectrum of pathogen-associated molecular patterns (PAMPs) from microorganisms able to trigger sepsis (endotoxin and NEPs). Like the RPT, the MAT provides a biological response to pyrogenic contamination. Thus, it is able to quantify those substances that activate human monocytes to release endogenous mediators that have a role in the human fever response (25). However, it should be noted that the LAL assay detects endotoxin pyrogens (e.g. lipopolysaccharide; LPS) very effectively, but is ineffective in determining NEPs (e.g. lipoteichoic acid; LTA). Thus, this selective specificity of the LAL assay results in a potential underestimation of the presence of NEPs, which is of great clinical significance (26). Therefore, the MAT can be viewed as a new-generation pyrogenic test that enhances the quality control of vaccines, by addressing this serious under-reporting of pyrogen presence.

The current study compared data from the pyrogenic detection methods described in the European

Pharmacopoeia (the RPT, the LAL assay and the MAT) with those recommended by the European Pharmacopoeia and WHO for the MenCC end-product, i.e. the RPT and the LAL assay (4, 8, 20). This study provides scientific support for the inclusion of non-animal alternative methods in the field of vaccine testing, as recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; 27, 28). It reinforces the power of the MAT, compared with pyrogen detection tests recommended in the MenCC guidelines, to evaluate pyrogenicity induced by endotoxin and NEPs in biological products. In addition, the use of the MAT contributes to make routine quality control more-specific, accurate, efficient and cruelty-free.

Materials and Methods

Samples

Three batches of MenCC (5 doses/vial), produced on an industrial scale and under clinical trial at the Immunobiological Technology Institute (Bio-Manguinhos), were used in this study (8–11). The MenCC final product consists of serogroup C meningococcal polysaccharide conjugated to tetanus toxoid (10µg/dose) as the carrier protein, aluminium hydroxide adjuvant (1mg/dose) with phosphate buffered saline (PBS) as the diluent. The human dose (HD) consists of 0.5ml of MenCC reconstituted according to the manufacturer's instructions. The same batches of MenCC were tested in the MAT, the LAL assay and the RPT. For the purposes of the current study, the MenCC samples were prepared as 10µg MenCC plus the following diluents:

- for the MAT: 2.5ml 0.9% w/v NaCl only, or 2.5ml aluminium hydroxide adjuvant only;
- for the LAL assay: 2.5ml aluminium hydroxide adjuvant only;
- for the RPT: 2.5ml pyrogen-free water only.

For clarity, the following acronyms are used throughout: MenCC when referring to the meningococcal C conjugate vaccine; MenCC–NaCl or MenCC–adjuvant when referring to the meningococcal C conjugate vaccine reconstituted in NaCl or adjuvant, respectively.

The Monocyte Activation Test

Pyrogenic stimuli

LPS from *Escherichia coli* O55:B5 (Cat. No. L2880; Sigma-Aldrich, Steinheim, Germany) was used as the endotoxin standard (endotoxin control), which was certifiably calibrated to the international reference endotoxin standard. The lyophilised contents of the endotoxin vial were reconstituted in Sterile Water For Injection, according to the manufacturer's instructions. The stock solution and subsequent dilutions were vortexed vigorously for at least 30 minutes immediately prior to use. The data were expressed in Endotoxin Units (EU), which are identical to International Units (IU), as defined by the WHO as a measure of pyrogen contaminant concentration in the industry. The standard endotoxin dose–response curve was constructed by using doses of 0.25, 0.5, 1.0 and 2.5EU/ml.

Lipoteichoic acid (LTA) from *Staphylococcus aureus* (Cat. No. L2515; Sigma-Aldrich, St Louis, MO, USA) and zymosan A from *Saccharomyces cerevisiae* (Cat. No. Z4250; Sigma-Aldrich, USA) were used as non-endotoxin pyrogen (NEP) stimuli. These NEPs cause different responses in the LAL assay: while LTA is not detected by this assay, zymosan A is able to trigger the LAL clotting cascade (similar to LPS stimuli), thus generating false negative and false positive results, respectively. The LTA dose–response curve was constructed from doses of 10, 50, 75 and 200µg/ml (29). The zymosan was used at a concentration of 5µg/ml (30). Endotoxin Equivalent Units (EEU) were used as the measure of NEP contaminant concentration, according to the endotoxin standard curve. To exclude the possibility of interference by endotoxin contamination in the LTA and zymosan commercial preparations, the solutions were pre-treated with 40mg/ml polymixin B (PMB; Sigma-Aldrich, USA) prior to use (31). A 0.9% NaCl pyrogen-free solution was used as a negative control (Halex Istar, Goiânia, Goiás, Brazil).

Determination of the maximum valid dilution

The maximum valid dilution (MVD), which is the maximum dilution factor at which it is still possible to detect the pyrogen, was estimated by dividing the endotoxin limit concentration (ELC) by the limit of detection (LOD). According to the MenCC monograph from the European Pharmacopoeia, the threshold for bacterial endotoxins (K) is 25IU, which corresponds to 25EU per HD (20). The maximum recommended bolus dose of product per kilogramme of body mass (M) is 0.5ml per HD. Therefore, the ELC for this product was calculated to be:

$$\text{ELC} = \frac{\text{K}}{\text{M}} = \frac{25\text{EU per HD}}{0.5\text{ml per HD}} = \frac{25\text{EU}}{0.5\text{ml}} = 50\text{EU/ml}$$

Based on the LOD calculation described in the European Pharmacopoeia, the LOD values ob-

tained were 0.045EU/ml for IL-1β and 0.042EU/ml for IL-6, so:

$$\text{MVD IL-1}\beta = \frac{K}{M} = \frac{\text{50EU per HD}}{\text{0.045ml per HD}} = 1111$$

$$\text{MVD IL-6} = \frac{K}{M} = \frac{\text{50EU per HD}}{\text{0.042ml per HD}} = 1190$$

Thus, the MVD established for the MenCC in the MAT was 1/1111 for IL-1β and 1/1190 for IL-6.

Interference test

Three batches of MenCC were used to determine the minimum valid dilution (MinVD) for the MAT, as recommended by the European Pharmacopoeia (23). As the aluminium hydroxide in the MenCC adjuvant is a known interfering factor, the European Pharmacopoeia recommends that, if necessary, it can be precluded from the vaccine and the test performed on samples from at least three batches (23). Thus, we performed the MAT with MenCC reconstituted in NaCl alone, and in adjuvant alone. The reconstitution in NaCl alone (without adjuvant) for the MAT followed the same rationale as that for the RPT (reconstitution in pyrogen-free water only). In parallel, MenCC was reconstituted in adjuvant only, as is the case for the LAL assay during routine quality control processes for MenCC production. The adjuvant alone was also analysed to verify its level of interference. The batches were mixed, serial dilutions were performed, and all dilutions were spiked with 0.5EU/ml of LPS. Endotoxin recovery (%) was then analysed for the spiked (S) and parallel unspiked (US) samples. Spike recovery was calculated by using the mean values of the endotoxin equivalent concentrations (EEU/ml) of the spiked (S) and the unspiked (US) MenCC according to the equation: $\% \text{ Recovery}_{MAT} = ([S - US]/0.5) \times 100$. Dilutions with endotoxin recovery within the 50–200% range were considered interference-free. The data are expressed as the mean ± SD of four replicates.

Blood samples

Heparinised blood samples were collected from ten healthy donors after informed consent was obtained, according to the requirements of the local ethics committee. Differential blood cell counts were routinely performed with a cell counter (BioClin®, Belo Horizonte, MG, Brazil), to exclude donors with acute infections. Fresh whole blood (WB) was used within four hours of collection. Cryopreserved whole blood (cryoWB) was prepared with endotoxin-free Sorënsen's phosphate buffer (Acila GmbH, Mörfelden-Walldorf, Hesse, Germany), by mixing with 20% v/v endotoxin-free dimethyl sulphoxide (DMSO; Cryosure-DMSO, Wak Chemie Medical GmbH, Steinbach, Germany). This solution was mixed with WB (1:2 v/v) and frozen at –80°C, as described by Schindler *et al.* (32). Three pooled lots of cryoWB and three pooled lots of WB were used in the experiments; each pooled lot was from four different donors.

Performance of the MAT

The MAT was performed as described in the European Pharmacopoeia (23). For the WB assay, 50μl of samples/controls were mixed with 500μl of saline and 50μl of pooled blood. For the cryoWB assay, 50μl of samples/controls were mixed with 450μl of saline and 100μl of thawed blood. The samples were spiked with 5ml LPS (0.5EU/ml in the final solution), which corresponds to the concentration in the middle of the endotoxin standard curve (23). Cell contact with the test samples was performed under sterile conditions in polypropylene vials (Eppendorf, Hamburg, Germany). The vials containing the blood with samples/controls were incubated in a humidified incubator (37°C, 5% CO_2) for 20 hours. After this period, the vials were stored at –20°C until needed for cytokine measurement. The endotoxin limit used as the pass/fail criterion for the MAT was 0.5EU/ml, which is based on the threshold pyrogenic dose in the RPT and on historical data as described by ICCVAM (28).

Cytokine measurement

IL-1β and IL-6 were measured with a commercially-available Quantikine® ELISA kit (R&D Systems, Wiesbaden, Germany), with a sensitivity range of 3.90–250pg/ml for IL-1β and 3.12–300pg/ml for IL-6, according to the manufacturer's instructions. The data are presented as the mean ± standard error (SEM) of four biological replicates.

To evaluate the donor blood in the assay with IL-1β or IL-6 detection, we used pooled blood samples from four different donors. To ensure the quality of the monocytic cells, the blood was collected and analysed, according to the MAT monograph (23). The pooled blood was challenged with *E. coli* LPS and the response data compared with a standard curve, with NaCl used as the negative response control.

The LAL assay

The endotoxin levels in batches of MenCC were determined by a kinetic turbidimetric LAL assay

(Endosafe; Charles River Laboratories, Lexington, MA, USA), by following the manufacturer's instructions. Prior to testing, the vaccine was reconstituted in the aluminium hydroxide adjuvant. The appropriate dilution for the LAL assay was determined by measuring the recovery of a known amount of endotoxin spiked in the MinVD of the product (1:100), according to the previous validation by the manufacturer. The change in turbidity was measured at 340nm with an ELx808IU microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The LPS content was analysed by using EndoScan-V™ endotoxin measuring software (Charles River Endosafe, Charleston, SC, USA). After correcting for the dilution, a 0.25EU/HD (or 0.25EU/ml) cut-off concentration was used to determine whether the MenCC sample was pyrogenic (P) or non-pyrogenic (NP).

The RPT

Animals were not used exclusively for the purpose of the current study. The RPT was performed during the MenCC development process, in order to determine pyrogenic contamination. The lyophilised contents of a MenCC vial were reconstituted in Sterile Water For Injection, to give a concentration of 10μg/0.5ml, which corresponds to the HD. Briefly, three healthy New Zealand white rabbits were inoculated with an intravenous dose of the reconstituted MenCC (1ml/kg of body weight). The animals used for these routine tests at Bio-Manguinhos were provided by the Laboratory Animals Breeding Center (Cecal) from the Oswaldo Cruz Foundation (Fiocruz). The tests were carried out in accordance with institutional protocols approved by the Fiocruz Ethics Committee on Animal Use (CEUA; protocol number LW65/14), and the recommendations outlined in the Brazilian Pharmacopoeia (33), which are similar to those in the United States Pharmacopoeia (34).

Statistical analysis

To ensure the precision and validity of the tests, preparatory assays were conducted to ensure that the criteria for the standard curves were satisfied, as described in the European Pharmacopoeia (23). The dose–response on a log scale was considered statistically significant ($p > 0.01$). The validity of the linear regression of the endotoxin standard curve was determined by calculating the coefficient of determination (r^2). Pearson's coefficient (r) was used to measure the strength of the correlation between two variables. The significance of the cytokine responses at the various LPS concentrations was determined by using one-way ANOVA; $p < 0.05$ was considered significant. For comparison between the LPS dose–response curves in the test (i.e. MenCC) and the control (i.e. NaCl), a non-parallelism analysis was performed by using the CombiStats™ statistical analysis software (version 5.0; EDQM/Council of Europe, Strasbourg, France); the assay was considered to be valid when $p < 0.05$. Comparisons among multiple groups were analysed by using one-way ANOVA, and the differences among groups were analysed with Student's t-test by using GraphPad Prism v4 software (GraphPad Software, La Jolla, CA, USA); values of $p < 0.05$ were considered statistically significant. The data were expressed as the mean ± SEM. Means and coefficients of variation (% CV) were calculated for each sample.

Results

The use of IL-1β and IL-6 as marker readouts for LPS detection in the MAT

The European Pharmacopoeia uses different variants of the MAT system for pyrogen detection (35). In order to confirm the applicability of the MAT for the testing of MenCC, the WB/IL-6, cryoWB/IL-1β and cryoWB/IL-6 MAT systems were evaluated, as detailed in the European Pharmacopoeia (23). First, we analysed the basal cytokine levels in these systems, and the relationship between cytokine inductions after stimulation by different LPS doses. The basal levels did not interfere with the LPS dose–response curve, as confirmed by the statistical analysis. These interpretations were based on the statistical parameters recommended by the European Pharmacopoeia (23), according to which the acceptance criteria for the regression of the LPS dose–response curve is $p < 0.01$. The LPS curves were satisfactory in terms of the regression for all of the MAT systems analysed, with p values lower than 0.001 (Figure 1). In addition, the coefficient of determination (r^2) was used to evaluate the linearity of the LPS curves ($r^2 > 0.95$ for all the MAT systems analysed; Figure 1).

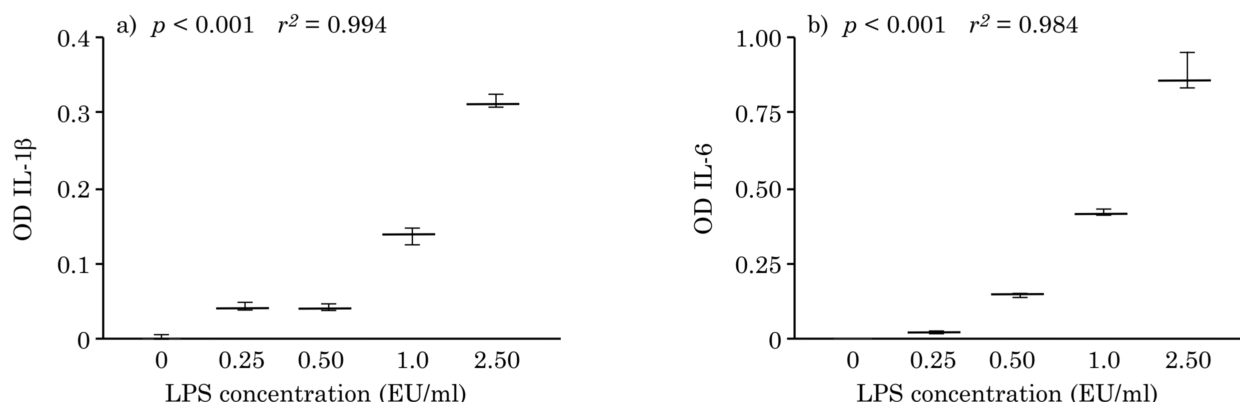
Applicability of the MAT WB and cryoWB systems in MenCC pyrogen testing

To determine the applicability of the MAT systems in MenCC pyrogen testing, a number of parameters were defined, as detailed in the European Pharmacopoeia (23, 24).

The interference test

For all MenCC–NaCl dilutions, LPS recovery was within the acceptable range (50–200%) when IL-1β

Figure 1: Standard endotoxin dose–response curve



The OD values corresponding to the cytokine readouts in relation to LPS concentration are shown for a) IL-1β and b) IL-6. The data are the mean of four replicates ± SEM of pooled cryopreserved human blood.

was used as the readout, and the MinVD was determined to be 1:4 (Figure 2a). Similarly, a 1:4 MenCC dilution was chosen for the IL-6 readout (Figure 2b, c and d). For MenCC–NaCl, LPS spike recovery at a 1:4 dilution was around 100% (85% for IL-1β and 103% for IL-6), and the acceptance parameters for LPS recovery were observed for all dilutions (Figure 2b).

To ensure the validity of the MAT for MenCC testing, if interfering substances (such as adjuvant) are detected in the product, then the substance should be precluded and the test should be performed on samples from at least three batches (22, 23). Thus, to determine whether MenCC in the presence of adjuvant (MenCC–adjuvant) could be successfully analysed with the MAT, the vaccine was reconstituted according to the manufacturer's instructions. It was observed that LPS recovery at a 1:4 dilution of MenCC–adjuvant was 120% with IL-6 as the readout (Figure 2c). Similarly, the vaccine adjuvant alone at a 1:4 dilution resulted in LPS recovery values of around 100% (99%; Figure 2d). A 1:4 dilution of MenCC was therefore chosen for use in the MAT, thus permitting a comparison of the levels of IL-1β and IL-6 in the supernatant from the same contact (i.e. blood plus vaccine). As only low dilutions of MenCC were within the acceptable range for the MAT, we limited our testing to dilutions below 1:10.

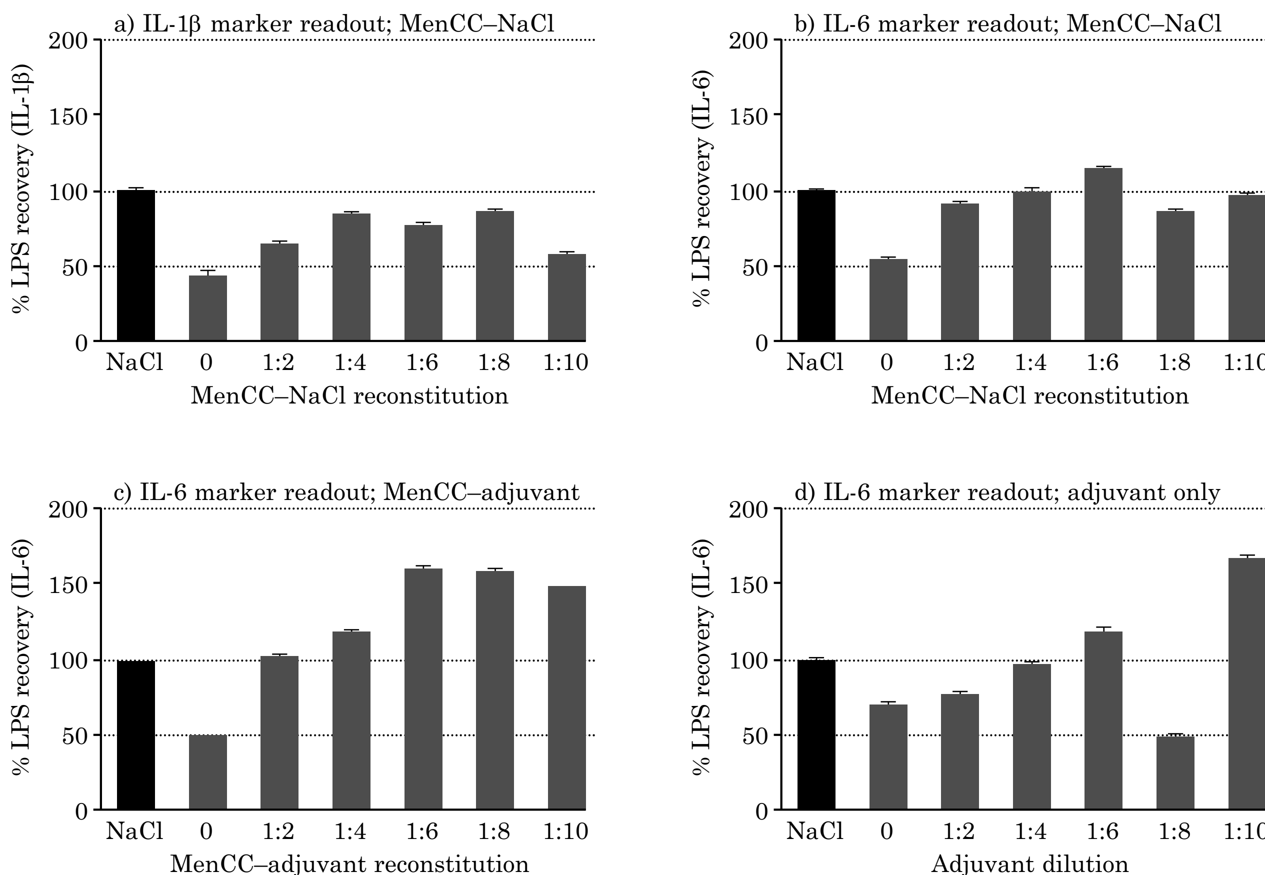
Comparison of IL-1β and IL-6 levels as marker readouts for the detection of LPS in MenCC

To determine the correlation between IL-1β and IL-6 as marker readouts in the MAT cryoWB system, the levels of both cytokines were measured in supernatants after exposure of cryoWB to LPS-spiked NaCl (control samples) and MenCC–NaCl (test samples). As shown in Figure 3, good correlations were observed between IL-1β and IL-6 induction by LPS in the NaCl control samples (r = 0.9918 and $p < 0.0001$) and in the MenCC–NaCl test samples (r = 0.9702 and p = 0.0028). A strong induction profile for both IL-1β and IL-6 in response to endotoxin was obtained both in NaCl and in MenCC–NaCl, confirming the suitability of these cytokines for use with the MAT cryoWB system for MenCC testing.

Endotoxin pyrogen detection in MenCC with the MAT cryoWB system

To investigate the ability of the MAT cryoWB system to quantify endotoxin pyrogens in MenCC, cryoWB was exposed to different concentrations of an LPS standard, and IL-1β and IL-6 levels were assessed as the readout. Potential interference of the vaccine product itself was assessed by comparing the OD values obtained (representing the IL-1β or IL-6 cytokine levels) in response to a range of LPS doses (0, 0.25, 0.5, 1.0 and 2.5EU/ml) diluted in the test (MenCC–NaCl) and control (NaCl) substances. Significant differences between the OD values are indicated by $p < 0.05$ (Figures 4a and 4b). The non-parallelism parameter, seen in the inset of Figures 4a and 4b, showed that the p values of the non-parallelism analysis were not significant ($p > 0.05$). Supporting the parallelism results, the correlation analysis showed that similar response profiles were obtained for IL-1β (r = 0.9793 and p = 0.0197) and IL-6 (r = 0.9608 and p = 0.0076) as a result of contact with LPS diluted in

Figure 2: The interference test



Endotoxin recovery was determined by using the MAT cryoWB system, to test for potential interfering substances in the MenCC, and to determine the minimum valid dilution of the test vaccine. For each dilution, the percentage LPS recovery was calculated as: $\% \, Recovery_{MAT} = ([S - US]/0.5) \times 100$*, where S = endotoxin equivalent concentration (EEU/ml) of 0.5EU/ml LPS-spiked test sample (NaCl, MenCC–NaCl, MenCC–adjuvant, or adjuvant), and US = endotoxin equivalent concentration (EEU/ml) of unspiked test sample.*

a) Shows LPS recovery from MenCC–NaCl, with the IL-1β readout; b) shows LPS recovery from MenCC–NaCl, with the IL-6 readout; c) shows LPS recovery from MenCC–adjuvant, with the IL-6 readout; and d) shows LPS recovery from adjuvant alone, with the IL-6 readout.

The lower and upper dotted lines represent the range within which a product is considered interference-free (50–200%). The middle dotted line represents the cut-off (100%) and corresponds to 0.5EU/ml of LPS in NaCl (control). Black bars = 0.5EU/ml of LPS in NaCl; grey bars = 0.5EU/ml of LPS in different MenCC dilutions. Error bars represent the standard deviation (SD) of the mean of four replicates.

NaCl *versus* LPS diluted in MenCC (Figures 4c and d).

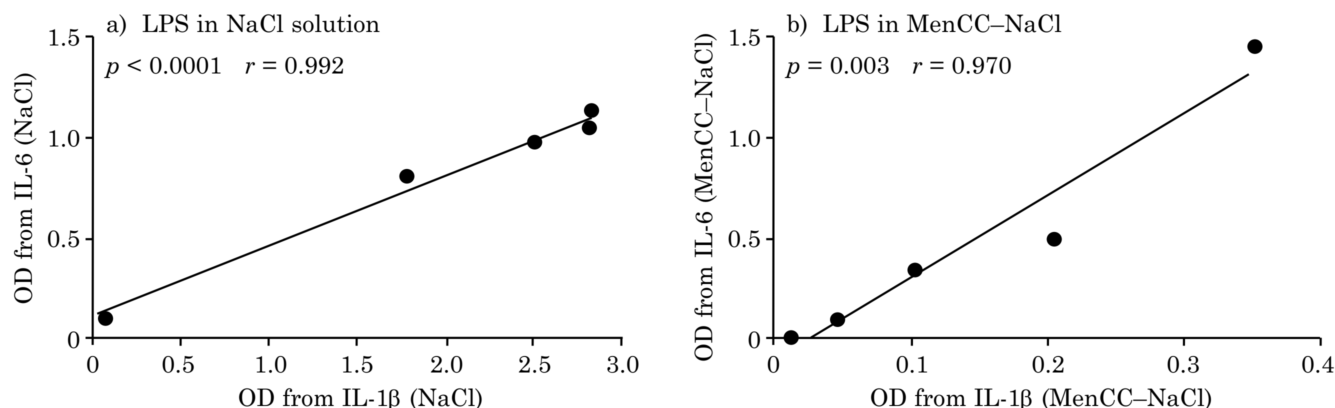
Intra-assay and inter-assay variability of LPS-induced IL-1β and IL-6 marker readouts

To evaluate the reproducibility of the results obtained in the different MAT systems — i.e. the two readouts (IL-1β and IL-6) and blood samples (cryoWB and WB) — intra-variability and inter-variability parameters were analysed for LPS-spiked NaCl and LPS-spiked MenCC–NaCl (Table 1).

The intra-assay CV values of the cytokine response induced by LPS in NaCl and LPS in MenCC–NaCl were calculated for the different MAT systems. LPS in NaCl was used as the variance control for the assay with the vaccine. For the NaCl and MenCC samples spiked with 0.5EU/ml LPS, the CV values were lower than 10%. The results obtained were consistent and acceptable, based on data from the literature (30).

Interestingly, the MAT cryoWB/IL-6 system showed lower CV values for LPS-spiked NaCl (2.98%) and LPS-spiked MenCC–NaCl samples (3.82–3.95%), as compared to the same samples with

Figure 3: Correlation between the induction of IL-6 and IL-1β in the MAT cryoWB system, in response to LPS



The relationship between the induction profiles of IL-6 and IL-1β in response to serial dilutions of LPS (0.125, 0.25, 0.5, 1 and 2.5EU/ml) in a) NaCl solution (control) and b) MenCC–NaCl was determined in the MAT cryoWB system. Optical density (OD) readouts were used to express the correlation between the two cytokine profiles. The solid line represents the line of best fit based on a simple regression model. r = Pearson's coefficient and p < 0.05 significance.

IL-1β as the readout (LPS in NaCl, 4.16%; and LPS in MenCC–NaCl, 5.05–9.29%), as shown in Table 1. These CV values are also within the range described in the literature (30).

These data were corroborated by analyses of inter-assay variability (i.e. when the assay was performed on a number of different days): the CV values for the MAT cryoWB/IL-6 system induced by LPS were lower (in NaCl, 4.35%; and in MenCC–NaCl, 4.66–5.44%), as compared to the MAT cryoWB/IL-1β system (in NaCl, 5.52%; and in MenCC–NaCl, 6.76–7.81%). Like the MAT cryoWB/IL-6 system, the MAT WB/IL-6 system showed CV values lower than those observed with the MAT cryoWB/IL-1β system (intra-assay: in NaCl, 2.80%; in MenCC–NaCl, 3.56–5.71%; and inter-assay: in NaCl, 2.13%; in MenCC–NaCl, 3.81–4.38%). Thus, the data revealed a similar trend, with higher intra-assay and inter-assay variability when IL-1β was used as the readout, when compared to IL-6 (Table 1).

Non-endotoxin pyrogen detection in MenCC with the MAT cryoWB system

The non-endotoxin pyrogen, lipoteichoic acid (LTA) from *S. aureus*, was used to investigate the capacity

Table 1: An overview of intra-assay and inter-assay variability of cytokine induction in blood in response to pyrogen contact

Sample	Intra-assay variability (% CV)			Inter-assay variability (% CV)		
	cryoWB system		WB system	cryoWB system		WB system
(0.5EU/ml LPS)	IL-1β	IL-6	IL-6	IL-1β	IL-6	IL-6
In NaCl	4.16	2.98	2.80	5.52	4.35	2.13
In MenCC–NaCl:						
Batch A	9.29	3.82	5.71	6.76	5.44	3.81
Batch B	5.05	3.86	3.56	7.21	4.84	4.38
Batch C	6.20	3.95	4.69	7.81	4.66	4.42

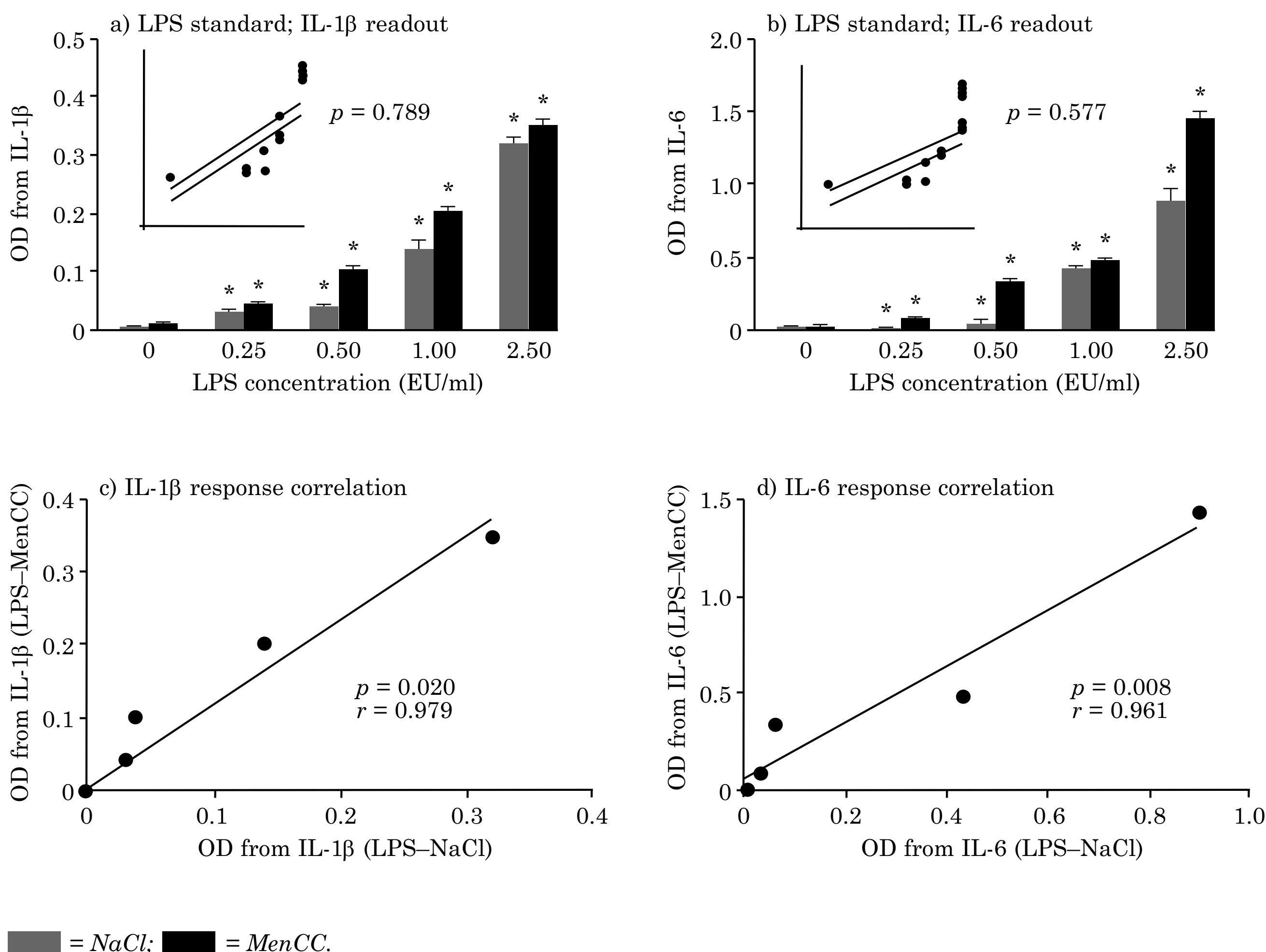
cryoWB = cryopreserved whole blood; WB = fresh whole blood; LPS = lipopolysaccharide; EU = endotoxin unit. The data were obtained in quadruplicate.

of the MAT to detect NEPs in MenCC. LTA is a classic example of an NEP, with great importance in the pathogenesis of sepsis (36) and as a potential source of pyrogenic contamination in manufacturing environments. Since past studies have identified LPS contamination in commercial LTA preparations (37), the LTA solution was pre-treated with PMB (a relatively specific inhibitor of LPS) prior to use. The efficacy of the PMB in inhibiting any LPS-induced reaction was determined in the LAL assay and in the MAT cryoWB system. For this purpose, a pyrogenic concentration of LTA solution (50μg/ml) was pre-treated with PMB and used to spike NaCl and MenCC–NaCl; an untreated LTA solution was also included for control purposes. The results obtained with the LAL assay indicate the presence of LPS in the commercial LTA solution (0.17EU/ml). The PMB was able to significantly reduce LPS contamination (0.036EU/ml, $p < 0.001$), reducing the level of LPS contamination by 77% (Figure 5a).

When the same experiment was performed with the MAT cryoWB system, MenCC–NaCl spiked with LTA without PMB pre-treatment had more than twice the EEU level of the LPS standard and of LTA after PMB pre-treatment (Figure 5b). These data showed that, in the MAT, LPS contamination induced a synergistic effect in the response to LTA, but after PMB pre-treatment, this synergistic effect was negligible in LTA-spiked MenCC–NaCl.

To determine the ability of the MAT cryoWB system to effectively quantify LTA in MenCC, we tested the response with a set of LTA standards

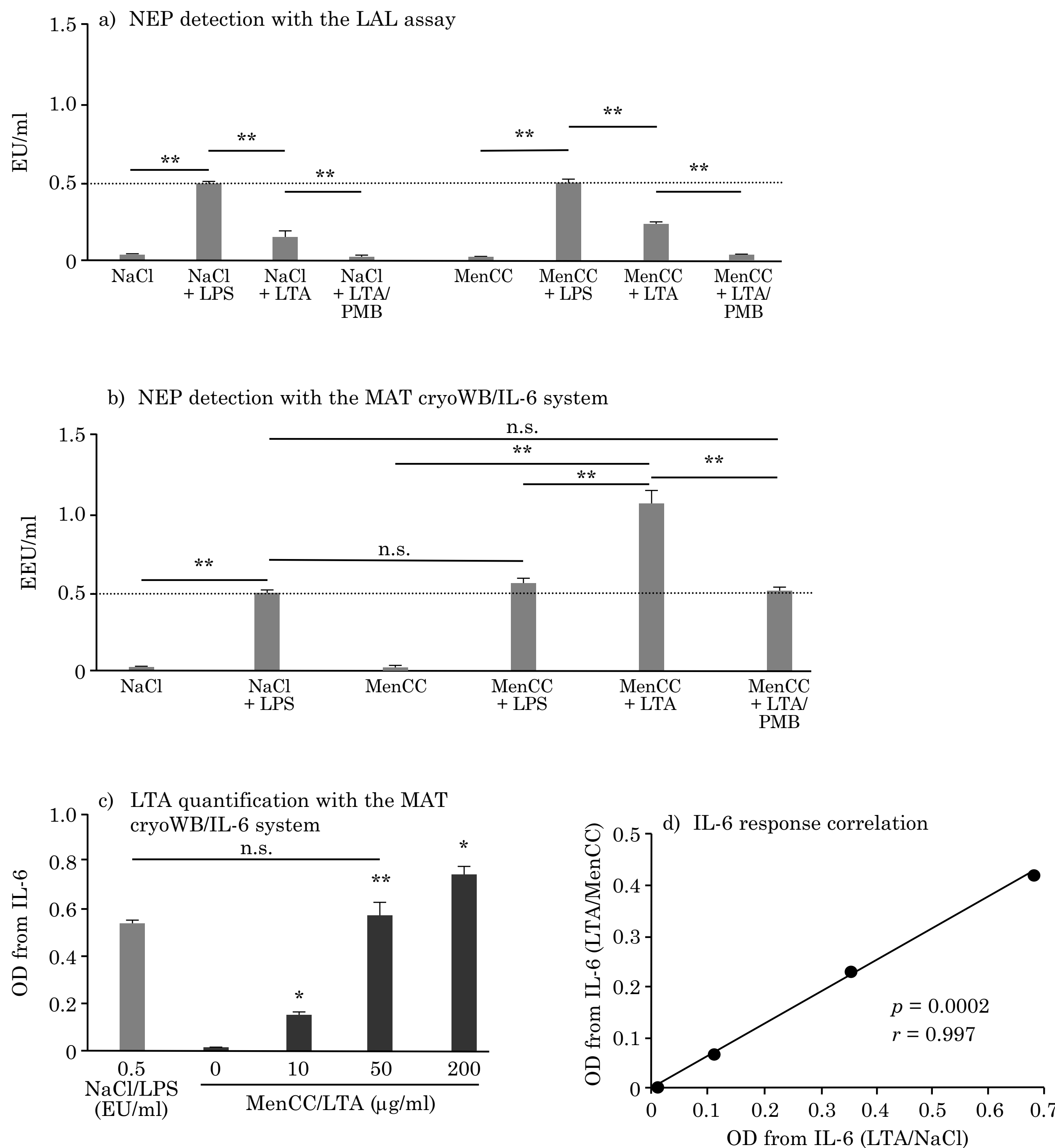
Figure 4: Ability of the MAT cryoWB system to detect pyrogens in MenCC



= NaCl; = MenCC.

CryoWB was spiked with 0.25–2.5EU/ml LPS standard (endotoxin pyrogen) and a) IL-1β and b) IL-6 were used as the readouts. LPS was diluted in NaCl (light grey bars) or in MenCC reconstituted in NaCl (MenCC–NaCl; dark grey bars). The insets in graphs a) and b) correspond to the non-parallelism analysis of the standard curves in NaCl solution and MenCC in NaCl. Lines are plotted on a fully specified logit-log scale. The error bars represent the standard error of the mean of four replicates. ANOVA was used to compare a sample with the previous dose;
p < 0.001. The correlation analysis between the cytokine induction profile of LPS-spiked NaCl control solution and LPS-spiked MenCC–NaCl are shown for c) IL-1β and d) IL-6.

Figure 5: Non-endotoxin pyrogen detection with the LAL assay and the MAT cryoWB/IL-6 system



The non-endotoxin pyrogen stimulant, LTA, was pre-treated with PMB to avoid the confounding effects of any LPS (endotoxin pyrogen) contamination on the LTA results. The NaCl control solution and samples of MenCC reconstituted in NaCl (MenCC–NaCl) were spiked with LPS (0.5EU/ml) and LTA (50μg/ml, with and without PMB treatment). The efficacy of LPS response inhibition by PMB was determined: a) in the LAL assay, which effectively detects endotoxin pyrogens such as LPS, but does not detect non-endotoxin pyrogens like LTA; and b) in the MAT cryoWB/IL-6 system. The dashed line represents the cut-off pyrogenic value, and corresponds to 0.5EU/ml LPS in NaCl.
c) The MAT cryoWB/IL-6 system was used to quantify the amount of LTA (pre-treated with PMB) spiked into MenCC–NaCl samples; the data were analysed by using one-way ANOVA. Correlation between the IL-6 induction profile of LTA-spiked NaCl control solution and LTA-spiked MenCC–NaCl is shown in d). The values are representative of three replicates. The Student's t*-test was used to compare the control samples (NaCl or MenCC–NaCl) with those spiked with LPS, LTA and LTA + PMB;* *p < 0.05, **p < 0.01, ***p < 0.001; n.s. = not significant.

(pre-treated with PMB), as shown in Figure 5c. The ODs obtained with the LTA standards were found to be significantly different ($p < 0.05$). Potential interference by MenCC in the quantitative response was determined by a correlation analysis performed with LTA standards diluted in NaCl and in MenCC–NaCl ($r = 0.9973$ and $p = 0.0002$). As Figure 5d shows, a high correlation was found between the LTA diluted in NaCl (control) and the LTA diluted in MenCC–NaCl (test).

Pyrogen detection in industrial batches of MenCC spiked with contaminants

As suggested by the monograph on the MAT for the validation of the method, endotoxin and NEPs contaminants should be spiked into the product to be tested. With this approach, LPS, as the endotoxin standard, and LTA and zymosan, as NEPs standards, were used for evaluating the pyrogenic potential of different biological sources of MenCC. Zymosan A (from *S. cerevisiae*) was included at this stage because the method validation for NEPs contaminants described in the European Pharmacopoeia (24) recommends at least two NEPs that are ligands for Toll-like receptors.

Three MenCC batches reconstituted in NaCl were spiked with an appropriate concentration of LPS, LTA and zymosan. The NEPs were pre-treated with the LPS inhibitor PMB, prior to performing the assay. The use of PMB suggested that any pyrogenic potency detected in samples spiked with LTA (Figure 5b) and zymosan (Table 2) was not caused by LPS contamination. Figure 6 shows that the MAT was able to detect the pyrogenic contaminants spiked into the test samples, with recovery rates that fell within the acceptable criteria of 50–200%. This result indicates that none of the MenCC batches were contaminated with pyrogenic contaminants.

Pyrogen detection with the MAT quantitative method

After successful product-specific validation with MenCC–NaCl, three MenCC batches were tested. For testing the vaccine, method A was chosen, as described in the European Pharmacopoeia (23). This method employs an LPS standard curve with a range of 0.25 to 2.5EEU/ml. A positive control that consisted of samples spiked with 0.5EU/ml of reference standard endotoxin was included, to ensure the suitability of the IL-1β and IL-6 readouts adopted for use in the MAT cryoWB system (the IL-6 and IL-1β readouts were validated according to the % recovery of spiked endotoxin). As the MAT WB/IL-6 system has been previously validated by ICCVAM, we analysed three batches of MenCC with this system, in order to compare the data with those obtained with the cryoWB/IL-6 system. The cytokine responses obtained were similar for these two systems (Table 3), corroborating our proposal that cryoWB/IL-6 could potentially be used as an alternative form of the MAT system for pyrogen detection.

As part of the experimental set-up, MenCC was reconstituted in adjuvant and in NaCl. However, the samples of MenCC–adjuvant revealed values that were higher than those for MenCC–NaCl (Table 3). In fact, MenCC batches reconstituted in adjuvant had the same profile as the adjuvant control. Interestingly, when IL-1β was used as readout, all batches of MenCC–adjuvant failed the criteria for test acceptance (i.e. % recovery). These observations support the view that IL-6 is the best readout for MenCC when reconstituted in adjuvant (Table 3).

Comparative evaluation of pyrogen tests for the MenCC

The same batches of MenCC that were evaluated with the MAT were analysed with other endotoxin

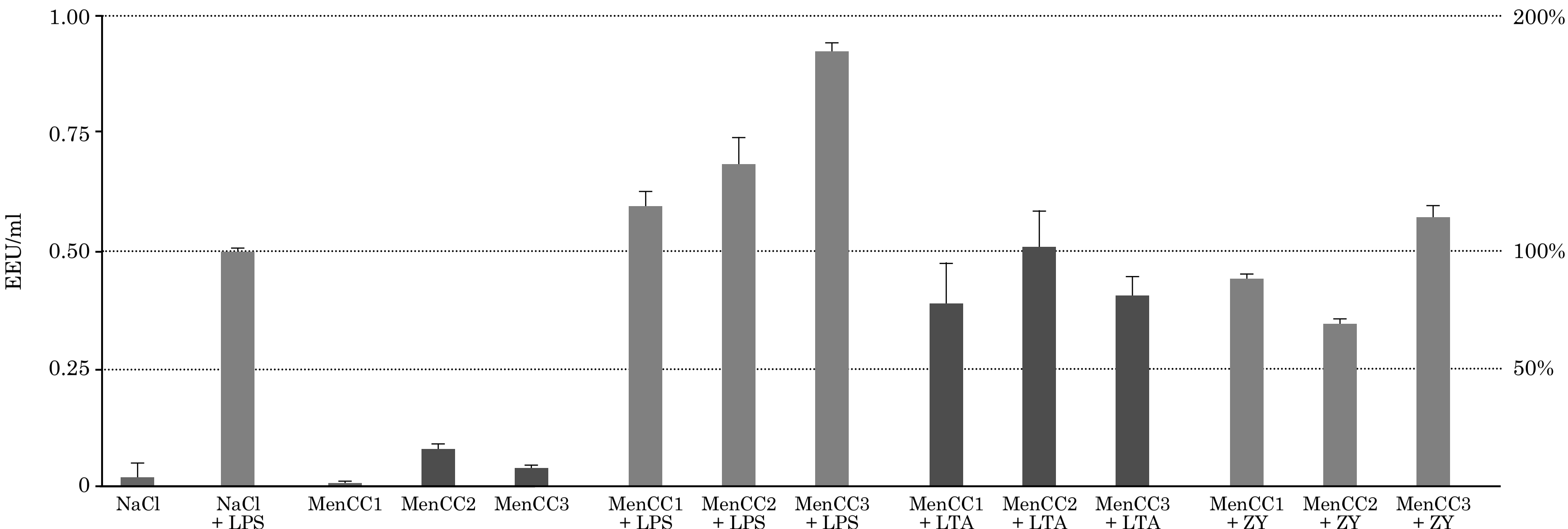
Table 2: The determination of LPS contamination in commercial zymosan preparations and the efficiency of PMB treatment

Pyrogen	Spike concentration (theoretical)	Pyrogen test: LAL assay (EU/ml)	Pyrogen test: MAT cryoWB/IL-6 (EEU/ml)
LPS	**0.5EU/ml**	**0.54 ± 0.04**	**0.54 ± 0.015**
Zymosan:			
without PMB pre-treatment	5μg/ml	> 50,000[a]	0.57 ± 0.017
with PMB pre-treatment	5μg/ml	> 50,000[a]	0.65 ± 0.060

LPS = lipopolysaccharide; PMB = polymixin B; EU = endotoxin unit; EEU = endotoxin equivalent unit.

[a]*Upper limit of 50,000EU/ml exceeded, the samples were not quantified.*

Figure 6: Pyrogen detection in batches of MenCC with the MAT cryoWB/IL-6 system



The applicability of the MAT cryoWB/IL-6 system for the detection of pyrogens in three MenCC batches was assessed by spiking MenCC batches reconstituted in NaCl (MenCC–NaCl) with endotoxin (LPS, 0.5EU/ml), and two non-endogen pyrogens (LTA, 50µg/ml; and zymosan [ZY], 5µg/ml). The NEPs were pre-treated with PMB to eliminate the possibility of LPS interference. The middle dotted line represents the cut-off and corresponds to the 0.5EU/ml of LPS in NaCl, which was used as a control of pyrogenic level. The lower and upper dotted lines represent the range (50–200%), within which the assay is considered valid. The values are representative of three replicates.

Table 3: A comparative analysis of pyrogen evaluation in MenCC batches by different methods

Method	MAT						LAL assay		
System	cryoWB/IL-1β		cryoWB/IL-6		WB/IL-6		kinetic chromogenic		RPT Σ (ΔT)
	EEU/ml	%	EEU/ml	%	EEU/ml	%	EU/ml	%	(°C)
NaCl	0.015 ± 0.003[NP]	100[V]	0.022 ± 0.002[NP]	100[V]	0.066 ± 0.002[NP]	100[V]	< 0.005[NP]	100[V]	nd
MenCC–NaCl:									
Batch A	0.056 ± 0.001[NP]	187[V]	0.016 ± 0.001[NP]	118[V]	0.056 ± 0.003[NP]	174[V]	0.019 ± 0.001[NP]	174[V]	0.65[NP]
Batch B	0.087 ± 0.004[NP]	184[V]	0.084 ± 0.005[NP]	137[V]	0.061 ± 0.005[NP]	163[V]	0.020 ± 0.002[NP]		0.40[NP]
Batch C	0.018 ± 0.006[NP]	86[V]	0.042 ± 0.003[NP]	193[V]	0.026 ± 0.002[NP]	169[V]	0.012 ± 0.001[NP]		0.05[NP]
Adjuvant only	0.149 ± 0.004[NP]	224[F]	0.115 ± 0.004[NP]	80[V]	nd	nd	nd	nd	nd
MenCC–adjuvant:									
Batch A	0.227 ± 0.005[NP]	304[F]	0.335 ± 0.027[NP]	124[V]	nd	nd	< 0.005[NP]	87[V]	nd
Batch B	0.258 ± 0.008[NP]	250[F]	0.397 ± 0.027[NP]	99[V]	nd	nd	< 0.005[NP]	153[V]	nd
Batch C	0.273 ± 0.014[NP]	261[F]	0.271 ± 0.007[NP]	141[V]	nd	nd	< 0.005[NP]	152[V]	nd

For the MAT (Method A), the values are the means of four replicates; NP values < 0.5EU/ml. For the LAL assay, the values are the means of four replicates; NP values < 0.5EU/ml; MenCC batches with pyrogen levels lower than the LOD (0.005EU/ml) were not quantified. For the RPT, the criteria are the sum (S) of the individual variation of temperature from three rabbits, NP ≤ 3.3°C and p *> 3.3°C. Historical RPT data from the same batches that were analysed with the LAL assay and the MAT was used.*

Recovery was calculated by using the mean values of the endotoxin equivalent concentrations (EEU/ml) of the LPS-spiked MenCC samples (S) and of the unspiked samples (US). The spike concentrations used were 0.5EU/ml for the MAT, and 5EU/ml for the LAL assay.

$\% \ Recovery_{MAT} = ([S - US]/0.5) \times 100$ *and* $\% \ Recovery_{LAL} = ([S - US]/5) \times 100$.

The adjuvant used was aluminium hydroxide, which was provided by the manufacturer.

[NP] = non-pyrogenic, [V] = valid assay, [F] = failed assay, nd = not determined.

tests — namely, the kinetic chromogenic LAL assay and the RPT, which are used as the standard pyrogen tests in industry, as recommended by the European Pharmacopoeia and WHO. Animals were not used exclusively for the purpose of the current study; already available historical data from the RPT were used for comparison. After correcting for the dilution, the cut-off at which MenCC batches were considered to be pyrogenic (P) or non-pyrogenic (NP) was 0.5EU/ml for the MAT, 0.5UE/ml for the LAL assay, and 3.3°C as the sum (S) of the individual variation of temperature for the RPT.

The P/NP classification of the MenCC batches analysed in the MAT cryoWB system were in accordance with the LAL assay results and the RPT data (Table 3), and confirmed the absence of pyrogenic contamination in the tested batches.

Discussion

The MAT is of significant interest as an animal replacement strategy in vaccine pyrogen testing. There is demand in the biological product industry for improved methods of detection of pyrogenic substances and microorganisms, and for their identification in a wide range of raw materials, intermediate products and final product batches (37, 38). For vaccines, every batch should be assessed for pyrogenic potential prior to marketing. Due to the high level of animal use required to ensure the quality of the final product, the implementation of alternative methods should be encouraged (39, 40).

The complexity, high aggregated value and unique features of biological products makes the product-specific validation of a given test mandatory, and reinforces the need for technical and scientific data support. The MAT has been used to evaluate a number of different biological products, such as: a) human serum albumin (HSA; 41); b) hyperimmune sera (HS; 42); c) bacterial vaccines (for example, the meningococcal OMV-based vaccine; 43); d) a group ACYW135 meningococcal polysaccharide vaccine (44); e) trivalent vaccines against diphtheria, tetanus and acellular pertussis (45); and f) viral vaccines (for example, the yellow fever vaccine 17DD-YFV; 46). Overall, the MAT has provided consistent batch-to-batch testing and yielded more-reliable data than the RPT *in vivo* assay (47).

Advantages of the MAT

Corroborating the previous successes of MAT use for biological products, the current study has shown that the MAT cryoWB system:

- is a suitable and reliable assay to determine the pyrogenic content in MenCC, which is a new conjugate vaccine developed by Bio-Manguinhos;
- is compatible with MenCC when it is diluted in the aluminium hydroxide-containing adjuvant (as indicated by the manufacturer), provided that IL-6 is used as the readout (IL-1β should not be used);
- gives data that correlate with similar data from the LAL assay and the RPT (which are the methods detailed in the European Pharmacopoeia and WHO guidelines relating to MenCC);
- is able to detect potential NEP contamination.

Thus, the MAT cryoWB system is a tool that can improve the safety of the MenCC, by confirming the absence of pyrogenic contaminants in the end-product, and can contribute to the replacement of the RPT assay in the MenCC quality control process.

In this study, different MAT systems were evaluated successfully, according to the criteria described by the European Pharmacopoeia (22), for use in product-specific verification tests with an endotoxin standard challenge. Based on the data obtained, it is recommended that the cryoWB system is used when employing the MAT in MenCC testing. Cryopreserved blood is best suited to an industrial setting, as obtaining fresh blood can be a limiting factor in the implementation of some tests (48). The data from the interference testing showed that the MAT cryoWB system is applicable to the MenCC, using both IL-1β and IL-6 readouts, under the conditions tested (dilution in NaCl) and with a minimum vaccine dilution of 1:4. The possibility of evaluating the MenCC in its final formulation (i.e. with the adjuvant), as recommended by the manufacturer, is a distinct advantage of the MAT cryoWB system, especially because the test samples were less diluted (1:4) when compared to the samples tested in the LAL assay (1:100). Dilution is by far the most important strategy for dealing with interference, and products can be diluted to overcome interference, while still permitting endotoxin detection. However, a product cannot be diluted beyond the endotoxin detection limit, i.e. the MVD. Although the dilution used in the LAL assay was valid (1:100), the lower dilution used in the MAT (1:4) is better able to detect potential pyrogenic contaminants.

Choice of marker readout

To evaluate the correlation of the pyrogenic response detected by using the IL-1β and IL-6 readouts with the MAT cryoWB system, an LPS standard curve was generated from dilutions in NaCl alone and in MenCC reconstituted in NaCl. An acceptable correlation was observed between the two cytokine readouts, suggesting that there was no discrepancy in the response between the readouts or MenCC interference in the readouts, as observed with the yellow fever vaccine 17DD-YFV (47).

To determine the best readout system for the MAT when testing the MenCC, the intra-assay and inter-assay coefficients of variation were evaluated for the MAT cryoWB and WB systems, with IL-6 and IL-1β as the marker readouts. The IL-6 cytokine readouts were found to have lower CV values than the IL-1β readouts. The CV values obtained in our study were lower than those described in the literature, which tend to be in the range of 20–45% (30, 32). Thus, according to the literature, we consider the variation in our study to be consistent with the test system, further supporting the reliability of the data obtained. These results prompted a minor change to the choice of cytokine marker, suggesting that IL-6 should be used as the readout for both the cryoWB and WB MAT systems. This has been described previously by our group for 17DD-YFV (47) and for a meningococcal OMV-based vaccine study (44). These data, in addition to the fact that IL-6 is secreted in large amounts in conditioned media, thus allowing better post-stimulation quantification when compared with IL-1β, have justified the choice of the IL-6 marker readout as the best option for the MAT pyrogen detection system (50, 51). This result was corroborated by Stoppelkamp *et al.* (52), who identified IL-6 as the readout best suited for the accelerated MAT as it produces a stronger signal and is linear over a longer range after LPS stimulus.

Evaluation of NEP detection

The capacity of the MAT to detect NEP quantitatively was determined by the generation of a LTA standard curve in the cryoWB/IL-6 system. LTA was used as a NEP stimulus because it: a) is a classic example of an NEP; b) is a ligand of Toll-like receptors; c) is of great importance in the pathogenesis of sepsis (36); d) induces false negatives in the LAL assay (30); and e) is an excellent control to demonstrate that possible contamination with NEPs might be overlooked by the biologicals industry (53). Prior to spiking the MenCC samples with LTA, we performed a LAL endotoxin assay on the commercial LTA preparation, to assess whether it was contaminated with residual LPS. As found in the literature (37), a high level of LPS contamination was identified when the LTA preparation was analysed without PMB pre-treatment. The response to this residual LPS contamination was potentiated by the LTA, in the presence of the MenCC preparations. This corroborates the data

on 17DD-YFV, and is in accordance with the finding by Kim *et al.* (54), who showed a priming effect of LTA on LPS IL-6 cytokine response in a monocyte cell line. Importantly, this priming potential was significantly reduced after PBM pre-treatment, as observed for 17DD-YFV (47).

In addition, when a quantitative assay was performed with LTA standards prepared in MenCC and with IL-6 as the readout, a significant difference was found between LTA doses, and 50μg/ml was defined as the pyrogenic dose for LTA, since it corresponded to 0.5EU/ml of LPS. These observations were in agreement with data described previously by Gimenes *et al.* (29), with IL-1β as the readout, and Mattos *et al.* (47), with IL-6 as the readout. The high correlation between the LTA standard curves in MenCC and in NaCl indicates that there was no vaccine interference in the MAT when used as a quantitative test.

Ability of the MAT to detect different pyrogen classes in MenCC

As recommended in the revised MAT monograph (24), the MAT should be subjected to product-specific validation by challenging it with endotoxin and at least two different NEPs spiked into the test samples. For this purpose, we quantitatively analysed the concentration of endotoxin (LPS) and NEPs (LTA and zymosan; expressed as EEU) in three MenCC batches. Zymosan was chosen because it: a) is detected in the LAL assay in a different way to that described for LTA, although producing more false positive results; b) is a ligand of Toll-like receptors; c) does not induce fever in the RPT (55); d) is regarded as an immunologically active pro-inflammatory molecule distinct from other glucan molecules (30), and detectable with the MAT; e) is able to induce only IL-6 and not IL-1β, as marker readouts (28, 30); and f) is an important control substance in the industry, since false positive results in the LAL assay could lead to an out-of-specification result (i.e. above the acceptable limit of 0.25EU/ml) (56). All stimuli spiked in MenCC were detectable, and the test was validated by quantifying NEPs in terms of EEU by using the LPS curve in NaCl. The use of PMB demonstrated that the pyrogenic potential of NEPs is not due to LPS contamination. In agreement with the literature (30, 42), this study showed that the MAT is capable of ensuring the safety of the MenCC when it comes to detecting pyrogenic contamination by molecules other than LPS.

The effects of adjuvant presence

The endotoxin spikes in MenCC–NaCl, which were detected with both IL-6 and IL-1β readouts in the MAT, were detected at similar levels to those in the LAL. For MenCC–adjuvant, the values of endotoxin detected were higher than those observed in NaCl. Furthermore, the use of the IL-1β readout was not valid, as the LPS recovery rate was higher than 200%. Previously, Ulanova *et al.* (57) demonstrated that exposure to aluminium hydroxide (i.e. adjuvant) directly stimulates monocytes to produce pro-inflammatory cytokines, such as IL-6, IL-1β and TNF. This observation corroborates our findings, in which the adjuvant control showed higher pyrogen values than the NaCl control, justifying the higher background values in MenCC–adjuvant. Interestingly, adjuvants promote phagocytosis, which enhances the immune response against antigens (58). Macrophages primed with LPS and exposed to aluminium salts *in vitro* produced higher levels of IL-1β when compared to a sample without adjuvant. However, this effect was not observed with the IL-6 readout under equivalent conditions, suggesting that the failure of the testing system with MenCC–adjuvant could be a result of the enhanced response to the LPS spike in the sample used as the control to validate the assay (59). This discrepancy induced by the adjuvant was not observed initially, since the interference test was performed with MenCC–NaCl and with IL-1β as the readout, and all MenCC testing conditions later focused on the use of IL-6 as the marker readout, based on our previous experience with IL-6 as the better readout system (43, 47) and the literature on the use of the MAT for meningococcal vaccines (44, 60). Although the values obtained with MenCC–adjuvant were lower than the endotoxin limit for MenCC quality control, they were higher than the endotoxin concentration determined by the LAL assay, in which the vaccine is also diluted in the adjuvant. Perhaps, as the dilution used for the MAT (four-fold) was lower than that for the LAL assay (100-fold), this difference could justify the discrepancy between the tests. Although both dilutions are in accordance with the MVD allowed for the product, a higher dilution might mask potential pyrogens, and the MinVD is recommended as it better reflects the real-life administration of the vaccine.

Comparison of the MAT with the RPT

MAT data were further compared with already available historical RPT data on the same batches. The satisfactory results obtained reinforce the absence of pyrogenic contamination in the MenCC batches tested, corroborating the possibility of using the MAT as a non-animal alternative method during the development of new products. Despite the consistency between the pyrogenic tests in the case of MenCC, the literature states that, specifically for biologicals, the MAT seems to

be a more reliable test than the RPT. This is based on several reports of clinical adverse fever reactions in products previously classified as 'pyrogen-free' by the RPT, but that were not sufficiently safe when administered to patients (42, 61). Vaccines are administered to humans in small volumes by intramuscular or subcutaneous injection. However, during the quality control process, pyrogenicity is evaluated by administering large volumes of product, by intravenous injection, to rabbits (48). Thus, the discrepancy between tests could be attributed to sensitivity, difference in the route of administration and species extrapolation. These are all important aspects to consider when choosing the best model for pyrogenic testing of vaccines for quality control purposes.

Conclusions

The MAT results were consistent and provided the same outcome as the two methods endorsed by MenCC guidelines to identify potential pyrogenic contamination. The MAT data also reinforced the absence of endotoxin and NEPs contamination in three batches of MenCC produced on an industrial scale by Bio-Manguinhos. These results are a compelling reason to replace the RPT by the MAT. This would contribute to the elimination of the use of rabbits for pyrogen detection during batch-to-batch routine quality control after clinical trials and as part of the tests for MenCC end-product licensing, focusing instead on a more-rigorous, specific and consistent quality control for healthy infants, adolescents and adults, who tend to be the targets of prophylactic programmes.

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