Applicability of the Monocyte Activation Test (MAT) in the Quality Control of the 17DD Yellow Fever Vaccine

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Summary — The need for alternatives to animal use in pyrogen testing has been driven by the Three Rs concept. This has resulted in the inclusion of the monocyte activation test (MAT) in the European Pharmacopoeia, 2010. However, some technical and regulatory obstacles must be overcome to ensure the effective implementation of the MAT by the industry, especially for the testing of biological products. The yellow fever (YF) vaccine (17DD-YFV) was chosen for evaluation in this study, in view of: a) the 2016–2018 outbreak of YF in Brazil; b) the increase in demand for 17DD-YFV doses; c) the complex production process with live attenuated virus; d) the presence of possible test interference factors, such as residual process components (e.g. ovalbumin); and e) the need for the investigation of other pyrogens that are not detectable by the methods prescribed in the YF vaccine monograph. The product-specific testing was carried out by using cryopreserved and fresh whole blood, and IL-6 and IL-1β levels were used as the marker readouts. After assessing the applicability of the MAT on a 1:10 dilution of 17DD-YFV, endotoxin and non-endotoxin pyrogens were quantified in spiked batches, by using the lipopolysaccharide and lipoteichoic acid standards, respectively. The quantitative analysis demonstrated the correlation between the MAT and the *Limulus* amoebocyte lysate (LAL) assays, with respect to the limits of endotoxin recovery in spiked batches and the detection of no pyrogenic contamination in commercial batches of 17DD-YFV. The data demonstrated the applicability of the MAT for 17DD-YFV pyrogen testing, and as an alternative method that can contribute to biological quality control studies.

Key words: *17DD yellow fever vaccine, alternative methods, health surveillance, monocyte activation test, pyrogen.*

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Introduction

The Brazilian authorities have recently reported the worst outbreak of yellow fever (YF) in decades, spreading into major population centres, killing hundreds of people and devastating wild monkey populations. In the period between December 2016 and November 2017, the outbreak of YF resulted in 3458 suspected cases, 795 confirmed cases and 436 deaths (1, 2), and new cases were detected between December 2017–January 2018 (2). To prevent further propagation and to control the disease, the Brazilian Ministry of Health recommended an emergency YF vaccination campaign. As part of this emergency strategy, in January 2017 more than 650,000 doses of 17DD-YF vaccine (17DD-YFV) were produced and supplied by the Immunobiological Technology Institute (Bio-Manguinhos, Brazil) and distributed throughout the country (3). In addition, the World Health Organisation (WHO) sent approximately 18 million 17DD-YFV doses to various countries in Africa (4), of which 5.6 million were provided by Bio-Manguinhos. Thus, due to the increase in demand and the prioritisation of this input into the emergency vaccination programme, the monthly Bio-Manguinhos production increased from four million to six million doses (5), culminating in the production of 61 million doses in 2017.

Closing the gap between supply and demand for 17DD-YFV involves efficient production and rigorous quality control processes. Careful attention is required, particularly when living organisms are involved in the complex vaccine production process, as this can result in inherent variability. In addition, the safety evaluation of vaccines is a very important step in quality control, as it ensures that potential adverse effects, such as low-grade fever (one of the mild reactions in vaccine-naïve subjects; 6), are not the result of pyrogenic contamination from the vaccine production process. Therefore, the regulatory authorities have recom-

mended that manufactured products are subjected to a pyrogen detection assay (7, 8), as part of the testing required to ensure the quality of systems and control processes, in accordance with Good Manufacturing Practice.

The pyrogen test is mandatory for pharmaceutical, biotechnological and medical devices, to guarantee the absence of contamination during the production process by microorganisms with pyrogenic activity potential (9). Particularly in Brazil, the quality control of biological products has become a priority target for investment (10, 11).

The Brazilian authorities have been looking for alternative methods to completely replace animal testing for the batch-to-batch release routine prior to commercialisation, a process that requires a great number of animals. Alternatives are needed, and the Monocyte Activation Test (MAT) was specifically developed as an alternative to animal pyrogen tests (12). The obvious advantages of the MAT, such as high sensitivity and the recognition of a broad spectrum of pyrogens (13), have led to its inclusion in the European Pharmacopoeia (12) as a third method with the ability to detect pyrogenic molecules. However, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has pointed out some limitations of the method as it included only Gram-negative bacteria-derived pyrogen (endotoxin) and no other potential contamination sources, such as non-endotoxin pyrogen (NEP), and the lack of studies assessing its suitability for use with other products such as biologicals and medical devices (14, 15).

There are a number of cases where substances inherent in the test vaccine might interfere with pyrogen testing. For example:

— Despite the usual absence from vaccines of constituents of Gram-negative bacteria, except as potential pyrogenic contaminants, there are certain exceptions to this rule. Gram-negative bacterial lipopolysaccharide (LPS) components are present in the outer membrane vesicle (OMV) vaccines against *Neisseria meningitides* (16–18);

— Some viral vaccines are inherently pyrogenic (19);

— Assay-interfering substances, such as aluminium hydroxide [$Al(OH)_3$], can be part of the vaccine formulation (20); and

— Multiple components can be present, with interaction between the substances (e.g. vaccines that contain protein and polysaccharide components from both Gram-positive and Gram-negative bacteria; 21).

In this context, the Bio-Manguinhos/Fiocruz, in collaboration with the National Institute for Quality Control in Health (INCQS/Fiocruz), embarked on this study, to consolidate the scientific development of alternative methods in the field of biologicals, following requirements outlined by the regulatory agencies. The 17DD-YFV was chosen for evaluation in this study in view of: a) the 2016–2018 outbreak of YF in Brazil; b) the increase in demand for 17DD-YFV doses; c) the complex production process with live attenuated virus; d) the presence of possible test interference factors, such as residual process components (e.g. ovalbumin); and e) the need for the investigation of other pyrogenic contaminants that are not detectable by the methods prescribed in the YF vaccine monograph (in the Brazilian and European Pharmacopoeias).

Materials and Methods

Vaccine samples

Ten batches of 17DD-YFV (10 doses/vial), manufactured by Bio-Manguinhos, were selected from samples tested during routine quality control. The vaccine was reconstituted in 5ml of diluent (0.9% w/v NaCl solution), with the human dose being 0.5ml. All ten batches used were classified as non-pyrogenic according to the *Limulus* amoebocyte lysate (LAL) assay. The MAT was performed on the same batches of 17DD-YFV.

The LAL assay

The endotoxin levels in the ten batches of 17DD-YFV were determined by a kinetic chromogenic LAL assay (Endosafe; Charles River Laboratories, Wilmington, MA, USA), according to the manufacturer's instructions. The vaccine was tested (as part of the quality control process), by measuring the recovery of a known amount of endotoxin spiked in the minimum valid dilution (MinVD) of the product (1:5), as previously established by the manufacturer. Optical density (OD) 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). The cut-off at which 17DD-YFV batches were considered pyrogenic (P) or non-pyrogenic (NP) was 10 endotoxin units (EU)/ml (= 5EU/human dose [HD]), as described in both the Brazilian Pharmacopoeia and the European Pharmacopoeia.

The Monocyte Activation Test (MAT)

Pyrogenic stimuli

LPS from *Escherichia coli* O55:B5 (Catalogue No. L2880; Sigma-Aldrich, Steinheim, Germany), which

was certifiably calibrated to the international reference endotoxin standard, was used as the control standard endotoxin. The lyophilised contents of the 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* (Sigma-Aldrich, St Louis, MO, USA) was used as a non-endotoxin pyrogen (NEP) stimulant. Endotoxin-Equivalent Units (EEU) were used to express the level of NEP contaminant concentration, according to the endotoxin standard curve. To exclude the possibility of confounding endotoxin contamination inherent in the LTA standard, pretreatment with 40µg/ml polymyxin B (PMB; 22), which is a relatively specific inhibitor of endotoxin biological activity, was performed in all the experiments. The LTA dose–response curve was constructed by using doses of 10, 50, 75, and 200µg/ml. A 0.9% w/v NaCl pyrogen-free solution (Halex Istar, Goiânia, Goiás, Brazil) was used as a negative control, and as the diluent for the pyrogenic stimulus and 17DD-YFV.

Determination of the maximum valid dilution

The maximum valid dilution (MVD) for 17DD-YFV was derived by dividing the endotoxin limit concentration (ELC) described in the YFV monograph (10EU/ml) by the limit of detection (LOD). Based on the LOD calculation described in the European Pharmacopoeia, the values obtained were 0.045EU/ml for IL-1β and 0.042EU/ml for IL-6.

Therefore, the MVD for 17DD-YFV in the MAT assay was determined to be 1:222 for IL-1β and 1:238 for IL-6.

Interference test

Three batches of 17DD-YFV were used to determine the vaccine minimum valid dilution (MinVD) for the MAT, to ensure that there was no assay interference from the vaccine. The batches were prepared and diluted as described above, with parallel samples either spiked (S) or unspiked (US) with 0.5EU/ml of LPS (added in a volume of 5µl).

Spike recovery was calculated by using the mean values of the endotoxin equivalent concentrations (EEU/ml) of the S and US 17DD-YFV, 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, as stipulated by the local ethics committee. Differential blood cell counts were routinely performed for every sample, with a Hemogram 60 cell counter (BioClin®, Belo Horizonte, MG, Brazil), in order to identify and exclude donors with an acute infection. Fresh whole blood (WB) was used within four hours. Cryopreserved whole blood (cryoWB) was prepared by using endotoxin-free Sorënsen's phosphate buffer (Acila GmbH, Mörfelden-Walldorf, Hesse, Germany), mixed 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*. (23). All the steps were carried out in a laminar flow cabinet. Three pooled lots of cryoWB and two 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 (12). For the WB assay, a 50µl sample (or control) was mixed with 500µl saline and 50µl of the pooled blood. For the cryoWB assay, 50µl of sample/control were mixed with 450µl saline and 100µl thawed blood. Cell contact with the test concentrations of the vaccine samples/controls took place in polypropylene vials (Eppendorf, Hamburg, Germany) for 20 hours, in a humidified incubator (37°C; 5% v/v CO_2). The vials containing the supernatants of the post-contact blood samples (obtained after centrifuging at 7000*g* for 5 minutes) were stored at –20°C until needed for cytokine measurement. The endotoxin level used as the pass/fail criterion for the MAT was 0.5EU/ml, which is based on historical data on the threshold pyrogenic dose in the rabbit pyrogen test (RPT), according to ICCVAM (14).

Cytokine measurement

Levels of IL-1β and IL-6 were measured by using commercially available Human Quantikine® ELISA kits (R&D Systems, Wiesbaden, Germany), with a detection range of 3.90–250pg/ml for IL-1β, and 3.12–300pg/ml for IL-6. The data are presented as the mean ± standard error of the mean (SEM) of four

biological replicates (i.e. biological replicates are parallel measurements of biologically distinct samples in the same experiment). The ELISA was carried out according to the manufacturer's instructions.

Statistical analysis

To ensure the precision and validity of the MAT, preparatory tests were conducted to ensure that the European Pharmacopoeia criteria for standard curve fitting were successfully met (12). The response curve for log-transformed dose data was considered statistically significant ($p > 0.01$; data not shown). The validity of the linear regression of the endotoxin standard curve was assessed by calculating the coefficient of determination (r^2). Correlation and contingency analyses were used for the MAT *versus* LAL assay comparison. The Pearson's correlation coefficient was used to measure the strength of the correlation between two variables. For comparison between the IL-6 dose–response curves following LPS and LTA dilution in either 17DD-YFV or NaCl solution, a non-parallelism analysis was performed by using the statistical analysis software CombiStats™ (version 5.0, EDQM/Council of Europe, Strasbourg, France), with the assay considered to be valid at $p < 0.05$. The comparisons between multiple groups were performed with a one-way ANOVA test; differences between the groups were analysed with Student's *t*-test by using GraphPad Prism v4 software (GraphPad Software, La Jolla, CA, USA), and values of $p < 0.05$ were considered to be statistically significant. The data were expressed as the mean ± SEM. Means and coefficients of variation (CV values) were calculated for each sample.

Results

The LAL assay

Batches of 17DD-YFV (10 doses/vial) were evaluated for the presence of endotoxin prior to performing the MAT, by using the kinetic chromogenic LAL assay, which is the accepted pyrogen test according to the YFV monograph (in the Brazilian and European Pharmacopoeias). All the vaccine samples used in this study were classified as non-pyrogenic (NP) in the LAL assay; results are only shown for comparative analysis with the MAT.

Comparison of IL-1β and IL-6 levels as MAT marker readouts for LPS detection

The LPS standard curves for all the MAT systems assessed were satisfactory, in terms of both response ($p < 0.001$) and linearity ($r^2 > 0.95$).

The use of the MAT for 17DD-YFV testing with cryopreserved whole blood and IL-1β or IL-6 as marker readouts

To determine the applicability of the MAT for pyrogen detection in 17DD-YFV, a number of parameters were defined prior to testing, as recommended in the European Pharmacopeia (12).

The interference test

All the dilutions prepared from the 17DD-YFV resulted in LPS spike recovery within the acceptable range of 50–200%, according to the IL-1β and IL-6 marker readouts (Figure 1), except for the 1:160 dilution of IL-6. However, the MinVD was determined as the dilution at which the recovery value was around 100%. Thus, the 1:10 dilution of 17DD-YFV was chosen for use in the MAT assay, for both the IL-1β marker (recovery 101%, Figure 1a) and the IL-6 marker (recovery 99%, Figure 1b). As a lower dilution factor was eventually chosen for the performance of the MAT, we only tested up to a 1:160 dilution.

Comparative analyses of LPS dose–response curves based on IL-1β and IL-6 levels as marker readouts

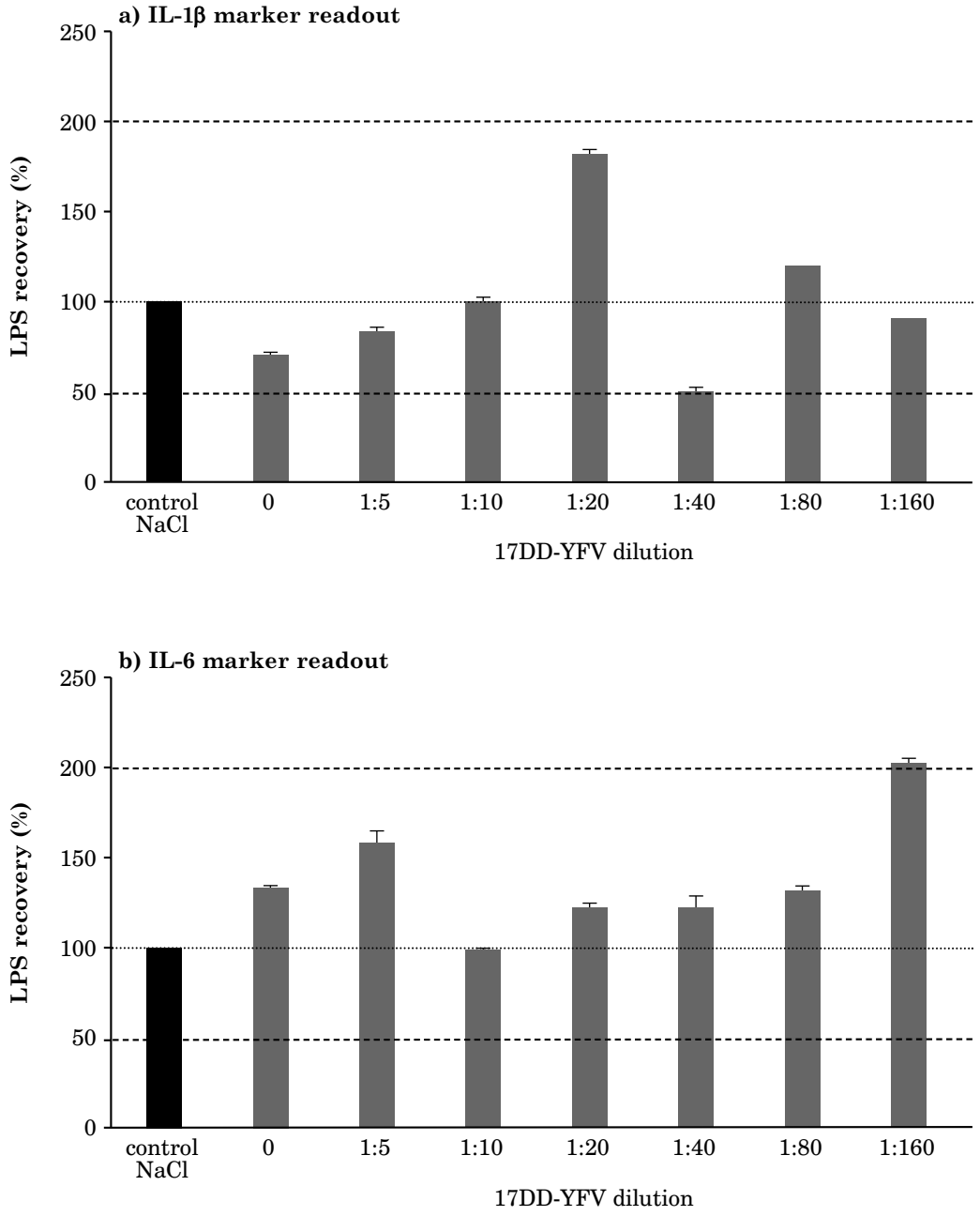
To determine the correlation between IL-1β and IL-6 levels as marker readouts, both cytokines were assessed within the same supernatants obtained from cryoWB samples exposed to LPS-spiked NaCl solution and LPS-spiked 117DD-YFV (with 0.25, 0.5, 1 and 2.5EU/ml LPS). As Figure 2 shows, acceptable correlations were observed between IL-1β and IL-6 induction by LPS for the NaCl solution control ($r = 0.9956$ and $p < 0.0001$) and 17DD-YFV ($r = 0.9972$ and $p = 0.0028$). A strong induction profile for both cytokines in response to endotoxin was obtained for the cryoWB, and this confirmed the suitability of both IL-6 and IL-1β levels for use as marker readouts in the cryoWB MAT system.

Ability of the MAT to detect different pyrogen classes in 17DD-YFV

The LTA solution (NEP stimulant) was pre-treated with a final concentration of 40μg/ml PMB, to avoid the confounding effects of LPS contamination on the NEP results. The efficacy of PMB inhibition of LPS activity in the LAL assay is shown in Figure 3.

The interference potential of the 17DD-YFV in the quantification of the different pyrogen classes was analysed by comparing the OD values obtained in

Figure 1: The interference test



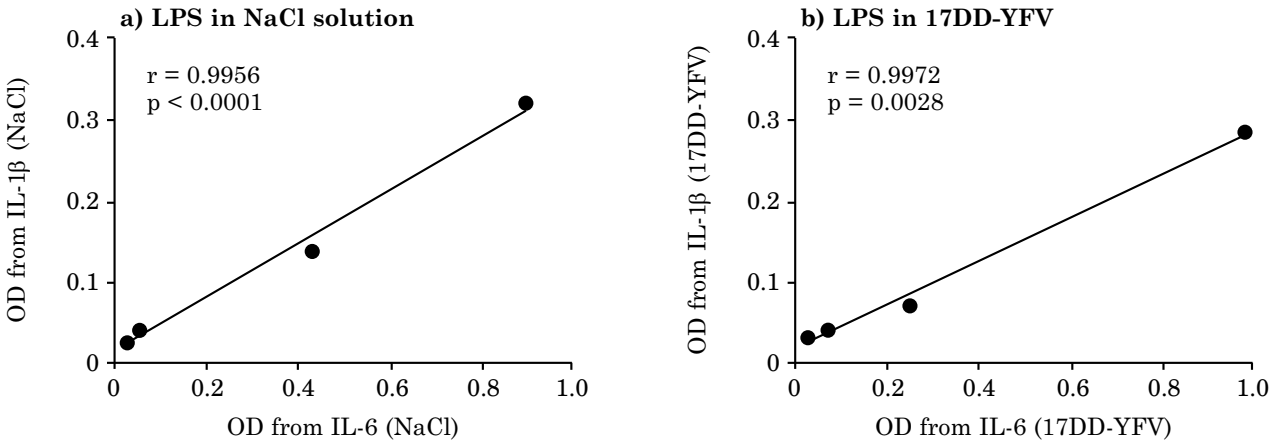
Endotoxin recovery was determined by using the MAT in the cryoWB system, to test for potential interference by 17DD-YFV and to determine the minimum valid dilution of the test vaccine. Both readouts were tested: a) shows LPS recovery with IL-1β; and b) shows LPS recovery with IL-6. For each vaccine dilution, the percentage LPS recovery was calculated as ([S – US]/0.5) × 100, where S = endotoxin equivalent concentration (EEU/ml) of 0.5EU/ml LPS-spiked 17DD-YFV, and US = endotoxin equivalent concentration (EEU/ml) of unspiked 17DD-YFV.

---------- = the range within which a product is considered interference-free (50–200%).

..... = the cut-off (100%) and corresponds to 0.5EU/ml of LPS in NaCl solution (control; black bars).

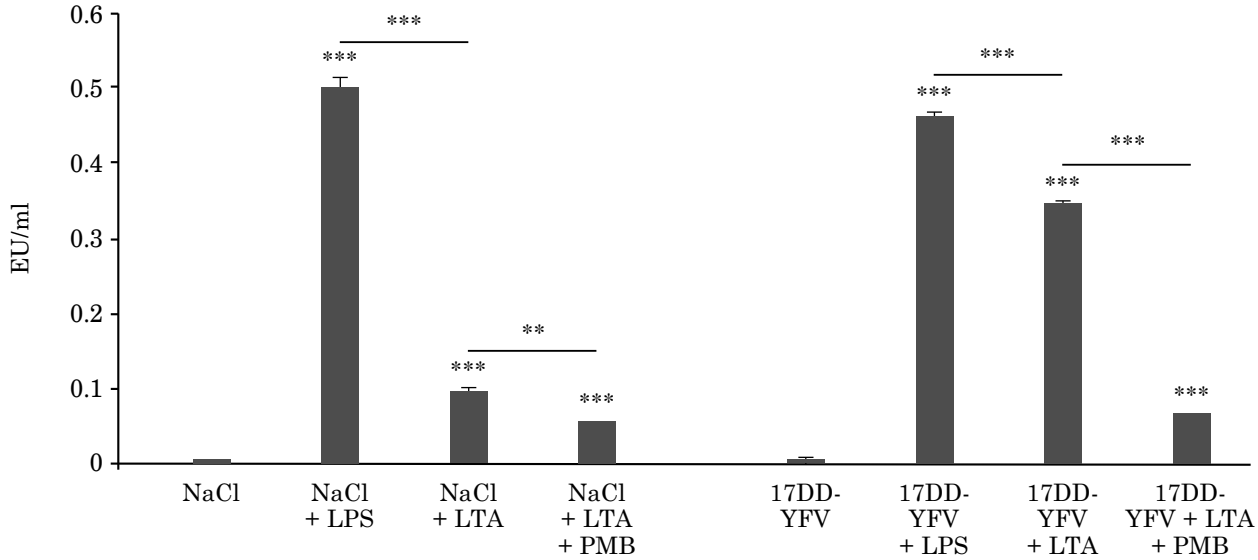
Error bars represent the standard deviation (SD) of the mean of four replicates.

Figure 2: Correlation between the induction of IL-6 and IL-1β in the cryoWB MAT system, in response to LPS



The relationship between the induction profiles of IL-6 and IL-1β in response to serial dilutions of LPS (0.25, 0.5, 1 and 2.5EU/ml) in a) NaCl solution (control) and b) 17DD-YFV was determined in the cryoWB MAT system. Optical density (OD) readouts from the cytokine ELISA assay 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.*

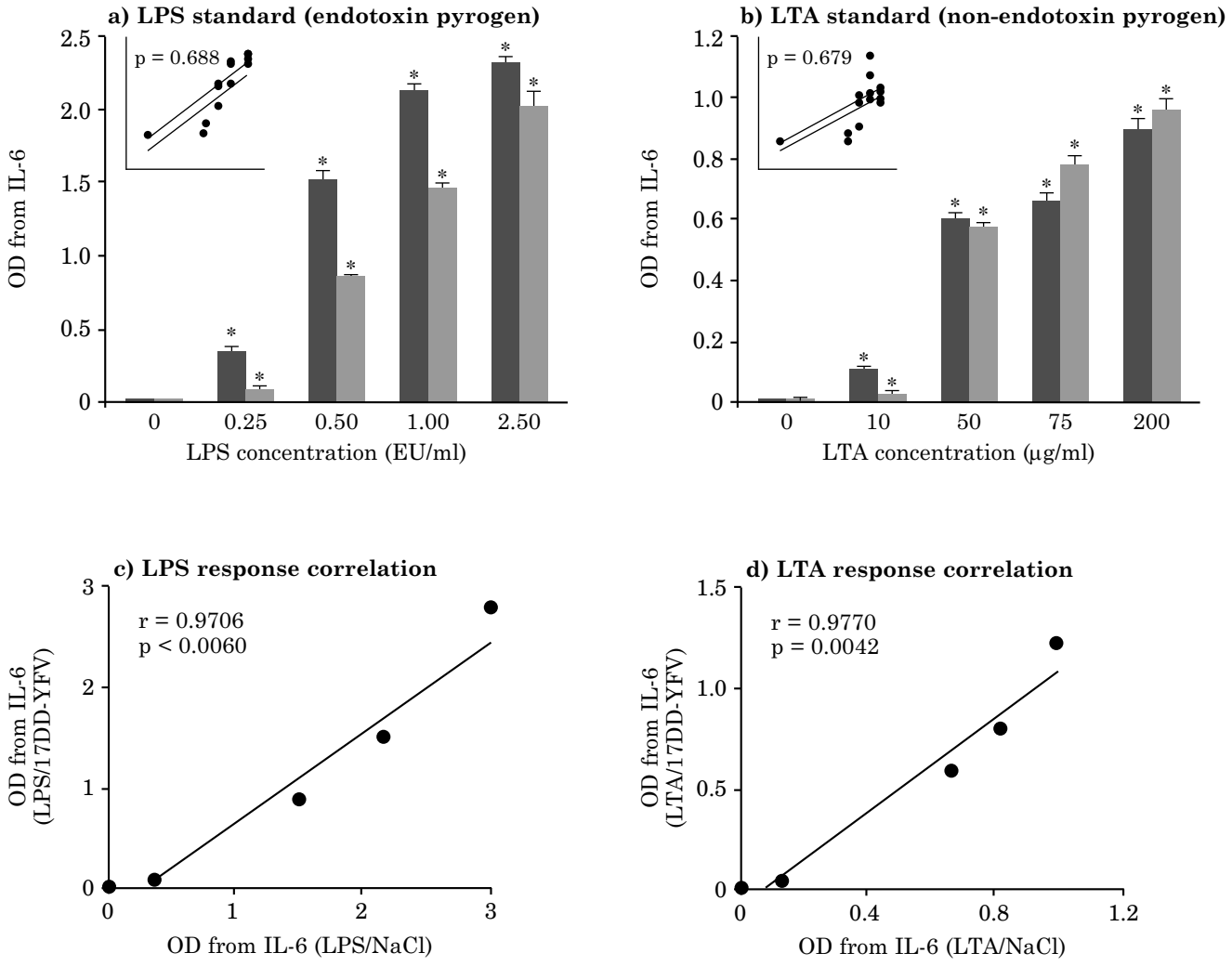
Figure 3: The inhibition of residual LPS activity by PMB, as determined in the LAL assay



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 obtained in the MAT. The efficacy of LPS response inhibition by PMB was determined in the LAL assay, which effectively detects endotoxin pyrogens such as LPS, but does not detect non-endotoxin pyrogens like LTA. The NaCl control solution and 17DD-YFV samples were spiked with LPS (0.5EU/ml) and LTA (50μg/ml, with and without PMB treatment) and analysed by using the LAL assay. The values are representative of three replicates. The Student's t*-test was used to compare the control samples (NaCl or 17DD-YFV) with those spiked with LPS, LTA and LTA + PMB;* **p < *0.01,* ***p < *0.001.*

the IL-6 ELISA, in response to LPS (0.25–2.5EU/ml; Figure 4a) and LTA (10–200µg/ml; Figure 4b) in the cyroWB MAT, when diluted in either 17DD-YFV or in the NaCl solution. 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, correlation analysis showed that similar IL-6 response profiles (as expressed by OD values obtained in the IL-6 ELISA) were obtained for LPS in 17DD-YFV and in NaCl solution (Figure 4c), and this was also evident for LTA (Figure 4d).

Figure 4: The ability of the MAT to detect different pyrogen classes in 17DD-YFV



*CryoWB was spiked with: a) 0.25–2.5EU/ml LPS standard (endotoxin pyrogen); and b) 10–200µg/ml LTA standard (non-endotoxin pyrogen). These pyrogens were used to spike the NaCl control solution (dark grey bars) and 17DD-YFV (light grey bars). OD values obtained in the IL-6 ELISA are plotted against pyrogen concentration. The insets in graphs a) and b) correspond to the non-parallelism analysis of the standard curves in NaCl solution and 17DD-YFV. 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. Graphs c) and d) show the correlation between the induction profile of IL-6 by c) LPS-spiked and d) LTA-spiked NaCl control solution and 17DD-YFV, expressed as OD values obtained in the IL-6 ELISA.*

Endotoxin pyrogen detection with the MAT Quantitative Method

After confirming that the MAT could successfully be used to assay the 17DD-YFV, samples of the different vaccine batches were tested. For the vaccine testing, the chosen methodology was Method A (Quantitative Method) from the European Pharmacopoeia (12), and an LPS standard curve range of 0.25–2.5EU/ml. Positive control samples spiked with 0.5EU/ml of the reference standard endotoxin were included, to ensure the suitability of cryoWB and IL-6 and IL-1β marker readouts for use in the MAT. Both cytokine readouts were in accordance with the results obtained in the LAL assay (Table 1).

The detection limits for the ELISA-based cytokine assays were 3.90pg/ml for IL-1β and 3.12pg/ml for IL-6, according to the manufacturer's data. The negative OD values were normally out of the linear phase of the dose–response curve for both cytokine standard curves and are indicated in Table 1 as values < LOD. Considering that the samples were diluted by a factor of ten (for the MAT) or by a factor of five (for the LAL assay) to minimise the risk of interference, in addition to a further sample dilution of 1:10 when added into the assay volume, we assumed that the lack of any apparent pyrogens in the unspiked samples was due to their amounts being below the LOD. The lack of detection of endotoxin in unspiked samples was also observed in the LAL assay (< 0.005EU/ml), which confirmed the low levels of endotoxin inherent in these vaccine batches.

As the WB/IL-6 system has been validated for pyrogen detection by ICCVAM, we analysed three 17DD-YFV batches by this means, and compared the results with those obtained with the cryoWB/IL-6 system proposed in this study. The responses observed with the two systems were similar, indicating that the cryoWB/IL-6 system can be used as an alternative means to detect pyrogens in the MAT.

Non-endotoxin pyrogen detection with the MAT Quantitative Method

In agreement with the findings of Gimenes *et al.* (24), the LTA concentration that corresponds to the threshold concentration in the *in vivo* rabbit pyrogen test was 50μg/ml when using IL-1β as the marker readout (Figure 5a); similar results were obtained when using IL-6 as the marker (Figure 5b).

In this study, the LTA solution was pre-treated with PMB prior to use in the assays. We tested the

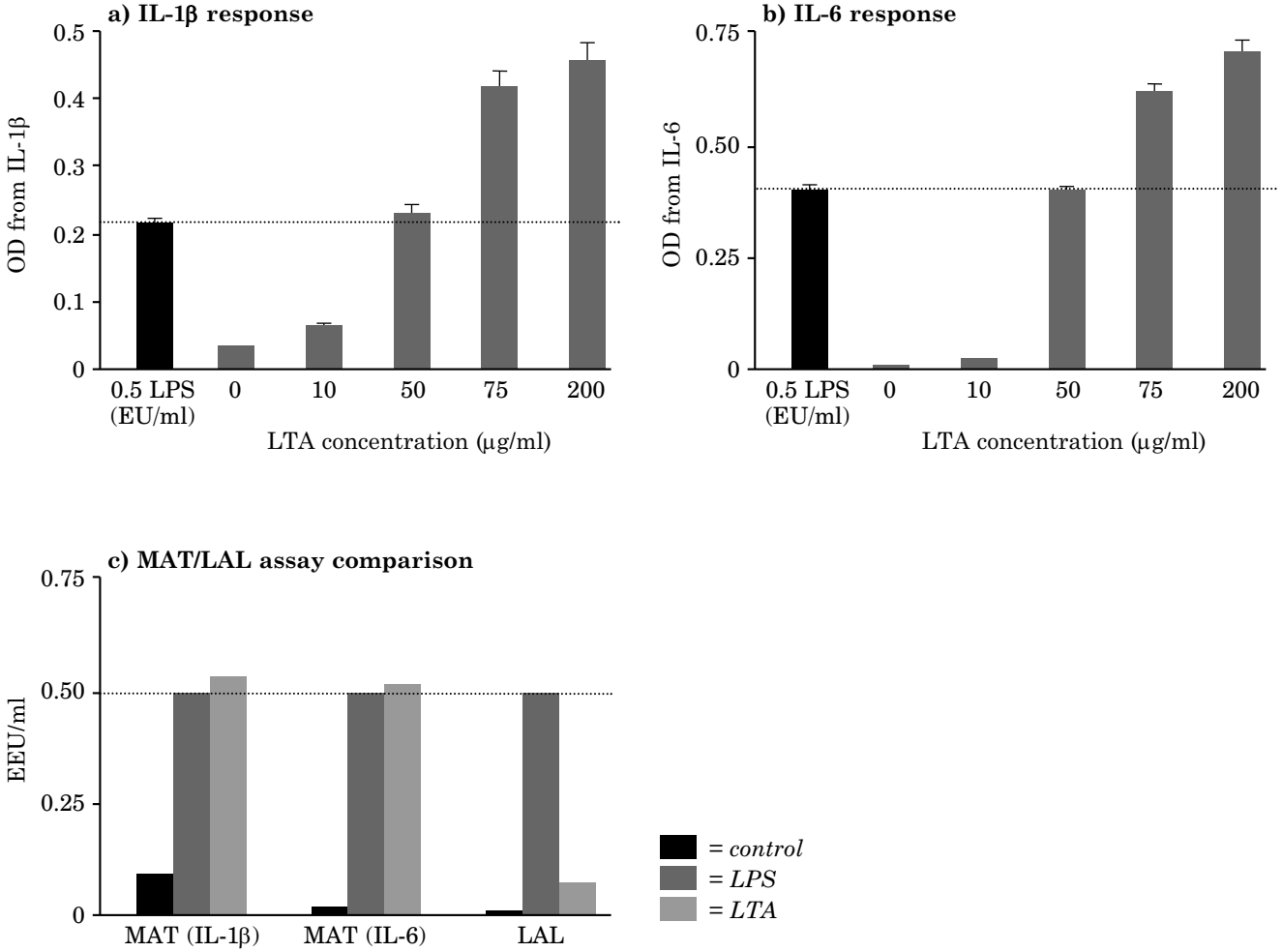
Table 1: A comparative analysis of pyrogen detection in 17DD-YFV by using the MAT and the LAL assay

	Method							
	MAT						LAL	
	cryoWB/IL-1β system		cryoWB/IL-6 system		WB/IL-6 system		Kinetic chromogenic method	
Batch	EEU/ml	% recovery[a]	EEU/ml	% recovery[a]	EEU/ml	% recovery[a]	EU/ml	% recovery[a]
A	< LOD[c]	109[b]	< LOD[c]	178[b]	< LOD[c]	188[b]	< LOD[c]	113[b]
B	< LOD[c]	132[b]	< LOD[c]	199[b]	< LOD[c]	163[b]	< LOD[c]	125[b]
C	< LOD[c]	148[b]	< LOD[c]	196[b]	< LOD[c]	173[b]	< LOD[c]	140[b]
D	< LOD[c]	117[b]	< LOD[c]	72[b]	nd	nd	0.0643[c]	68[b]
E	< LOD[c]	149[b]	< LOD[c]	177[b]	nd	nd	0.1012[c]	87[b]
F	< LOD[c]	108[b]	< LOD[c]	80[b]	nd	nd	< LOD[c]	61[b]
G	< LOD[c]	146[b]	< LOD[c]	166[b]	nd	nd	< LOD[c]	89[b]
H	< LOD[c]	146[b]	< LOD[c]	71[b]	nd	nd	< LOD[c]	103[b]
I	< LOD[c]	166[b]	< LOD[c]	172[b]	nd	nd	0.1627[c]	151[b]
J	< LOD[c]	199[b]	< LOD[c]	192[b]	nd	nd	0.1603[c]	150[b]

[a]The values are the means of four replicates; [b]valid assay (recovery in the 50–200% range); [c]non-pyrogenic; cryoWB = cryopreserved whole blood; WB = fresh whole blood; LAL = Limulus *amoebocyte lysate; MAT = monocyte activation test; nd = not determined.*

Recovery was calculated by using the mean values of the endotoxin equivalent concentrations (EEU/ml) of the LPS-spiked (S) 17DD-YFV and the unspiked samples (US). The spike concentrations used were 0.5EEU/ml for the MAT, and 5EEU/ml for the LAL. % Recovery$_{MAT}$ = ([S – US]/0.5) × 100 and % Recovery$_{LAL}$ = ([S – US]/5) × 100. For samples labelled < LOD (limit of detection), it was not possible to quantify endotoxin recovery, since the US dilutions were below the sensitivity limit of the ELISA (3.12pg/ml for IL-6 and 3.90pg/ml for IL-1β). The LOD for the LAL was < 0.005EU/ml.

Figure 5: Non-endotoxin pyrogen detection in the cryoWB MAT system, with IL-6 and IL-1β as the marker readouts



To assess NEP detection, 17DD-YFV samples were spiked with a range of LTA concentrations (10–200µg/ml) and the OD values obtained in the cytokine ELISA were plotted against the concentrations for a) the IL-β marker and b) the IL-6 marker. The dotted line represents the cut-off corresponding to 0.5EEU/ml LPS in the same assay system. The bars represent the mean ± SD.
Graph c) shows a comparative analysis of pyrogen recovery (EEU/ml) for LPS (endotoxin pyrogen) and LTA (non-endotoxin pyrogen) in 17DD-YFV samples, by using the MAT cryoWB system (with both cytokine marker readouts) and the LAL assay. The graph is representative of three independent experiments performed in quadruplicate. The control sample in graph c) was 17DD-YFV. The dotted line represents the cut-off corresponding to 0.5EEU/ml LPS.

efficacy of PMB in inhibiting any LPS-induced response, by analysing the LTA response, before and after PMB treatment, in the LAL assay. The LAL assay detects endotoxin pyrogens (LPS) very effectively, but it is ineffective in detecting NEPs (such as LTA). PMB treatment was shown to significantly reduce the response to LPS contamination in the LTA (see Figure 3; NaCl + LTA *versus* NaCl + LTA + PMB and 17DD-YFV + LTA *versus* 17DD-YFV + LTA + PMB).

However, it was also apparent that the response to any residual LPS contamination in the LTA standard was potentiated (Figure 3; NaCl *versus* NaCl + LTA and 17DD-YFV *versus* 17DD-YFV + LTA). This synergistic effect of LTA and LPS was successfully blocked by pre-treatment with PMB (Figure 3; 17DD-YFV + LTA *versus* 17DD-YFV + LTA + PMB and NaCl + LTA *versus* NaCl + LTA + PMB), and thus it would not confound the quantitative analysis of LTA in the MAT (Figure 5a and 5b).

In parallel, we showed that both the LTA and LPS stimuli could be similarly quantified in the MAT by using IL-6 and IL-1β as marker readouts

(Figure 5a and 5b). This was shown not to be the case for the LAL assay (Figure 5c), confirming the ineffectiveness of the LAL method in detecting NEPs. The low LTA signal apparent in the LAL assay (Figure 5c) was most likely related to residual LPS contamination in the LTA solution after PMB treatment.

Intra-assay and inter-assay variability of pyrogen-induced IL-1β and IL-6 marker readouts in the MAT

In order to evaluate the reproducibility of the MAT with the two marker readouts, i.e. IL-1β and IL-6, the intra-assay and inter-assay variability was analysed by assaying the 17DD-YFV test vaccine and NaCl control solution spiked with 0.5 EU/ml LPS and 50μg/ml LTA (Table 2).

Interestingly, the intra-assay CV values for the control and test solutions spiked with LPS in the cryoWB/IL-6 assay were lower (NaCl = 2.3%; 17DD-YFV = 5.1–6.2%) than those for the cryoWB/IL-1β assay (NaCl = 17.5%; 17DD-YFV = 6.3–14.6%). However, the results obtained were consistent and acceptable, based on the MAT data available in the literature (25).

Inter-assay variability was also analysed, which showed that the CV values for the cytokine marker readouts induced by LPS in the control and test solutions on different days were lower in the case of cryoWB/IL-6 (NaCl = 4.2%; 17DD-YFV = 5.6–6.7%), as compared to cryoWB/IL-1β (NaCl = 22.4%; 17DD-YFV = 11.1–16.9%).

Generally, both the cryoWB/IL-6 and WB/IL-6 systems gave intra-assay and inter-assay CV values for LPS that were below those of cryoWB/IL-1β, as shown in Table 2. Thus, the results trend showed a lower intra-assay and inter-assay variability when IL-6 was used as the marker readout, as compared to IL-1β. This trend toward lower variability when using IL-6 as the marker readout was mirrored in the case of the LTA-spiked control and test solutions (Table 2).

Correlation between endotoxin detection in the MAT and in the LAL assay

A comparative analysis between the LAL assay and the MAT was performed, to demonstrate the correlation between the results from the two methods. This analysis was carried out according to the exogenous LPS recovery data for each assay. A statistical analysis showed a positive correlation between the LAL assay and MAT results with IL-1β and IL-6 as the marker readouts, with values of $r = 0.571$ and $r = 0.740$, respectively. Corroborating these analyses, no differences were observed between the two MAT readout systems (i.e. IL-6 and IL-1β) with regard to the number of false negatives obtained. False positive results were not considered, since the selection of all the samples was based on having negative LAL assay results.

Discussion

The Three Rs principles can potentially be applied in various areas of scientific research and testing (26). In the quality control of biologicals, reduction of animal use is a priority, as the demand for routine tests that check batch-to-batch consistency before marketing lead to the use of large numbers

Table 2: An overview of intra-assay and inter-assay variability of cytokine induction in fresh and cryopreserved blood in response to two classes of pyrogens

		Intra-assay variability (% CV)			Inter-assay variability (% CV)		
		CryoWB system		WB system	CryoWB system		WB system
Pyrogen stimulant	Sample	IL-1β	IL-6	IL-6	IL-1β	IL-6	IL-6
0.5EU/ml LPS	NaCl control solution	17.5	2.3	2.9	22.4	4.2	5.5
	17DD-YFV A	6.3	5.1	5.6	16.9	6.5	5.9
	17DD-YFV B	14.6	5.3	4.5	11.1	5.6	4.9
	17DD-YFV C	9.1	6.2	2.9	14.6	6.7	4.2
50μg/ml LTA	NaCl control solution	12.5	3.8	nd	15.0	3.9	nd
	17DD-YFV A	10.3	1.9	nd	12.3	3.4	nd
	17DD-YFV B	6.4	5.1	nd	11.4	2.5	nd
	17DD-YFV C	7.7	4.7	nd	13.6	6.6	nd

EU = endotoxin unit; LPS = lipopolysaccharide; LTA = lipoteichoic acid; nd = value not determined; cryoWB = cryopreserved whole blood; WB = fresh whole blood. The CV values are expressed as percentages. The data were obtained in quadruplicate.

of animals (27). Among the tests required for quality control purposes, regulators require a pyrogen detection test to be performed on products manufactured for human health (28).

Pyrogen detection in biological products remains a critical issue in production and quality control, and requires testing methods of extreme sensitivity. Although all bacteria have some association with the presence of endotoxins, the greatest sources are Gram-negative bacteria (29). Thus, from a regulatory point of view, the LAL assay has been regarded as the official substitute test for endotoxin detection whenever possible. The United States Pharmacopoeia states that: *The pyrogen test on rabbits may be used only if a product is incompatible with the LAL test* (30).

The LAL assay is accepted for the detection of endotoxins in vaccines against hepatitis A, *Haemophilus influenzae* type B, influenza, rabies, typhoid fever and yellow fever (31). However, the assay only detects endotoxin pyrogens (LPS), which means that NEPs in these products remain unassessed, the clinical importance of which is probably underestimated. Therefore, appropriate quality controls should be carried out to address this serious underreporting of NEP detection.

Advantages of the MAT

In addition to being a true animal *replacement* method according to the Three Rs principles, the MAT also serves as an opportunity to improve product quality control, and to contribute to the expansion of technology and scientific knowledge of biological assessments with human endpoints (32). In this context, the test has been used to evaluate human serum albumin (HSA) (33), hyperimmune sera (HS) (34), and bacterial vaccines, such as the *Shigella sonnei* vaccine (35) and the meningococcal OMV-based vaccine (36). Overall, it has ensured better consistency of batches, by providing more reliable data than the *in vivo* assay (37). In the current study, we showed that the MAT:

— is a suitable and reliable assay for determining the pyrogenic content in 17DD-YFV;

— correlates with the endotoxin assay (LAL) described in the yellow fever monograph (in the Brazilian and European Pharmacopoeias);

— is able to detect potential contamination with NEPs; and

— serves to enhance the safety of 17DD-YFV through better quality control, by confirming the absence of two classes of pyrogenic contaminants.

We have also shown that the MAT is not confounded by interactions with residual ovalbumin from the embryonated eggs used in the vaccine production, or by the influence of live virus on the cytokine marker readouts.

To the best of our knowledge, this is the first report of the MAT being used to test a viral vaccine. However, there is still much to learn about the test, especially with regard to its use in biological product testing. Biological products pose particular challenges, due to their complexity (e.g. in the case of YFV, it comprises attenuated virus grown in embryonated eggs) and high aggregated value (influenced by technology, demand, etc.), which leads to stricter product-specific validation requirements and reinforces the need for high-level technical and scientific data support.

Rationale for choosing IL-6 as a marker readout in the cryoWB system

We analysed the applicability of the MAT for detecting pyrogens in 17DD-YFV, with WB and cryoWB used as the source of monocytes, and IL-6 and IL-1β as the marker readouts. Various sources of monocytes have been previously validated by ICCVAM (15), and WB was seen as a good option when considering the time-to-sample processing and reproducibility of the assay (23, 38). However, we initially focused on the evaluation of the cryoWB system — a method that is best suited to an industrial setting, as obtaining fresh blood for assay purposes becomes a limiting factor.

When testing the biological product, 17DD-YFV, in the cryoWB system in this study, two marker readouts were chosen — IL-1β and IL-6. Only the cryoWB/IL-1β system has been validated internationally (14). The cryoWB/IL-6 system is another potential alternative system for use by industry, but this system has yet to be validated. We compared the cryoWB/IL-6 system with WB/IL-6, which has been validated internationally (23). As expected, all of the internationally-validated systems tested were able to detect LPS, as did the MAT cryoWB/IL-6 approach.

We opted to include IL-6 as a marker readout in our cryoWB model, because this cytokine had been previously evaluated in four of the five international validation studies on the MAT (14). Additionally, previous studies have shown that IL-6 was induced by minor amounts of pyrogen, as compared to those required to induce TNF-α and IL-1β (39). Corroborating these data, after the administration of endotoxin to humans, the concentration of IL-6 in peripheral blood was higher than that of TNF-α and IL-1β (40, 41) — indeed, the pyrogenic activity of TNF-α and IL-1β depends on the initial release of IL-6 (42). Interestingly, IL-6 is the main circulating endogenous cytokine in humans (and in experimental animals), and this

cytokine is the one that best correlates with pyrogenic activity in the blood (43).

Evaluation of the MAT for 17DD-YFV testing in the cryoWB/IL-1β and cryoWB/IL-6 systems

All the MAT systems evaluated in this study, when challenged with endotoxin standards, were successfully verified for their potential use in product-specific testing according to the criteria outlined in the European Pharmacopoeia (12). Based on these data, we believe that cryoWB is suitable for use in the MAT for assessing the presence of pyrogens in 17DD-YFV.

The interference test

The interference test results showed that it was possible to evaluate 17DD-YFV, with IL-1β and IL-6 as the marker readouts, by using a minimum vaccine preparation dilution of 1:10. Similarly, other complex biological products, such as HSA (33) and HS (34), have been found to be suitable for analysis by using the MAT. However, in the case of HSA, the apparent IL-1β response was lowered by glucan contamination in the less-diluted samples, whereas the IL-6 response was unaffected. In the case of HS, there was no evident interference associated with either readout (i.e. IL-6 or IL-1β).

Comparative analyses of LPS dose–response curves based on IL-1β and IL-6 levels as marker readouts

LPS spike recovery in 17DD-YFV was used to evaluate the MAT, with a specified acceptable range of 50–200%. We showed that LPS recovery was within the acceptable range for all batches, and with both cytokine readouts (i.e. cryoWB/IL-1β and cryoWB/IL-6). To verify the cryoWB/IL-6 results, three batches were analysed in parallel in the WB/IL-6 system. In both systems, the control samples were found to be pyrogen negative, and LPS-spike recovery was similar, which confirms the applicability of cryoWB/IL-6 system in the MAT.

Ability of the MAT to detect different pyrogen classes in 17DD-YFV

In the current study, we took advantage of the capacity of LTA to induce cytokine release by using it as an NEP stimulus in the MAT. LTA is a classic example of an NEP, in that it has a key role in the pathogenesis of sepsis (44), induces false negatives in the LAL assay, but is detected by the MAT and the RPT (25). It is an excellent control to demonstrate that possible contamination of biological products with NEPs can be overlooked by industry. As described in Hasiwa *et al.* (25), LTA is the key NEP for the detection of Gram-positive bacteria by human monocytes in the MAT, making it an ideal NEP reference material.

Prior to their use in this study, the commercial LTA preparations were assessed for potential LPS contamination by using the LAL assay. We identified residual LPS contamination in the preparations of LTA, when the samples were analysed without PBM pre-treatment, and the response to this residual LPS contamination was potentiated by the LTA. This observation is in accordance with the findings of Kim *et al.* (45), who showed that LTA pre-treatment had a priming effect on LPS-induced IL-6 cytokine production in a monocytic cell line. Importantly, however, the priming action of LTA on the LPS response was significantly reduced after PBM treatment.

Our data corroborate other reports in the literature, by showing that LTA is detectable in the MAT, and that it was possible to identify and quantify this contamination in LTA-spiked 17DD-YFV samples.

Correlation between endotoxin detection in the MAT and in the LAL assay

The results of the quantitative MAT analyses with cryoWB were comparable to those obtained with the LAL assay, as used in product quality control procedures. An acceptable correlation was observed between the two cytokine marker readouts used for assessing pyrogenic response with the MAT cryoWB system, when comparing the induction profiles of IL-1β and IL-6 in LPS-spiked NaCl and LPS-spiked 17DD-YFV (see earlier *Discussion* section). In fact, IL-6/IL-1β responses and LAL activity were detected at similar levels of endotoxin. As the induction of IL-6 and IL-1β in the cryoWB system showed a strong correlation, this confirmed the applicability of IL-6 as a marker readout with cryoWB in the MAT, an observation that corroborates the results of Silva *et al.* (34).

Intra-assay and inter-assay variability of pyrogen-induced IL-1β and IL-6 marker readouts in the MAT

In order to evaluate the variability of the two systems, the intra-assay and inter-assay CV values were calculated for the cryoWB/IL-6, cryoWB/IL-1β and WB/IL-6 systems, based on the LPS percentage recovery data. According to Schindler *et al.* (23), CV values should not exceed 45%, and values less than this figure were obtained for our data.

After induction by LPS-spiked NaCl and 17DD-YFV, the CV values of the cryoWB/IL-6 and WB/IL-6 data (in both the intra-assay and inter-assay analyses) were below the CV values of the cryoWB/IL-1β data. Hasiwa *et al.* (25) found that the intra-assay CV values obtained with the cryoWB system were less than 20%, for all of the cytokines evaluated in their study. This CV value of 20% is higher than any that were calculated from our assay data. Thus, based on the available literature, we consider that the variation found in our study is consistent with this test system, reinforcing the reliability of the data obtained. These results reflect the smaller variation observed with the IL-6 readout compared to the IL-1β readout with cryoWB and WB. A review of the literature has also endorsed IL-6 detection as the best option for pyrogen detection with the MAT, because it is secreted in large amounts in conditioned media, and thus permits better post-stimulation quantification, as compared with IL-1β and TNF (46).

Conclusions

The YFV monograph recommends testing only for endotoxin pyrogens in vaccine products. However, assays for the presence of other pyrogens are important, in order to exclude potential contamination with NEPs. Therefore, we investigated the ability of the MAT to detect NEPs in LTA-spiked 17DD-YFV. Our findings reinforced the view that possible NEP contaminants that would not be detected by the assay described in the YFV monograph (i.e. the endotoxin test), could be detected with the MAT. In this respect, no discrepancy was observed between the results obtained with the LAL assay and the MAT (Quantitative Method format), confirming the absence of endotoxins and NEP contamination in the tested commercial batches of 17DD-YFV.

Implementing new technological advances, such as the incorporation of the MAT as a safety test for the 17DD-YFV, would improve quality control and further guarantee vaccine safety. It would also open avenues of opportunity for testing other portfolio products from Bio-Manguinhos that currently rely on rabbits for the detection of pyrogens, thus making quality control more specific, accurate and efficient across the product range.

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