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Applicability of the Monocyte Activation Test (MAT) for hyperimmune sera in the routine of the quality control laboratory: Comparison with the Rabbit Pyrogen Test (RPT)

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ABSTRACT

Pyrogen tests are safety assays performed during the routine quality control of injectable products required by regulatory agencies. Currently, there are three available testing possibilities: 1) the Rabbit Pyrogen Test (RPT); 2) the Bacterial Endotoxin Test (BET); and 3) test systems using human whole-blood or monocytes, termed Monocyte Activation Test (MAT). Although BET is often considered as a replacement for the animal test, it is unable to detect pyrogens other than endotoxin. MAT is based on the human fever reaction and thus, most closely reflects the human response. The aim of this study was to conduct a parallel comparison of the RPT and MAT for hyperimmune sera (HS) batches analyzed during the routine of a quality control laboratory. MAT was performed in the same 43 batches of HS previously tested using RPT. The results showed that MAT presented 100% sensitivity and approximately 85% specificity as compared to RPT, i.e., no false-negative results were obtained. Few suspicious samples, which were negative in the RPT after retesting, provided divergent positive results suggesting a lower limit of detection of MAT. MAT is thus able to detect contaminants in biological products such as HS batches.

1. Introduction

Pyrogens comprise a chemically heterogeneous group of fever-inducing compounds derived from microorganisms, particularly bacteria, viruses, and fungi. This kind of microbial contamination is considered a serious public health problem and can result in symptoms ranging from vascular alterations to shock and death (Dinarello et al., 1984; Hoffmann et al., 2005). Pyrogenic tests are safety assays performed during the routine quality control of injectable products by regulatory agencies as well as during the manufacturing process (Dinarello et al., 1984; Daneshian et al., 2009). Currently, there are three available testing possibilities: 1) the Rabbit Pyrogen Test (RPT); 2) the Bacterial Endotoxin Test (BET), additionally known as the *Limulus* Amebocyte Lysate (LAL) test; and 3) the Monocyte Activation Test (MAT) (Hartung et al., 2001; Melandri et al., 2010; Perdomo-Morales et al., 2011; Hasiwa et al., 2013).

Several countries have published laws for regulating the use of animals during experimentation and education. These laws firmly call attention to the principle of the "3Rs" (replacement, reduction, and refinement) and strongly recommend the use of validated methods. When it is not possible to replace animals, ethical procedures must be applied during the handling and use of these animals (European Commission, 2010; Hartung, 2010). Taking into account that MAT is already validated, this method may be used as a replacement for RPT.

RPT, considered as the gold standard of pyrogenic tests, covers a broader range of substances and is able to detect endotoxin and non-endotoxin pyrogens (NEPs). However, this method is less sensitive than in vitro tests and results in the use of many rabbits (Hartung et al., 2001; Nakagawa et al., 2002). The LAL test is widely used as a simple and highly sensitive in vitro method for the detection of endotoxin; however, this test is associated with several inherent limitations and it has not been able to fully replace the rabbit test. This test fails to detect NEP, and consequently, other significant sources of pyrogen, which are undetectable, may present a risk for human health (Schindler et al., 2009).

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Moreover, certain products that are tested on rabbits, such as various biological substances and vaccines, cannot be tested using the LAL test (Schindler et al., 2009) since they can induce false-positive results. It was found that all human serum albumin (HSA) batches were contaminated with (1,3)-β-glucans, which interfere with the conventional LAL test (Perdomo-Morales et al., 2011).

Studies on the development of a method for replacing RPT, which began during the 1980s, have been based on isolated peripheral-blood mononuclear cells (PBMCs) and their cytokine release inducible by pyrogens (Poole et al., 1988). Subsequently, the whole-blood (WB) assay was developed, showing its effectiveness in detecting exogenous pyrogens other than endotoxin (Hartung and Wendel, 1996; Fennrich et al., 1999; Hartung et al., 2001; Schindler et al., 2009; Hasiwa et al., 2013).

Five test systems using human WB or monocytes, called Monocyte Activation Tests (MATs), have been internationally validated and may be a potential replacement for the RPT (Hoffmann et al., 2005). The MAT can be used to detect cytokines released by activated human monocytes or monocytic cells, such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα), which have important roles in fever pathogenesis. With the use of cryopreserved human WB by the introduction of a standardized freezing procedure using dimethyl sulfoxide (DMSO) as a cryoprotective agent, the MAT became more standardized and more widely available, and it was demonstrated that it can be used as a tool to study details of the fever reaction pathway in the innate human immune response (Schindler et al., 2004; Schindler et al., 2006).

Despite the number of scientific studies on the MAT (Hasiwa et al., 2013), data produced by the validation study were not sufficient to support the broader application of MAT, such as its use for a wide variety of products and its capability to detect NEP-mediated pyrogenicity (ICCVAM, 2008).

The ESAC statement 'On the Validity of *In-Vitro* Pyrogen Tests of 2006' (ECVAM, 2006) endorsed MATs that were included in the European Pharmacopeia during 2010 (EDQM, 2010) as a third method to detect endotoxin contamination in human parenteral drugs on a case-by-case basis, subject to validation for each specific product to demonstrate equivalence to RPT (ICCVAM, 2008). For the majority of biological products, the Brazilian Pharmacopeia still recommends the RPT as a mandatory toxicological assay within routine quality control (Presgrave, 2003; Melandri et al., 2010).

Some reports on biological products have indicated that although these products passed the RPT and LAL test, they resulted in pyrogenic episodes in patients, indicating that these tests do not always provide sufficient safety support (Perdomo-Morales et al., 2011; Hasiwa et al., 2013). Therefore, the development of MAT using human blood provided a convenient and validated *in vitro* alternative for the testing of biological products. The US Interagency Coordinating Committee for the Validation of Alternative Test Methods (ICCVAM) declared that "the MAT is suitable after a product-specific validation as a replacement for the Rabbit Pyrogen Test" (ICCVAM, 2008); therefore, it is necessary to compare the MAT and RPT for biological products.

Hyperimmune sera (HS) are an important class of biological products subject to health surveillance. Many of them, such as antivenom sera, are used on a large scale for the treatment for poisoning by snakebite, scorpions, spiders, and centipedes. Other kinds of HS include those used in the treatment of patients with diseases caused by microorganisms (bacteria or viruses), such as antitetanus (ATS) and antirabies (ARS) sera. All HS produced in Brazil are analyzed by the National Institute of Quality Control in Health (INCQS) before being distributed to Vaccination Centers as a part of the National Immunization Program. These analyzed HS are mainly antibothropic (ABS), ARS, anticrotalic (ACS), and ATS, and these types represent >90% of the products tested by the RPT. The aim of this study was to carry out a parallel comparison of RPT and MAT for HS batches analyzed during the routine of a quality control laboratory.

2. Animals, materials, and methods

2.1. Samples

Forty-three batches of HS were selected from samples tested during routine of the quality control. All 18 samples that needed to be repeated were used for the current study and 25 nonpyrogenic (NP) samples were randomly selected for RPT. The HS analyzed from two important manufacturers (A and B) included the following: ABS, anti-bothropic-lachetic serum (ABLS), ACS, ATS, anti-elapidic serum (AELS), anti-escorpionic serum (AES), and ARS. The MAT was performed on the same batches of HS as those previously tested during the RPT.

2.2. Rabbit Pyrogen Test

No animal was used solely for the purpose of the current study, since they were additionally used during the routine assay for detection of pyrogenic contamination of injectable products. For the current study, data from samples represent the results of 165 rabbits provided by the Laboratory Animals Breeding Center (Cecal) from the Oswaldo Cruz Foundation (Fiocruz) and used for analyses by the INCQS. The RPT was performed in healthy male New Zealand white rabbits weighing ≥1.5 kg. All animal experimentations were carried out in accordance with approved Institutional protocols by the Ethics Committee on Animal Use (CEUA) from Fiocruz and the recommendations outlined in the Brazilian Pharmacopeia that follow the same directions of the United States Pharmacopeia. Animals were kept in individual cages, in ventilated racks, with room temperature 20 ± 2 °C and 50%–70% humidity, and received autoclaved hay as environmental enrichment. HS samples were injected (1 mL/kg of body weight) into a marginal ear vein of each of three rabbits. After the injection, the rectal temperatures of the animals were recorded using a PyroMon® system (Ellab, Hillrod, Denmark) over a 3-hour period. When none of the rabbits showed a temperature increase of ≥0.5 °C from their basal temperature, the sample tested was classified as NP. When only one rabbit showed a temperature increase of ≥0.5 °C, the test was repeated (R) using five different rabbits. If no more than three of the eight rabbits tested showed an individual temperature increase of ≥0.5 °C and if the sum of the temperature increases for the eight rabbits did not exceed 3.3 °C, the sample tested was classified as NP. Otherwise, the sample was classified as pyrogenic (P).

2.3. Monocyte Activation Test

2.3.1. Immune stimuli

The standard endotoxin stock solution was prepared from lipopolysaccharide (LPS) of *Escherichia coli* O55:B5 (Sigma–Aldrich, Steinheim, Germany) diluted in pyrogenic-free 0.9% sodium chloride solution (Halex Istar, Goiânia, Brazil). We used endotoxin equivalents units (EEU) as values for the contaminant concentration, as read off the standard endotoxin dose–response curve range from 0.125 to 5 EU/mL (method A) or estimated by comparison with responses to standard endotoxin solution (method B). For both methods, the endotoxin limit of 5 EU/mL was used as the pass/fail criterion for the MAT, which is the limit of the LPS concentration taken as the lowest dose that produces a fever in rabbits and humans (Hochstein et al., 1990). This limit was used since we can inject up to 5 EU/kg/10 mL for each animal. In the case of HS, the animal dose is 1 mL/kg, so, taking into account the human threshold as 5 EU/kg, HS dose for animals was taken as 5 EU/mL/kg.

2.3.2. Determination of the maximum valid dilution

The maximum valid dilution (MVD) was calculated for ABS by multiplying the endotoxin limit concentration (ELC) by the concentration of the test solution (C) and dividing this by the limit of detection (LOD). The ELC was calculated as K/M, where K is the threshold pyrogenic dose of endotoxin per kilogram of body mass per day (5 EEU/kg/24 h)

and M is the daily maximum recommended bolus dose of product per kg body mass. The maximum dose of ABS is 120 mL/70 kg/24 h. The ELC for ABS was found to be 2.9 EEU/mL.

2.3.3. Determination of the limit of detection

LOD was determined using the endotoxin standard curve (0.125–5 EEU/mL) by determining the concentration of endotoxin corresponding to the cut-off value. The cut-off value was expressed as an optical density (OD) and it was calculated using the mean of the four replicates for the responses to the blank plus three-fold the standard deviation (SD) of the four replicates of the responses to the blank.

2.3.4. Test for interferences

Three batches of pyrogenic-free ABS (manufacturer A, Brazil) were used. The test was conducted by incubating diluted WB with geometric sample dilutions spiked with 0.5 EEU/mL and assayed in parallel with the corresponding unspiked dilution. Dilutions with endotoxin recovery within the 50–200% range were considered interference-free, and the first dilution was defined as the minimum valid dilution (MinVD). The data are expressed as mean ± SD of four replicates.

2.3.5. Blood samples

Heparinized blood samples were collected from four healthy donors. Differential blood-cell counts were routinely performed with a cell counter (Hemogram 60 BioClin) to exclude donors with acute infection. Under sterile conditions, endotoxin-free Sorënsen's phosphate buffer (Acila GMNmbh, Mörfelden-Walldorf, Germany) was mixed with 20% (*v*/*v*) endotoxin-free DMSO (Wak Chemie Medical GmbH, Steinbach, Germany), and this solution was mixed with fresh blood (1:2) as described by Schindler et al. (2006). The blood from the four volunteers was aliquoted in pre-cooled cryotubes (Eppendorf, Hamburg, Germany) and directly frozen at −80 °C. Thawed blood samples were pooled at the time of testing. The same pooled cryopreserved blood was used for all experiments.

2.3.6. Whole blood incubation

Human WB incubations were performed as described previously in European Pharmacopeia (EDQM, 2010). Thawed, heparinized cryopreserved WB was diluted with 900 μL saline and stimulated with 100 μL of LPS in polypropylene vials (Eppendorf, Hamburg, Germany). After incubation for 16 h at 37 °C in a humidified atmosphere with 5% CO_2, the vials were closed and stored at −20 °C until cytokine measurement.

2.3.7. Cytokine measurement

Commercially available IL-1β and IL-6 kits (R&D Systems, Wiesbaden, Germany) with a sensitivity range of 3.9–250 pg/mL for IL-1β, and 12–300 pg/mL for IL-6 were used. The data are presented as mean ± standard error of the mean (SEM) of four replicates.

2.3.8. Statistical analyses

The results obtained in the current study were analyzed by performance comparisons between the *in vitro* (MAT) and the *in vivo* tests (RPT), derived from the contingency table. The following variables were calculated: sensitivity (the ratio of *in vivo* pyrogenic classified *in vitro* as pyrogenic); specificity (the ratio of *in vivo* NP classified *in vitro* as NP); accuracy (the ratio of product classes [pyrogenic and NP] correctly classified *in vitro*); false-positives and false-negatives.

3. Results

The interference test for ABS in the WB-MAT was conducted in clean samples of four different batches. The quantification range in the MAT was up to 0.125 EEU/mL LPS and the spikes were 0.5 EEU/mL. For ABS, it was possible to quantify IL-1 and IL-6 recovery to LPS between 50% and 200% in almost all diluted samples, except a 1:5 dilution (Table 1). Therefore, we chose the MinVD 1:10 for the current study.

Table 2 summarizes the relationship between the results of the HS samples (1:10 diluted) performed in RPT and MAT with IL-1β readout. Batches 1 to 25 passed the RPT, 26 to 40 passed the RPT after the retest, and batches 41 to 43 failed the RPT according to the Brazilian Pharmacopeia guidelines, showing pyrogenic contamination. When the results of RPT were compared to those of MAT, we observed that batches 36 to 43 clearly failed MAT during the application of both methods, i.e., these batches showed pyrogenic contamination. However, two batches, 34 and 35, failed MAT only for method B, although in method A, the endotoxin equivalent concentration was very close to the limit dose. This difference between the methods is likely due to the adjustment of the dose–response curve in method A, which involves a comparison of the batches being examined with a standard endotoxin dose–response curve. This way, depending on the slope, the endotoxin equivalent concentration can be modified.

To understand the differences found between methods A and B for the HS routine, five batches of ABS were artificially spiked with endotoxin at the limit dose (5 EEU/mL) and analyzed for methods A and B for IL-1β and IL-6 as readouts (Table 3). When the results were evaluated by the IL-1β readouts, batches A and E passed the MAT using method A and failed using method B, thereby ratifying the results summarized in Table 1. For the IL-6 readout, only batch A passed the MAT using method A, and it failed using method B. This comparison between the methods should be further investigated in future studies since there is little data in the literature comparing the two methods for the same sets of any injectable products.

3.1. Predictive abilities

Tables 4 and 5 show the concordance and discordance frequency observations between the *in vivo* and *in vitro* assays. We observed differences between the two methods (A and B) with regard to the number of false negatives, i.e., products classified as NP *in vivo* and pyrogenic *in vitro*. Among the 43 products, only five were considered false positives for method A and seven for method B. This result could have occurred due the higher sensitivity of the MAT than the RPT to detect pyrogen contamination. The differences between methods A and B could be due to adjusting the endotoxin standard curve (method A) determining the concentration of endotoxin corresponding to the OD, whereas method B is a yes-or-no type. The implications of these methods are that if the sample response is below the positive control response, it is considered NP, whereas when the sample response is equal to or higher than the positive control, it is considered pyrogenic.

We found 100% sensitivity for both methods A and B (Table 6). Therefore, MAT was able to detect all cases of products considered pyrogenic in the RPT. Due to the differences observed between methods A and B regarding false-positive results, we found 87,5% and 82,5% specificity, respectively. Additionally, we analyzed the accuracy, which refers to the degree of agreement between the results from the diagnostic test

Table 1
Endotoxin recoveries (%) obtained in the interference test with anti-bothropic serum in whole-blood monocyte-activation test using IL-1β and IL-6 as readouts.

Readout	Batches	Dilutions							
		0	1/5	1/10	1/20	1/40	1/80	1/160	1/320
IL-1β	A	36	47	199	132	112	105	111	74
	B	5	56	103	149	100	152	161	147
	C	6	115	119	115	134	105	110	146
	D	20	51	181	110	129	176	111	113
IL-6	A	10	60	100	170	137	78	149	178
	B	8	78	167	≥200	130	90	159	184
	C	1	65	99	≥200	≥200	169	134	176
	D	1	59	132	≥200	137	179	149	178

Recovery was calculated using the mean value of endotoxin equivalent concentrations (EEU/mL) of the spiked sample. Anti-bothropic serum (ABS) at 0.5 EEU/mL (S) and the un-spiked samples (US) were calculated as follows: % recovery = ((S − US) / 0.5) × 100.

Table 2
Comparative results of pyrogen evaluation of hyperimmune sera (HS) by Monocyte Activation Test (MAT) and Rabbit Pyrogen Test (RPT).

Manufacturer	Batches	Samples	Individual temperature variation	Animals[a] ≥0.5 °C	RPT (Σ ΔT)[b] (°C)	MAT (EEU/mL)[c]	MAT[d]
A	1	ATS	0.20; 0.20; 0.00	0	0.4^{NP}	0.31 ± 0.15^{NP}	NP
A	2	ABS	0.30; 0.00; 0.00	0	0.3^{NP}	0.16 ± 0.03^{NP}	NP
A	3	ABS	0.00; 0.20; 0.20	0	0.4^{NP}	0.16 ± 0.12^{NP}	NP
A	4	ABS	0.00; 0.10; 0.00	0	0.1^{NP}	0.18 ± 0.04^{NP}	NP
A	5	ABS	0.30; 0.20; 0.20	0	0.7^{NP}	0.43 ± 0.2^{NP}	NP
A	6	ABS	0.00; 0.20;0.20	0	0.4^{NP}	0.82 ± 0.01^{NP}	NP
B	7	ABS	0.00; 0.10; 0.20	0	0.3^{NP}	2.85 ± 0.10^{NP}	NP
B	8	ABS	0.10; 0.00; 0.00	0	0.1^{NP}	0.16 ± 0.80^{NP}	NP
B	9	ABS	0.20; 0.20; 0.00	0	0.4^{NP}	0.31 ± 0.05^{NP}	NP
B	10	ABS	0.10.0.10.0.20	0	0.4^{NP}	0.18 ± 0.02^{NP}	NP
B	11	ABS	0.00; 0.00; 0.00	0	0^{NP}	0.82 ± 0.01^{NP}	NP
A	12	ABS	0.40; 0.20; 0.20	0	0.3^{NP}	0.25 ± 0.01^{NP}	NP
B	13	ABS	0.10; 0.10; 0.20	0	0.4^{NP}	0.02 ± 0.08^{NP}	NP
A	14	ABS	0.00 0.00; 0.00	0	0^{NP}	0.82 ± 0.01^{NP}	NP
B	15	ABS	0.00; 0.00; 0.10	0	0.1^{NP}	0.8 ± 0.01^{NP}	NP
B	16	ABS	0.00; 0.00; 0.00	0	0^{NP}	0.05 ± 0.02^{NP}	NP
A	17	ABS	0.00; 0.00; 0.20	0	0.2^{NP}	0.85 ± 0.012^{NP}	NP
B	18	ABS	0.00; 0.40; 0.00	0	0.4^{NP}	0.15 ± 0.03^{NP}	NP
B	19	ARS	0.20; 0.00; 0.00	0	0.2^{NP}	0.55 ± 0.04^{NP}	NP
B	20	ABS	0.00; 0.10; 0.00	0	0.1^{NP}	0.49 ± 0.01^{NP}	NP
B	21	ACS	0.00; 0.10; 0.10	0	0.2^{NP}	0.05 ± 0.02^{NP}	NP
B	22	ABLS	0.00; 0.30; 0.10	0	0.4^{NP}	0.64 ± 0.01^{NP}	NP
B	23	ABS	0.00; 0.20; 0.20	0	0.4^{NP}	0.12 ± 0.02^{NP}	NP
B	24	ARS	0.00; 0.00; 0.00	0	0.1^{NP}	0.15 ± 0.02^{NP}	NP
B	25	ARS	0.10; 0.00; 0.00	0	0.1^{NP}	0.55 ± 0.43^{NP}	NP
B	26	ACS	0.50; 0.10; 0.00; 0.20; 0.00; 0.20; 0.00; 0.10	1	$1.1^{R/NP}$	0.18 ± 0.09^{NP}	NP
B	27	ABS	0.60; 0.30; 0.30; 0.10; 0.40; 0.60; 0.20; 0.00	2	$2.5^{R/NP}$	0.59 ± 0.08^{NP}	NP
B	28	ABLS	0.60; 0.20; 0.40; 0.50; 0.50; 0.30; 0.20; 0.30	3	$3^{R/NP}$	0.19 ± 0.03^{NP}	NP
B	29	ARS	0.50; 0.30; 0.40; 0.10; 0.10; 0.20; 0.20; 0.30	1	$2.1^{R/NP}$	2.34 ± 0.06^{NP}	NP
B	30	ABLS	0.20; 0.10; 0.60; 0.10; 0.10; 0.20; 0.30; 0.40	1	$2^{R/NP}$	2.34 ± 0.05^{NP}	NP
B	31	ABLS	0.30; 0.20; 0.60; 0.00; 0.10; 0.20; 0.30; 0.30	1	$2^{R/NP}$	3.08 ± 0.05^{NP}	NP
A	32	AELS	0.50; 0.20; 0.10; 0.10; 0.10; 0.30; 0.00; 0.10	1	$1.5^{R/NP}$	2.66 ± 0.02^{NP}	NP
A	33	ABS	0.00; 0.60; 0.00; 0.20; 0.40; 0.10; 0.20; 0.20	1	$1.7^{R/NP}$	0.12 ± 0.05^{NP}	NP
B	34	ABLS	0.30; 0.50; 0.10; 0.20; 0.50; 0.30; 0.30; 0.50	3	$2.7^{R/NP}$	4.65 ± 0.37^{NP}	**P**
B	35	ABS	0.40; 0.40; 0.50; 0.00; 0.10; 0.00; 0.10; 0.00	1	$1.5^{R/NP}$	4.53 ± 0.20^{NP}	**P**
B	36	ATS	0.80; 0.10; 0.50; 0.30; 0.00; 0.10; 0.20; 0.60	3	$2.6^{R/NP}$	$\mathbf{5.47 \pm 0.34^{P}}$	**P**
B	37	ABS	0.10; 0.50; 0.10; 0.10; 0.10; 0; 0; 0.40	1	$1.3^{R/NP}$	$\mathbf{5.92 \pm 0.51^{P}}$	**P**
A	38	ARS	0.30;0.10; 0.60; 0.10; 0.15; 0.00; 0.10; 0.00	1	$1.35^{R/NP}$	$\mathbf{5.3 \pm 0.08^{P}}$	**P**
A	39	AES	0.35; 0.00; 0.50; 0.20; 0.45; 0.05; 0.10; 0.2	1	$1.85^{R/NP}$	$\mathbf{5.8 \pm 0.32^{P}}$	**P**
A	40	AES	0.35; 0.00; 0.50; 0.20; 0.45; 0.05; 0.10; 0.3	1	$1.85^{R/NP}$	$\mathbf{5.4 \pm 0.23^{P}}$	**P**
A	41	ABLS	0.30; 0.40; 0.70; 0.50; 0.50; 0.40; 0.50;0.60	6	$\mathbf{3.9^{R/P}}$	$\mathbf{6.22 \pm 0.46^{P}}$	**P**
A	42	ABLS	0.30; 0.70; 0.50; 0.30; 0.50; 0.60; 0.40;0.40	4	$\mathbf{3.7^{R/P}}$	$\mathbf{6.08 \pm 0.44^{P}}$	**P**
A	43	ARS	0.75; 0.30; 1.05; 0.30; 0.40; 0.30; 0.40; 0.65	3	$\mathbf{4.15^{R/P}}$	$\mathbf{7.2 \pm 0.95^{P}}$	**P**

NP = nonpyrogenic; R/NP = repeat/nonpyrogenic; R/P = repeat/pyrogenic; P = pyrogenic. Antitetanic serum (ATS); antibothropic serum (ABS); anti-bothropic-lachetic serum (ABLS), anticrotalic serum (ACS); antitetanic serum (ATS); anti-elapidic serum (AELS); anti-escorpionic serum (AES); and antirabies serum (ARS).

[a] Pharmacopeia Criteria for the number of animals: ≤3 (NP), ≥4 (P).

[b] Pharmacopeia criteria: Sum (∑) of the individual variation of temperature (VIT): ≤3.3 °C (NP); >3.3 °C (P).

[c] Method A. Values are means of three replicates with standard deviation (SD).

[d] Method B. It was considered that 5 EEU/mL was a positive control endotoxin.

under study and those from a reference test. The concordance between the MAT and the RPT was 88,3% and 83,7% for methods A and B, respectively.

Table 3
Comparative results of pyrogen evaluation of hyperimmune sera (HS) by the Monocyte Activation Test (MAT) in methods A and B.

Batch	IL-1β		IL-6	
	Method A EEU/mL	Method B	Method A EEU/mL	Method B
A	4.136 ± 0.28^{NP}	P	4.904 ± 0.035	P
B	5.373 ± 0.25^{P}	P	$5.934 \pm \mathbf{0.07^{P}}$	P
C	5.264 ± 0.16^{P}	P	5.254 ± 0.127^{P}	P
D	5.423 ± 0.31^{P}	P	5.654 ± 0.043^{P}	P
E	4.956 ± 0.13^{NP}	P	5.823 ± 0.021^{P}	P

Method A: The values are the means and standard deviation of three replicates. Method B: It had been considered as a positive control 5 EU/mL of endotoxin. NP, nonpyrogenic; P, pyrogenic.

4. Discussion

Biologicals are an important class of products evaluated during the routine the quality control in the laboratory and are responsible for the use of a great number of animals during RPT. Directive 2010/63/

Table 4
Contingency table using the method A whole-blood Monocyte Activation Test (WB-MAT) values.

		In vivo classification	
		Pyrogenic	Nonpyrogenic
In vitro classification	Pyrogenic	3 (a)	5 (b)
	Nonpyrogenic	0 (c)	35 (d)

a = the number of samples for which the Rabbit Pyrogen Test (RPT) and MAT were positive (true positive); b = the number of samples for which the RPT was negative and the MAT was positive (false positive); c = the number of samples for which the RPT was positive and the MAT was negative (false negative); d = the number of samples for which the RPT and the MAT were negative (true negative).

Table 5
Contingency table using the method B whole-blood Monocyte Activation Test (WB-MAT) values.

		In vivo classification	
		Pyrogenic	Nonpyrogenic
In vitro classification	Pyrogenic	3 (a)	7 (b)
	Nonpyrogenic	0 (c)	33 (d)

a = the number of samples for which the Rabbit Pyrogen Test (RPT) and MAT were positive (true positive); b = the number of samples for which the RPT was negative and the MAT was positive (false positive); c = the number of samples for which the RPT was positive and the MAT was negative (false negative); d = the number of samples for which the RPT and MAT were negative (true negative).

EU is firmly based on the principle of the Three Rs and enforces the replacement of animal tests when validated alternatives exist (European Commission, 2010). The RPT has to be evaluated with regard to the Three Rs, since for HS, none of the alternative methods are considered as a full substitute for rabbits because it is not recommended to re-use animals due to the possibility of cross-reaction (Williams, 2007). In this case, Freitas et al. (2011) proposed a reduction of the number of rabbits used in RPT by their re-use up to four times in one week without interfering in the reaction, while the RPT has to be considered as a mild pain test. Directive 2010/63/EU (article 16) imposes restrictions on animal re-use, which were considered to limit research because the least pain, suffering, distress, or lasting harm and which are most likely to provide satisfactory results shall be selected. Although the RPT can be considered as a mild pain test and the re-use contributes to the reduction of the use of animals during RPT for HS quality control, quite a number of rabbits continue to be used.

When evaluating the use of LAL test in HS quality control, Fingola et al. (2013) proposed the use of the kinetic chromogenic LAL (C-LAL) assay for the determination of bacterial endotoxin in samples of ABS. However, the LAL test is not considered a substitute for RPT since it is not able to detect other kinds of pyrogens. Perdomo-Morales et al. (2011) indicated that despite the LAL assay being implemented for pyrogen testing of some biological products, they encountered problems with endotoxin recovery in the interference test with C-LAL that invalidate the use of LAL test as an end-product endotoxin test for HSA. Failure to recover endotoxin from biological products with LAL test has been documented in previous studies (Hochstein et al., 1979; Jürgens et al., 2002; Perdomo-Morales et al., 2011).

The MAT was established as a true alternative for pyrogen testing, detecting LPS, NEP, and mixtures. This test mimics the human fever reaction and reflects the reactions of the human innate immune system more accurately than the other two methods (RPT and LAL test), and thus allows a more realistic prediction of the pyrogenic activity. Our results showed that MAT already demonstrated its effectiveness and sensitivity in detecting pyrogens for HS batches analyzed during the quality control routine, thereby providing a real-life picture of the performance of MAT.

Any decision regarding specific criteria for acceptable levels of sensitivity and specificity in a given situation involves weighing the consequences of leaving samples undetected (false negatives) against "erroneously" classifying satisfactory samples as pyrogenic (false positives). Regarding these factors, there were no false-negative results. The positive findings for some negative RPT assays demonstrates that the MAT is capable of detecting some limit endotoxin concentration that the test in rabbits cannot detect due to biological variation.

Table 6
Predictability of the Monocyte Activation Test (MAT) for 43 products based on methods A and B.

Parameter	MAT values (%)	
	Method A	Method B
Sensitivity	100	100
Specificity	87.5	82.5
Accuracy	88.3	83.7
False negatives	0	0
False positives	11.6	16.3

One of the problems in comparing *in vivo* and *in vitro* tests was related to biological intrinsic variation of the temperature of the animals. In the first test using three animals, when the RPT showed positive or negative results, the MAT showed the same profile. However, when the RPT showed suspicious results and it was necessary to repeat the test, MAT could show discordant results, probably due to the biological variation of the rabbits. Perdomo-Morales et al. (2011) have supported the replacement of the RPT with MAT as a final release test for pyrogens in HSA, and probably additionally in other blood-derived products and therapeutic proteins. In our study, the results showed that MAT has good sensitivity and specificity and is able to detect contaminants in biological products such as HS batches.

Recently, some authors have suggested that MAT is not widely used due to the need for a large volume of blood in this test, even if it is cryopreserved. Koryakina et al. (2014) have developed and qualified a procedure to prepare functional monocytes as cryopreserved PBMCs from the leukocyte filters. These filters are used in the separation of blood in blood donation centers and are normally treated as biological waste. Solati et al. (2015) additionally used the MAT, using frozen pooled human MNC, and showed that it is a highly sensitive, specific, and reproducible pyrogen test, able to detect and quantify endotoxin and non-endotoxin pyrogenic contaminants in parenteral pharmaceuticals.

An additional study has suggested detection of endotoxin and non-endotoxin pyrogens using a bovine whole-blood assay; however, they stated that "further efforts are indispensable to improve the method's functionality, detection limits, and robustness, as well as to verify whether it can detect further pyrogens including lipopeptides" (Wunderlich et al., 2014; Wunderlich et al., 2015). Animal experimentation is criticized not only for ethical reasons, but also because the animal model may not correspond exactly to the human response. However, in methods that use human cells, such as the MAT, a human response is produced and the problem of the extrapolation of data between species is avoided.

The results of the current study are very important since we demonstrated the applicability of the MAT to HS, which was not an object of its validation (Directive 2010/63/EU). This fact may allow the MAT to replace the use of rabbits, as in contrast what was initially thought, since the official acceptance of the MAT, the number of animals used for pyrogen testing did not fall, but increased by approximately 10,000 to a total of 170,000 (Hartung, 2015). Brazil does not as yet have an official count of experimental animals used. However, as an example, the Brazilian INCQS uses approximately 600 rabbits per year only for routine pyrogen testing, whereas HS represents approximately 90% of samples tested (Caldeira, 2015).

5. Conclusions

The current study showed that MAT presented the same results as RPT when RPT was definitively negative or positive. In cases where RPT result was suspicious (required a repeated test with five different rabbits), MAT could present a different result, and a false negative result never occurred with MAT. These findings show that the MAT is more sensitive than RPT and may detect pyrogenicity earlier than RPT. Taking into account the safety for users, a false positive is preferable to a false negative. Besides, in this case, MAT may not be understood as giving false positive responses, since rabbits have already shown that there was an amount of pyrogenic substance that led to a repeat of the test. However, that was not sufficient to be detected as a fever response, possibly due to biological variation. These results show that MAT may replace the use of rabbits in pyrogen tests of HS, providing information for this specific product that was previously lacking in the literature.

Conflict of interest statement

Thomas Hartung, a co-investigator in this study, holds patents as inventor of the whole-blood pyrogen test and the use of cryopreserved blood, which are licensed to Merck-Millipore; he receives royalties from Merck-Millipore from sales of the kit version.

Transparency document

The Transparency document associated with this article can be found, in online version.

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