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# Evaluation of recombinant factor C assay for the detection of divergent lipopolysaccharide structural species and comparison with *Limulus* amoebocyte lysate-based assays and a human monocyte activity assay

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## Abstract

**Purpose.** The *Limulus* amoebocyte lysate (LAL) assay is widely used for the screening of lipopolysaccharide (LPS) in parenteral pharmaceuticals. However, correlation of LPS in Gram-negative bacterial infections by LAL assay has been problematic, partly due to the variable reactivity of different LPS structures. Recombinant factor C (rFC) has allowed the development of a new simple, specific and sensitive LPS detection system (PyroGene). In this work, the potential of the new assay for detecting various LPS structures has been investigated and compared with two LAL-based assays and a human monocyte activity assay.

**Methodology.** The activity of the various LPS structures has been investigated by PyroGene and two LAL-based assays and a human monocyte activity assay.

**Results.** The rFC assay detected most LPS structures in picogram quantities and the potency of *E. coli*, *B. cepacia*, *Salmonella* smooth and *Salmonella* R345 LPS was no different when measured with PyroGene or LAL assays. However, the reactivity of *K. pneumoniae*, *S. marcescens*, *B. pertussis* and *P. aeruginosa* LPS differed significantly between these assays. Importantly, pairwise correlation analysis revealed that only the PyroGene assay produced a significant positive correlation with the release of IL-6 from a monocytic cell line.

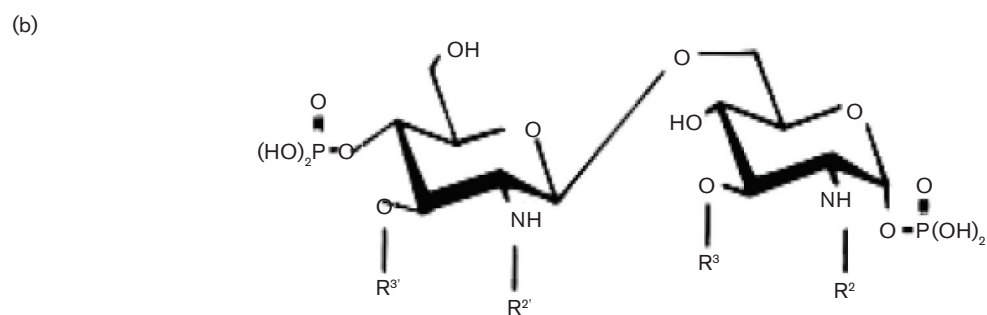
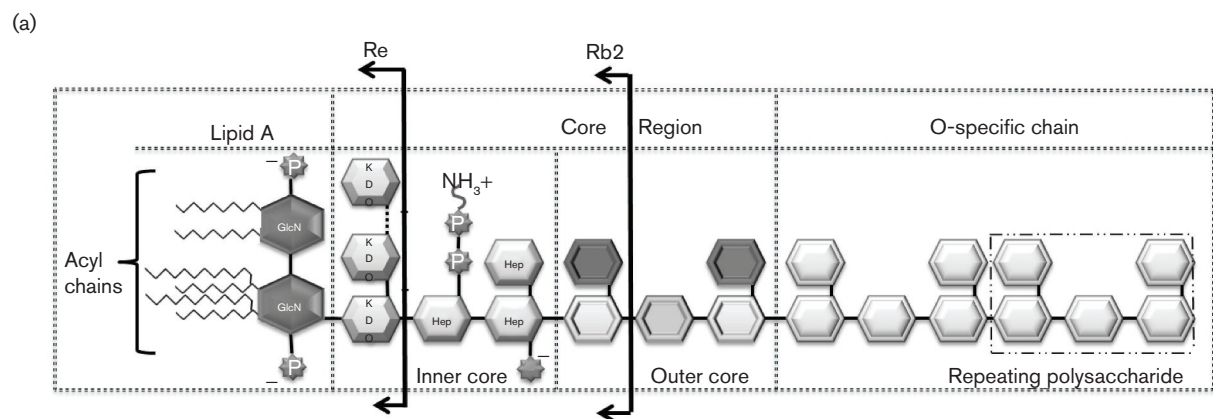
**Conclusion.** We conclude that the rFC-based assay is a good replacement for conventional LAL assays and as it correlates significantly with IL-6 produced by a human monocyte cell line it could potentially be more useful for detecting LPS in a clinical setting.

## INTRODUCTION

Parenteral pharmaceutical products and medical devices such as pacemakers, catheters and other invasive devices are required to be free from pyrogenic contamination [1], as potentially life-threatening septic shock could be elicited if pyrogens are administered inadvertently to the human body [2]. Bacterial endotoxin (lipopolysaccharide; LPS) is the pyrogen of main concern to the pharmaceutical and medical industries because it is the most common contaminant and is stable and shows high pyrogenicity [3–5].

LPS, which is a complex glycolipid embedded within the outer membrane of Gram-negative bacteria, is ubiquitous and shows manifold biological activities [6]. It comprises a

conserved acylated disaccharide, known as lipid A, which is attached to a core oligosaccharide moiety (Fig. 1a). The core region is further extended by additional glycosylation to give the O-specific antigen [7]. The nature and number of sugars, as well as the number of repeat units within the O-polysaccharide region, determine the serotype specificity of each bacterial strain within a species. The biologically active part of LPS, lipid A, also shows structural diversity due to the pattern of substitution of the two phosphate groups on lipid A, the type of fatty acids and the degree of acylation [7, 8]. Lipid A from *E. coli* LPS typically has a hexa-acyl structure (similar to the one shown in Fig. 1a), while LPS from different Gram-negative bacteria may have different numbers and arrangements of acyl chains (Fig. 1b) [9].



Bacterial species	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	References
<i>Salmonella minnesota</i>	C14-O-(C14)	C14-O-(C12)	C14-OH	C14-O-(C16)	[51]
<i>E. coli</i>	C14-O-(C14)	C14-O-(C12)	C14-OH	C14-OH	[52]
<i>F. tularensis</i>	H (with one P group)	C18-O-(C16)	C18-OH	C18-OH	[35]
<i>S. marcescens</i>	C14-O-(C14)	C14-O-(C14)	H	C14-OH	[53]
<i>B. pertussis</i>	C14-OH	C14-O-(C14)	C10-OH	C14-OH	[7]
<i>K. pneumoniae</i>	C10-O-(C12)	C10-O-(C12)	C10-OH	C10-OH	[52]
<i>P. aeruginosa</i>	C10-OH	C12-O-(C12)	C10-OH	C12-OH	[54]
<i>B. cepacia</i>	C14-OH	C14-O-(C16)	C14-OH	C16-OH	[55]

**Fig. 1.** (a) Schematic diagram of lipopolysaccharide from *Salmonella minnesota* showing the main structural components. The regions with truncation of the core sugars in the rough mutants Rb2 (*S. minnesota* R345) and Re (*S. minnesota* R595) are shown. GlcN, glucosamine; Kdo, '2-keto-3-deoxyoctulosonic acid' (3-deoxy-D-manno-octulosonic acid); Hep, D-glycero-D-manno-heptose. Adapted from Alexander and Rietschel [51]. (b) Lipid A structures of the LPS from the different Gram-negative bacteria used in this study. The table shows the number, length and type of acyl chains for each lipid A species.

LPS is an important inflammatory molecule that has been shown to induce responses in many different cell types [8]. Humans are extremely sensitive to endotoxin and nanomolar quantities of LPS are sufficient to induce an acute fever response [10] and sepsis [11]. The extremely high potency of LPS in affecting biological systems, along with its

ubiquity and stability, have required the development of a highly reliable, sensitive and quantitative test for the pharmaceutical and medical industries [12].

Currently, the rabbit pyrogen test and the bacterial endotoxin test (BET), often referred to as the *Limulus* amoebocyte lysate (LAL) test, are prescribed by health authorities and

pharmacopoeias to test parenteral medicinal products. The adoption of the Russell and Burch 3R concept [13] by the European Union in 1986 [14], which aims to refine, reduce and replace the use of animals in diagnostic and research testing, led to a marked reduction in use of the rabbit pyrogen test in subsequent years and the adoption of the LAL test as an *in vitro* alternative pyrogen test for many parenteral products.

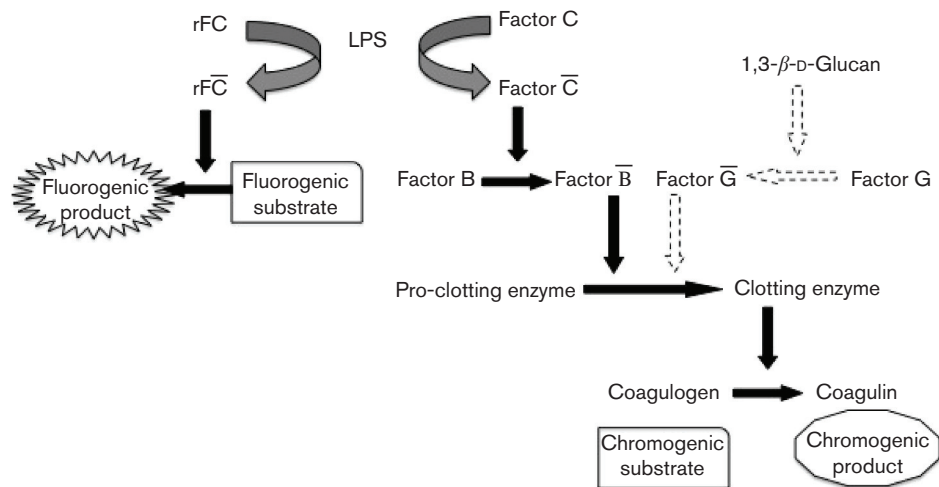
The principle of the LAL assay is based on the observation that endotoxin causes serine protease-mediated extracellular coagulation of the haemolymph of the horseshoe crab [15]. The enzymatic components and the molecular events that are responsible for the clotting cascade of *Limulus polyphemus* and other related species (namely *Tachyplesus tridentatus* [16] and *Carcinoscorpius rotundicauda* [17]) are well characterized (Fig. 2). Factor C, the first component of the cascade, is a serine protease that is activated by endotoxin binding [16]. The cascade, initiated by LPS, culminates in the activation of the pro-clotting enzyme to its active form, the clotting enzyme, which in turn acts on coagulogen to convert it into the coagulin clot (Fig. 2) [16, 18, 19]. The kinetic chromogenic LAL assay uses a synthetic peptide-p-nitroaniline substrate that is cleaved by the clotting enzyme, resulting in a product that exhibits a yellow colour (Fig. 2). The intensity of the yellow colour or the rate of colour formation correlates with the concentration of LPS in the assayed samples. The coagulation cascade of the LAL system can also be activated via activation of factor G by fungal glucans [20] (Fig. 2, upper right), although the activation of factor C by LPS is many times more sensitive [21].

In addition, the LAL assay shows batch-to-batch fluctuations due to seasonal and geographical differences in the

starting materials [12]. Furthermore, the dwindling horseshoe crab population, mainly due to extensive harvesting as a bait for commercial fishing [22], habitat loss and pollution, mandates the conservation of this organism, as it has important roles in the ecology of its marine habitat [2]. The commercial exploitation of the horseshoe crab for the LAL assay, therefore, adds to the threats to its populations [2, 12]. To overcome all these issues, an alternative source of LAL assay was required.

This led to the production of factor C (FC) by recombinant DNA technology. FC has been cloned and expressed in *Escherichia coli* [23], yeast [23, 24] and mammalian cells [25], and recombinant FC (rFC) is capable of strongly binding LPS. The production of an rFC has enabled the development of a simple, specific and sensitive LPS detection assay that could potentially replace routine LAL-based assays (Fig. 2, upper left), particularly in cases where fungal contamination might be high.

Despite the applicability of the LAL assay for the detection of LPS from many bacterial sources, there has been no study to investigate the reactivity of rFC with different LPS structures. The range and sensitivity of rFC to different LPS structures will be important to determine before rFC-based assays become more widely adopted for different test samples. Moreover, the comparison of LPS detection capabilities between rFC and current LAL assays is also important to ascertain. We believe this is the first study to determine the reactivity of different LPS chemotypes with the PyroGene (rFC) assay and then to compare the rFC-based assay with two other commercial kinetic chromogenic LAL-based assays in detecting LPS structures from various bacterial species. Furthermore, the inflammatory potential of selected



**Fig. 2.** The LPS reactive coagulation cascade system in the *Limulus* horseshoe crab that results in the formation of a coagulin gel. The coagulogen chromogenic substrate and chromogenic product used in the LAL assays (adapted from [18, 28]) are shown. The scheme also shows the activation of the LAL clotting system by factor G through interaction with fungal 1,3- $\beta$ -D-glucan (top right). Activation of recombinant factor C (rFC) by LPS and subsequent reaction to form a fluorogenic product as used in the PyroGene assay is shown at the top left.

LPS structural species was determined by the induction and release of IL-6 from a human monocytic cell line to allow comparison and assess the clinical relevance of rFC-based and LAL assays.

## METHODS

LPS from *Salmonella minnesota* smooth strain, *Salmonella minnesota* rough strain (R345), *Escherichia coli* O111:B4, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* serotype 10 were purchased from Sigma-Aldrich (Poole, UK). ['Premium Grade' (protein, nucleic acids <1%)]. LPS from *E. coli* J5, *Salmonella minnesota* lipid A, *Salmonella minnesota* rough strain (R595), *Bordetella pertussis* and *Francisella tularensis* were from List Biologicals Laboratories, Inc. (Campbell, CA, USA) ['Ultra Pure' grade (protein, nucleic acids <1%)]. LPS from *Burkholderia cepacia* and *Serratia marcescens* was extracted from bacteria and treated with DNase and proteinase K as described previously by Bamford *et al.* [26]. The Kinetic-QCL LAL assay kit, WinKQCL V 3.0.1 software, pyrogene-free water, dilution tubes and pipette tips were purchased from Lonza (Verviers, Belgium). The PyroGene recombinant Factor C endotoxin detection kit was kindly provided by Lonza (Verviers, Belgium). The Endosafe Endochrome-K Kinetic LAL assay kit was purchased from Charles River (Charleston, SC, USA). The FLx800 TBIE fluorescence microplate reader and the ELx-808 IUBWI spectrophotometer were supplied by Bio-Tek Instruments (Bedfordshire, UK). The human monocytic cell line, Mono-Mac 6 (MM6), was obtained from the German Cell Collection (DSMZ, Braunschweig, Germany) and all tissue culture consumables were purchased from Lonza (Verviers, Belgium). A DuoSet ELISA kit for IL-6 ELISA was obtained from R & D systems (Oxford, UK) and reverse transcription RT-PCR kits and primers were purchased from Invitrogen Ltd (Paisley, UK). An iCycler thermal cycler was supplied by Bio-Rad Laboratories (Hertfordshire, UK).

### Cell lines

Mono-Mac 6 (MM6) cells were maintained in RPMI 1640 medium, supplemented with 200 mM L-glutamine, 1% penicillin/streptomycin, 10% (v/v) foetal bovine serum, 1 mM sodium pyruvate and 1% (v/v) non-essential amino acids. The cells were seeded at a density recommended by the supplier and grown at 37 °C in humidified air and 5% CO<sub>2</sub>.

### Measurement of cytokines at the protein and mRNA levels

MM6 cells ( $5 \times 10^5$  for protein and  $2.5 \times 10^6$  for the expression of mRNA) were incubated with various LPS structural species for 4 h (RT-PCR) or 24 h (ELISA). The released IL-6 in the cell culture supernatants following stimulation with various endotoxins was measured by ELISA using a DuoSet ELISA kit, whereas the level of mRNA expression was measured by RT-PCR using an iCycler thermal cycler (Bio-Rad, UK) according to the manufacturer's instructions. The primers for IL-6 and the internal control GAPDH were

designed using OligoPerfect™ online software (<http://tools.invitrogen.com>). The primers for IL-6 were forward 5'-TAC CCC CAG GAG AAG ATT CC-3', reverse 5'-TTT TCT GCC AGT GCC TCT TT-3' and GAPDH forward 5'-ACA GTC AGC CGC ATC TTC TT-3' and reverse 5'-GAC AAG CTT CCC GTT CTC AG-3'.

### Endotoxin detection assays

The PyroGene recombinant factor C endotoxin detection end-point assay was performed as described in the manufacturer's instructions and analysed using WINKQCL V 3.0.1 software. The sensitivity or gating setting of the FLx800 TBIE reader for individual kits was also determined as outlined by the supplier using WinKQCL software. The assays were performed according to the manufacturer's instructions using WinKQCL V 3.0.1 software for both Kinetic QCL (KQCL) and Endosafe Endochrome-K assays.

The following US Pharmacopeia (USP) reference standard endotoxin (RSE) (LPS from *E. coli* O55:B5) dilutions were made: 0.01, 0.1, 1 and 10 EU ml<sup>-1</sup> in each experiment as a standard for all the assays. Since different LPS structures did not have similar potency, i.e. endotoxin activity as measured by LAL or rFC-based assays, their activity in a LAL- or rFC-based assay is expressed using an endotoxin unit (EU). The EU is the activity of a specific endotoxin preparation, defined as one-fifth of the amount of *E. coli* O55:B5 endotoxin required to bring about the threshold pyrogenic response when injected into man and rabbit on a per kilogram basis (1 EU=100 pg of *E. coli* O55:B5 endotoxin) [27]. The amount of the various LPS structures and lipid A (from *Salmonella minnesota*) that were tested in the LPS detection assays varied from a picogram up to 50 ng, however, for comparison the results were expressed as EU ng<sup>-1</sup> of LPS for both rFC-based and LAL assays.

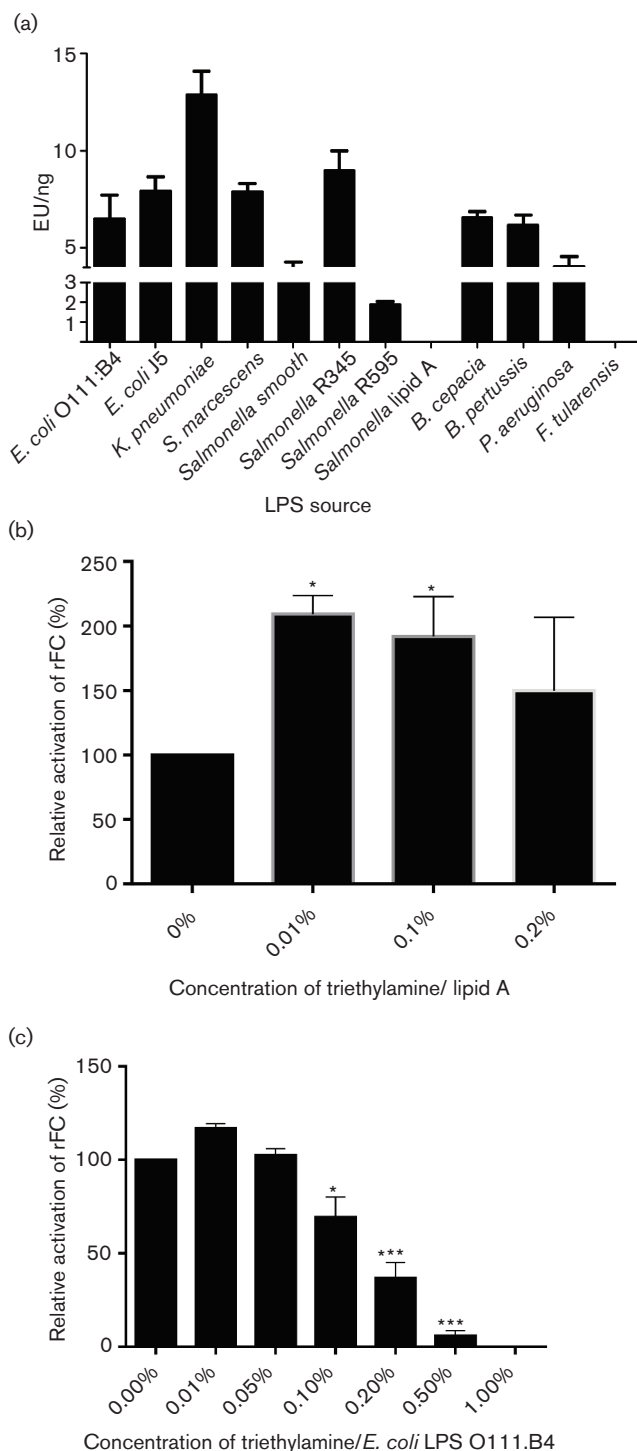
### Data analysis

Statistical comparisons among groups were determined by Student's *t*-test and one-way ANOVA, or Pearson's correlation using GraphPad PRISM software v 4.03 (GraphPad Software, Inc).

## RESULTS

### Detection of various LPS structural species by rFC-based assay

In the present work, the reactivity of 11 different LPS structural species and a lipid A portion with rFC were analysed and the results from the rFC-based assay were compared with those from two LAL-based assays. The rFC-based assay is designed to detect the LPS potency in the range of 0.01 to 10 EU/ml, and this assay was able to detect all the LPS structures in picogram quantities, except for the lipid A and LPS from *F. tularensis* (Fig. 3a). However, there is a significant variation in the activity of the different LPS structures as measured by this assay (Fig. 3a). The most rFC-reactive, i.e. potent, LPS structure detected was LPS from *K. pneumoniae*, while the LPS from *F. tularensis* was the least



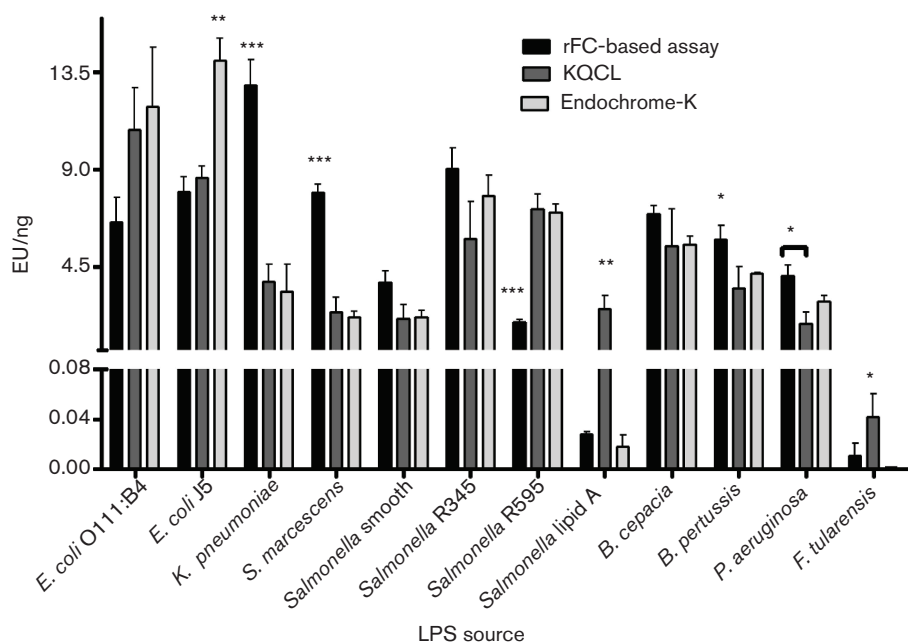
**Fig. 3.** The reactivity of different LPS structures as determined with the rFC-based assay. (a) The activity of each LPS structure is expressed as endotoxin units (EU)/ng of LPS ( $n$ =at least three independent experiments conducted in duplicate). \* $P$ <0.05; \*\*\* $P$ <0.001 vs LPS from *E. coli* O111:B4. Effect of triethylamine on the reactivity of lipid A (b) and *E. coli* O111:B4 (c). For the *E. coli* O111:B4 the reactivity of LPS with triethylamine was normalized against the LPS without the addition of triethylamine. \* $P$ <0.05; \*\*\* $P$ <0.001 versus LPS from *E. coli* O111:B4.

reactive of all the structures investigated in this work. Indeed, on an equal-weight basis, the LPS from the most active species (*K. pneumoniae*) was 35 687 times more reactive than the least reactive LPS (*F. tularensis*). The assay was only able to detect LPS from *F. tularensis* when its concentration was greater than or equal to  $50 \text{ ng ml}^{-1}$ , considerably higher than the amounts detected for the other species.

To investigate the detection of the different chemotypes of LPS from the enteric bacteria, LPS from *Salmonella* smooth type, R345 (Rb2), R595 (Re) and lipid A from *Salmonella* (Fig. 1a), and smooth-type *E. coli* O111:B4 and *E. coli* J5 were used (Fig. 3a). Chemotypes containing the core saccharide region (*Salmonella* R345 and *E. coli* J5) showed no marked difference in rFC reactivity compared with the smooth strain chemotype. However, chemotypes with a truncated core region (R595) and lipid A showed significantly reduced potency. The mean reactivity of *Salmonella* smooth-type LPS was 134 times greater than that of the lipid A. This marked difference in rFC reactivity between smooth- and rough-strain LPS chemotypes is probably due to differences in solubility. It was previously suggested that the detergent additives of the PyroGene assay buffer, Zwittergent, makes lipid A more insoluble and unreactive [28]. To aid the solubility of lipid A and improve its reactivity with rFC, lipid A was treated with triethylamine, which is a known LPS dispersion agent [29], and Triton X-100, which is also reported to increase the reactivity with factor C [30]. The activation of rFC by lipid A was doubled when triethylamine at a concentration range of 0.01 to 0.2 % was used (Fig. 3b). The activity of the smooth LPS structures was also slightly increased with low levels of triethylamine, but higher concentrations of triethylamine (>0.1 %) reduced its activity. Likewise, when other LPS structures were treated with triethylamine at a concentration of 0.01 %, it either slightly increased or had no effect on their activation of rFC, but higher concentrations of triethylamine negatively affected their activity (Fig. 3c). Treatment of samples which contained lipid A with low concentrations of Triton X-100 also enhanced the reactivity of lipid A-spiked samples (results not shown).

### Comparison of rFC-based assay with LAL-based assays

The reactivity of the rFC-based assay with LPS was compared with that for two LAL-based assays provided by two different suppliers. The rFC-based assay showed greater sensitivity to a number of LPS from different organisms, while it gave approximately equal sensitivity in the detection of LPS from others (Fig. 4). The activity of LPS from *E. coli* O111:B4, *Burkholderia cepacia*, *Salmonella* smooth and *Salmonella* rough (R345) when analysed by rFC-based assay showed no significant difference compared with the LAL assays (KQCL and Endosafe Endochrome-K assays) ( $P=0.1392$ ). There was no marked variation between the rFC-based assay and KQCL in the detection of LPS from *E. coli* J5, but the Endochrome-K assay gave a significantly higher reading for this structure ( $P=0.0016$ ).



**Fig. 4.** Comparison of the reactivity of the different LPS structures in the LAL assays and rFC assay ( $n =$  at least three independent experiments conducted in duplicate). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$  rFC response vs LAL responses.

The potency of LPS from *Klebsiella pneumoniae* and *S. marcescens* measured by rFC-based assay was significantly higher than that measured by KQCL and Endochrome-K ( $P = 0.0001$ ). The potency of the LPS from *P. aeruginosa* and *B. pertussis* was significantly higher when assayed by rFC-based assay compared with the KQCL assay ( $P = 0.0159$  and  $P = 0.00001$ , respectively). However, the PyroGene (rFC) assay was less sensitive to *Salmonella* R595 compared with the LAL assays. The rFC-based assay also showed less reactivity to lipid A ( $P = 0.0007$ ) and LPS from *F. tularensis* compared with the KQCL assay ( $P = 0.00001$ ). The Endochrome-K assay was the least sensitive of the three assays in detecting lipid A; it only detected lipid A when the concentrations were  $\geq 10 \text{ ng ml}^{-1}$ . Furthermore, the effect of the concentration gradient on these assays was also investigated by using the three strains of *E. coli* LPS, namely O55:B5, O111:B4 and J5. A dilution ranging from 1000 to  $1 \text{ pg ml}^{-1}$  was prepared and assayed. The results showed there is a classical linear relationship between the activity from the individual assays and the LPS concentrations in the concentration range that was tested (see Fig. S1, available in the online Supplementary Material).

#### Induction of IL-6 by various LPS structural species

The various LPS structures used to study the reactivity of rFC were also investigated for their potential to induce IL-6 from a human monocyte cell line. The induction of IL-6 at the mRNA and protein levels was studied after the cells were stimulated with various LPS structures for 4 and 24 h, respectively. The various LPS structural species when added to the cells at equal concentrations induced a markedly different level of IL-6 (Fig. 5). Previous studies have shown

that production of IL-6 by MM6 cells in response to the LPS used is almost completely blocked by polymyxin B, and that MM6 cells are almost uniquely sensitive to LPS at the concentrations used here, indicating that activation of the cell assay was due to LPS rather than other contaminants [31, 32]. A very high level of IL-6 was released from the cells when they were stimulated with LPS from *E. coli*, *K. pneumoniae*, *B. cepacia*, *Salmonella* (both the smooth and rough R345 chemotypes) and *S. marcescens*, and there was no marked difference amongst these structures in the level of IL-6 induction. However, LPS from these bacteria induced significantly higher amounts of IL-6 compared with LPS from *B. pertussis*, *P. aeruginosa* and lipid A. The level of IL-6 mRNA induction following stimulation of the cells with selected LPS structures for 4 h was also studied. The result obtained from RT-PCR was very similar to the ELISA result (data not shown). The rFC-based assay showed a significant positive correlation with the induction and release of IL-6 from the monocyte cell line ( $r^2 = 0.62$ ,  $P = 0.039$ ) (Table 1). In contrast, the KQCL and Endochrome-K assays showed a positive correlation with IL-6, but the correlation was not significant, as shown in Table 1.

#### DISCUSSION

Because of its ubiquity and biological potency, LPS is the most important contaminant of pharmaceutical products [3, 4, 33]. Human beings are extremely sensitive to endotoxin and its effects range from fever to shock and death [2, 11, 34]. This necessitated the development of a highly reliable, sensitive quantitative test for the pharmaceutical and medical industries to avert any potential risks [12]. The LAL-based assay has been

**Table 1.** The Pearson's correlation analysis matrix for the various LPS structural species and lipid A for induction of IL-6 and pyrogenicity as determined by PyroGene, KQCL and Endosafe Endochrome-K assays. ( $n$  = at least three independent experiments conducted in duplicates).

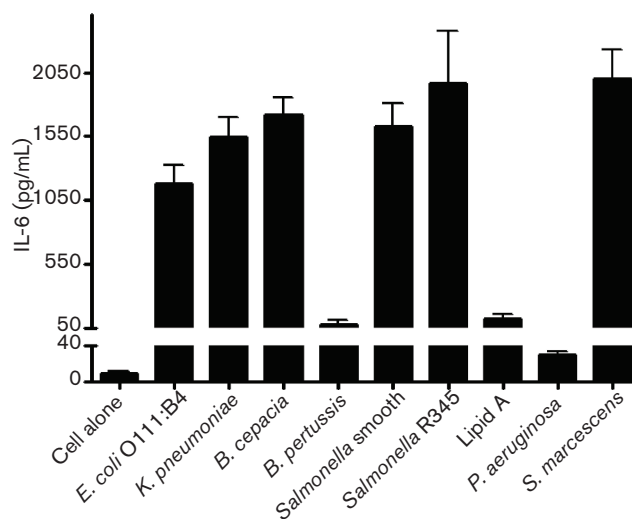
	PyroGene	KQCL	Endosafe	IL-6
KQCL	0.34	-	-	-
<i>P</i> -value	0.14	-	-	-
Endosafe	0.41	0.93	-	-
<i>P</i> -value	0.09	0.00	-	-
IL-6	0.62	0.24	0.28	-
<i>P</i> -value	0.04	0.27	0.23	-

used to detect endotoxin successfully from pharmaceuticals, parenteral fluids, medical devices, water and foods [12], and supplanted the time-consuming and expensive rabbit pyrogen test [2]. However, several groups [1, 2, 20, 35, 36] have questioned the specificity and sensitivity of the LAL-based assay, as the alternative pathway (factor G) can react with fungal contaminants ( $\beta$ -D-glucan) [20] and also give a positive result. In addition, some LAL-reactive materials that co-purify with clot proteins in the *Limulus* lysate may also give false-positive or -negative results in some batches of LAL [20, 35]. Moreover, the commercial procurement of the horseshoe crabs for LAL assays might exacerbate ecological concerns regarding horseshoe crab populations, which are threatened with extinction [2, 12].

A simple and rapid fluorimetric assay for the detection of endotoxin that is based on rFC and overcomes the shortcomings of LPS detection based on clot proteins derived from the haemolymph of horseshoe crabs has been recently introduced [12]. In the present work, the ability of the rFC

assay to detect various LPS structural species and lipid A – which vary in the length of their O-chain, core region, number and length of acyl chains, and presence or absence of phosphate groups on their lipid A – was evaluated. Although factor C has been shown to be uniquely sensitive to LPS, in order to avoid any possible reactivity to potential contaminants, highly purified commercial LPS was utilized, and at the concentrations used ( $100 \text{ ng ml}^{-1}$ ) there was minimal protein or nucleic acid. In addition, the rFC-based method was compared with two other LAL-based assays in the detection of these different LPS structures.

The rFC-based endotoxin assay is able to detect all of the LPS structures in picogram quantities, with the exception of LPS from *F. tularensis* and lipid A from *Salmonella minnesota*. Within the LPS structures, there is a striking range of reactivity, where the most reactive LPS (from *K. pneumonia*) is nearly 36 000 times more reactive than the least reactive LPS (from *F. tularensis*). This difference could perhaps arise from the structural characteristics of the lipid A moiety of *F. tularensis* [37–39]. Unlike typical potent endotoxins, which are equipped with two phosphate groups on the diglucosamine backbone, *F. tularensis* lipid A has no phosphate groups [39], or perhaps only one [38]. Using synthetic lipid A analogues (LA-14-PP), Takada *et al.* [40] showed that dephosphorylation of both phosphate groups reduced the *Limulus* activity by approximately 32 000-fold. This is almost the same degree of reduction in activity that was noted in this study using the rFC-based assay. It is worth pointing out here that factor C has also been reported to interact with acidic phospholipids such as phosphatidylglycerol and phosphatidylserine (but not with neutral phospholipids) in a manner comparable to synthetic lipid A



**Fig. 5.** The release of IL-6 induced by LPS structures. MonoMac 6 cells ( $5 \times 10^5$  per well) were stimulated with the various LPS structural species at a concentration of  $100 \text{ ng ml}^{-1}$  for 24 h ( $n$  = at least three independent experiments conducted in duplicate). The released IL-6 in the cell culture supernatants was measured by ELISA using a DuoSet ELISA kit following the manufacturer's instructions. \* $P < 0.05$  versus mean IL-6 production in LPS-stimulated cells.



analogues [19, 41, 42]. Therefore, it could be speculated that electrostatic interaction is very important for the binding and activation of factor C by LPS, and the reduction of negative charge due to lack of the phosphate groups could potentially affect this interaction. The absence of phosphate groups on the lipid A of *F. tularensis* might also indirectly affect the interaction with factor C due to poor solubility of the LPS species in the reagent mixture [40].

It has been demonstrated that the nature of the acyl groups linked to the diglucosamine backbone of lipid A plays a key role in the potency of lipopolysaccharide activation of factor C [19, 40, 43]. The low reactivity of the LPS from *F. tularensis* could thus be further explained by its significant deviation in terms of the number and chain length of the fatty acids on lipid A. *F. tularensis* lipid A is tetraacylated with three C-18 and a C-16 fatty acid [38, 39], resembling less potent lipid A structures from *Helicobacter pylori* and *Porphyromonas gingivalis* [44, 45] (as assessed by their potential in inducing pro-inflammatory cytokines from macrophages), while a typical biologically potent endotoxin has six C-12 to C-14 acyl chains. Therefore, we would expect the reactivity of the LPS of *Helicobacter pylori* and *Porphyromonas gingivalis* to be similar to that of *F. tularensis*. Using synthetic lipid A and its analogues, Kanegasaki *et al.* [43] also showed a significant reduction in *Limulus* activity when the number of fatty acids was reduced from six to four.

The low reactivity of the lipid A of *Salmonella minnesota* in all assays could be caused by its low solubility in aqueous medium. Furthermore, there is a report [28] which claims that lipid A is probably made more insoluble and unreactive in the presence of Zwittergent, which is a detergent additive of the rFC PyroGene Assay buffer. This detergent is added to assay buffer to disperse the LPS aggregate and make it more reactive to rFC. Since lipid A and lipid A-rich endotoxins are most likely to occur in naturally contaminated solutions, the chance of missing these when using buffer containing Zwittergent as a detergent could be high, although the spike recovery with the O-antigen rich endotoxin results appears to be acceptable [21]. The solubility of lipid A was enhanced using triethylamine, which is a known LPS dispersion agent [29], and Triton X-100, which is also reported to increase the activity of factor C [19]. It is not known why the kinetic chromogenic LAL assay was more sensitive to *F. tularensis* and lipid A, but it may be due to differences in the buffer contents which aid the solubility of more hydrophobic LPS structures. This suggestion is supported by the results using less soluble LPS from *Salmonella* R595, which also gave a higher reactivity with the KQCL LAL assay than the PyroGene rFC-based assay. However, the more soluble penta- and hexa-acylated LPS structures that were tested in this study were seen to be at least as potent, if not more potent, when assayed by rFC-based assay compared with the LAL-based tests.

Evaluation of assays that use non-human *in vitro* systems (i.e. rFC or LAL) to assess potential human health risks requires that comparisons be made with systems that use

human cells. Production of inflammatory cytokines, such as IL-6, from a human monocyte cell line, MonoMac 6 (MM6), which has been shown to be a good model of inflammatory potential in humans [46–48]. This cell line expresses all the receptor components for LPS signalling and is phenotypically and functionally similar to human peripheral blood monocytes [32, 49]. Several investigators [46–48] have also tried to use this cell line as an *in vitro* test for pyrogens. Moesby *et al.* [50] made a comparative study of MM6 cells with isolated mononuclear cells and the LAL assay in respect of their potential to detect pyrogens. IL-6 was the preferred readout in the present project, since IL-6 is, in contrast to IL-1 $\beta$  or TNF- $\alpha$ , released entirely into the cell-conditioned medium, allowing its complete estimation [1], and this cytokine has been shown to be released by low levels of LPS [47]. IL-6 is also the main endogenous pyrogen in humans and experimental animals and thus shows a better correlation with the pyrogenic activity of LPS in the blood. The various LPS structures used at a concentration of 100 ng ml<sup>-1</sup> in the present study induced a significant level of IL-6 from MM6 cells. Previous studies have shown that MM6 cells are very sensitive to LPS [31, 32] and that compared with LPS, putative pyrogens from Gram-positive organisms are much less potent inducers of cytokines in MM6 cells [46]. Moreover, in studies to identify microbial contaminants in biological products using MM6 cells and HEK293 cells transfected with different TLRs, only MM6 cells and HEK293 cells transfected with TLR4 responded to LPS [34]. Therefore, MM6 cells are useful models for assessing cell responses to LPS and our results with MM6 cells with LPS at the concentrations used in the current work are unlikely to be due to non-LPS contaminants. The same LPS structures also produced significant activity in the rFC-based assay and there was a significant positive correlation between rFC and the MM6 cell system ( $r^2=0.62$ ,  $P=0.039$ ). This suggests that rFC may react to lipid A/LPS structural variations in a similar manner to the MD-2-Toll-like receptor 4 receptor of the host immune system and, theoretically, it would be a useful assay for the assessment of LPS as a potential biomarker of Gram-negative bacterial infections and sepsis.

In conclusion, the rFC-based assay can be a viable alternative assay for the detection of endotoxin for pharmaceuticals, medical devices, water and foods, and could replace LAL-based assays and preclude the need to harvest horseshoe crabs, which are considered to be ‘living fossils’, and so contribute significantly to their conservation.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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