J Fluoresc (2014) 24:795–801
DOI 10.1007/s10895-014-1354-7

ORIGINAL PAPER

Rapid Homogenous Time-Resolved Fluorescence (HTRF) Immunoassay for Anthrax Detection

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Received: 19 December 2013 / Accepted: 27 January 2014 / Published online: 12 February 2014

Abstract Infection with *Bacillus anthracsis* spores induces an acute anthrax disease that can cause casualties and death in untreated cases. Thus rapid diagnosis of anthrax at early stage of the disease is essential to allow an effective treatment. Here we present the development of rapid and sensitive homogenous time-resolved fluorescence (HTRF) immunoassays based on the energy transfer process of europium cryptate (EuK) donor to AlexaFluor647 acceptor. The energy transfer process is limited to $d<10$ nm, making the HTRF an ideal assay for examination of homogenous and complex samples, since only mutual binding of the donor and acceptor antibodies to the analyte would result in positive signal. HTRF assay was developed for the detection of the bacterial Protective Antigen (PA) toxin, a serological marker that correlates with bacteremia in infected hosts, using two monoclonal anti-PA antibodies that specifically recognize two different epitopes on the PA molecule. The assay was sensitive enabling detection of 2 ng/ml PA in the serum of *B. anthracsis*-infected rabbits in only 15 min assay. Additionally, HTRF assay was developed for the detection of bacterial spores using polyclonal anti-spore antibodies that recognize many epitopes on the bacterial surface. The assay enabled the detection of 2×10^6 spores/ml in 30 min assay and was specific, showing no cross reactivity with closely related non-virulent *bacillus cereus* strain. This study describes the use of the HTRF assay for the detection of both singled-epitope (proteins) and multi-epitope (particles) as rapid, simple and sensitive method that can be used at the time that fast results are needed to allow an effective medical care.

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Keywords *Bacillus anthracis* · PA · Homogenous Time-resolved fluorescence · FRET · Antibodies · Immunodiagnosis

Introduction

Bacillus anthracis, the etiological agent of anthrax is one of the major bioterrorism-associated bacteria. Anthrax spores are easily delivered and can cause casualties as was reported in the anthrax attack in 2001 [1]. Inhaled anthrax is the most hazardous form of exposure leading to bacteremia and to the secretion of bacterial exotoxins, i.e. protective antigen (PA) edema and lethal factors that induce tissue damage and even death in untreated cases [2]. Therefore early diagnosis of anthrax is essential to prevent public catastrophe and allow an effective treatment. Diagnosis of anthrax can be achieved either by detection of the bacterial spore in contaminated fields or by monitoring the levels of the bacterial toxin—PA in clinical serum samples [3] that was shown to be in high correlation with bacteremia [4]. Previously, different immunodiagnostic methods have been developed to detect *B. anthracis* spores such as classic sandwich enzyme-linked immunosorbent assay (ELISA)[5], flow cytometry [6] and resonance energy transfer flow cytometry assays [7] that included specific antibodies (Abs) against the bacterial surface antigens. The detection of PA based on ELISA sandwich format could detect ng/ml [8, 9] or pg/ml [10]. However the sandwich immunoassays lasted few hours and required several incubation steps, making them not ideal for continuous medical monitoring of the disease progression in exposed patients. Thus, detection of PA in a direct method that includes all the assay components—Abs and analyte homogenously in one incubation step would make the assay short and simple.

Recently we reported a sensitive homogeneous assay for the detection of PA in serum samples of *B. anthracis* infected

rabbits using luminescent oxygen channeling effect (LOCI). However this method required several addition steps of reagents hence it prolonged ca ~1 h [11]. In this study we present the development of a one-step highly rapid (15–30 min) assay in a different homogeneous assay format based on fluorescence resonance transfer effect (FRET) of lanthanide complex. The principle of homogenous FRET-based assays is that two Abs labeled with donor and acceptor fluorophores mutually bind the analyte, inducing the energy transfer between them in a distance dependent manner of up to 10 nm in diameter (Fig. 1). To increase the sensitivity of the FRET immunoassay, lanthanide complexes were incorporated as donors [12]. Due to the time–resolved fluorescence (TRF) effect, this lanthanide complex has long decay times of luminescence (1 ms) [13] and large spectral separation (stock shift) between absorption and emission wavelengths of ~300 nm [14]. Both characteristics prevent background noise arising from auto-fluorescence of chemical and biological impurities in the samples characterized by short lifetime (ns) and minimal stock shift (10–20 nm) [15]. An example of lanthanide complex used as a donor is europium (Eu^{III}) tris-bipyridine cryptate (EuK) [16], in which the europium is encapsulated inside the cavity of the cryptate cage, and protected from drastic chemical conditions [17]. Thus, the combination of FRET and time-resolved fluorescence of lanthanide complexes enabled the development of homogeneous time-resolved fluorescence (HTRF) assays [18] with increased signal to noise ratios and higher sensitivity and selectivity.

Inhere we demonstrate new HTRF assays for the detection of the bacterial toxin—PA and bacterial spores that are fast, simple and sensitive.

Materials and Methods

Reagents and Abs

Protective Antigen protein, 83 kD (PA) was purified by Q-Sepharose chromatography, as described previously [19].

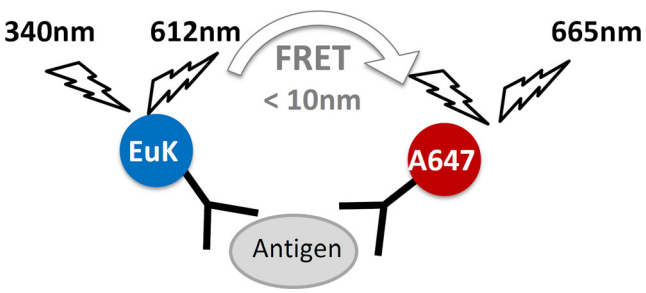

Fig. 1 Schematic illustration of the HTRF system: two Abs that recognize different epitopes on the antigen surface are directly conjugated to the donor europium tribipyridin cryptate (EuK) or the acceptor AlexaFluor647. The donor excitation at 340 nm leads to energy emission at 612 nm that induces the acceptor excitation and emission at 665 nm only when the donor and the acceptor are in close proximity (distance<10 nm)

Mouse IgG anti-PA monoclonal Abs (Mab2, Mab3, Mab5) were produced as was done previously [20]. Anti-*B. anthracis* spores Abs were raised against a soluble exosporium fraction as described previously [7] and purified from hyper-immune serum with Amino-link columns according to the manufacturer's protocol (Pierce, Rockford, IL). Abs were stained with the Europium Cryptate labeling kit (Cisbio Bioassays, Codolet Cedex, France, cat # 62EUSPEA) and with the AlexaFluor 647 (A647) carboxyl acid succinimidyl ester labeling kit (invitrogen, Eugene, Oregon, USA) according to the manufacturer's instructions. Strepvidin-A647 was obtained from Invitrogen (#S-21374) with labeling of dye to protein ratio of 3. Normal Rabbit Serum (NRS) was obtained from the Israeli Biological Industries (Israel).

Bacillus Strains

Bacillus anthracis Δ14185, a non-toxinogenic, non-encapsulated ($Tox^{-}Cap^{-}$) derivative of ATCC 14185 [21] (Bacillus Genetic Stock center) and *Bacillus cereus* were obtained from the Israel Institute for Biological Research collection. Spores were produced in sporulation medium SSM, as previously described [22].

Biotinylation

Biotinylation of IgG purified Ab fractions was carried out using sulfo-NHS-SS-biotin [sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate; Pierce 21331] according to the manufacturer's instructions. The number of biotins per Ab has been calculated by the HABA ([2-(4-hydroxyazobenzene] benzoic acid) method (Pierce 28050) and was shown to be four biotin molecules per Ab in average.

Binding Assays

Binding studies were carried out using the Octet Red system (Forte Bio) that measures biolayer interferometry (BLI). The biosensor electrode in the instrument senses interference pattern of white light reflected from a ligand bound to the biosensor tip that is translated to a shift in wavelength (Δλ) measured in nanometers. Mab2 were conjugated to biotin and were used as a probe to bind to the streptavidin-coated biosensor for 150 s followed by wash. Then, the biosensor was incubated with PA, Mab3 and Mab5 sequentially for 150 s each followed by wash. The assay buffer was PBS containing 1 mg/ml BSA (Sigma) and 0.1 % tween 20 (Sigma). All the steps were performed at 30ºC with shaking at 1,500 rpm in 96-well plates. Bindings and dissociations were measured as changes over time in the light interference, and curves were presented after subtraction of control unloaded biosensors.

HTRF Assay

HTRF assays were performed using the donor, Europium^{III} tris-bipyridine cryptate (EuK), and the acceptor, AlexaFluor647 (A647) conjugated to the assay Abs. The donor excitation at 340 nm leads to energy emission at 612 nm that induces in close proximity (d<10 nm) the acceptor excitation and emission at 665 nm (Fig. 1). The assays were carried out in a white (non-Maxisorp) micro plate (Nunc, Roskide, Denmark). In the assay development process the donor and acceptor Abs were incubated with the analyte diluted in assay buffer containing 50 nM Phosphate buffer (pH=7), potassium fluoride (KF) 400 mM (Sigma, Rehovot, Israel) and 0.1 % BSA (Israeli Biological Industries, Israel) in a final volume of 60 μl for different time periods. While reading unknown samples all the reagents were pre incubated in the micro-plate wells (40 μl) and the samples (20 μl) were added for 15 min incubation before the reading. The results were read in the Infinite F200 reader (Tecan, Switzerland) in the following settings: Excitation (340 nm), Emission (612 nm or 665 nm), Lag time 100 μs, Integration time 400 μs.

Calculation of ΔF

The HTRF signals were calculated as normalized fluorescence transfer value (ΔF) [23]: the fluorescence value at 665 nm (F665nm) of the sample was divided by the fluorescence value at 612 nm (F612nm) of the sample. The resulting value was subtracted by the background and divided by the background (see equation below).

$$\Delta F = \frac{\frac{F(665nm)Sample}{F(612nm)Sample} - \frac{F(665nm)control}{F(612nm)control}}{\frac{F(665nm)control}{F(612nm)control}}$$

Limit of detection (LOD) was determined as 3 standard deviations above the control background without antigen.

Statistics

The InStat 3 program (GraphPad) was used for statistical analysis. Statistical analysis was performed using the Welch's corrected T test. Differences were considered statistically significant at p<0.05.

Results and Discussion

Development of HTRF Assay for PA Detection

The aim of the study was to develop a HTRF assay for fast and sensitive detection of PA in clinical samples of *B. anthracis*-infected hosts. The HTRF process requires simultaneous binding of donor and acceptor pair Abs to non-overlapping epitopes on the target antigen. To that end, we prepared three monoclonal mouse anti-PA Abs: Mab2, Mab3 and Mab5 and investigated them in a pairwise-mapping analysis using the Octet Red biolayer interferometry (see Materials and Methods). In this system, binding of an analyte to the biosensor tip causes a wavelength shift leading to an interference pattern measured in real time. Here, the streptavidin-coated biosensor tip was incubated with biotinylated Mab2 that served as an anchor to the PA on the tip. Then after a washing step, the Mab2-coated tip was incubated with PA to form Mab2-PA complex resulting in a wavelength shift with classic association/dissociation kinetics (Fig. 2). Following a short wash, the Mab2-PA complex was incubated with Mab3, resulting in another wavelength shift that quickly reached saturation and was not easily dissociated (Fig. 2). Finally the Mab2-PA-Mab3 complex was incubated with Mab5, resulting in a third shift. This experiment indicates that the three Abs bind different and non-overlapping epitopes on the PA molecule. MAb3 and Mab5 had the highest affinity to PA (data not shown), thus were used in the following HTRF assay.

Mab5 and Mab3 were covalently labeled with europium cryptate (EuK) and AlexaFluor647 (A647) respectively to form a FRET pair in a direct assay set up, and were examined for their ability to simultaneously bind PA for the induction of an efficient measurable fluorescence energy transfer. Mab5-EuK and Mab3-A647 were mixed with 100 ng/ml PA and the FRET signal was measured. The results are presented in Fig. 3 (left column) as ΔF values—the calculated positive signals subtracted from the background without PA (see Materials and Methods). The results demonstrate the possible use of these pair Abs in the HTRF assay. To confirm that the FRET signal

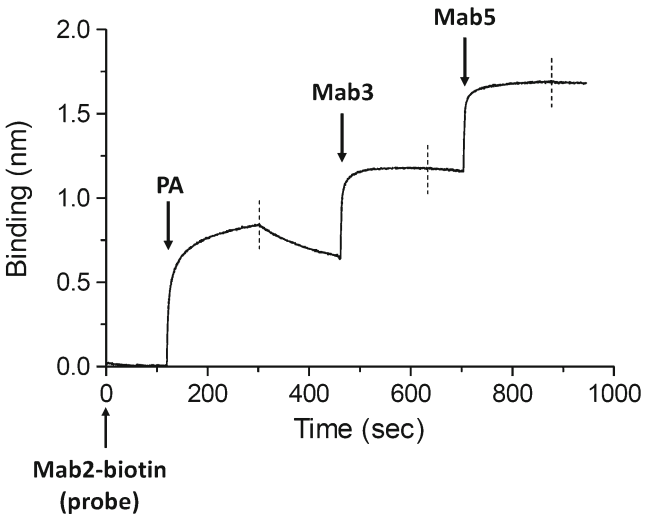

Fig. 2 Binding curve: each of the analytes (Mab2, PA, Mab3 and Mab5) were incubated separately in sequential steps as presented in *black arrows*. Bindings and dissociations were measured as changes over time in the light interference (nm) using the Octet Red biolayer interferometry

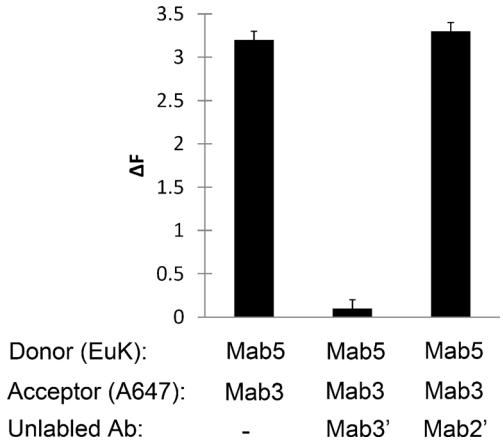

Fig. 3 HTRF assay for the detection of PA: Detection of PA (100 ng/ml) using Mab5-EuK (donor) and Mab3-A647 (acceptor) in the presence of unlabeled competing Ab (Mab3′ or Mab2′) or without unlabeled Ab (-)

is specifically induced by the simultaneous binding of the pair Abs to PA, a competition assay was performed; unlabeled Mab3 (Mab3′) was added to the assay mixture at the same concentration as the A647-labeled Mab3 (1:1). As expected, the unlabeled Mab3 competed with the binding of the labeled-Mab3 leading to a complete abolishment of the FRET signal. In contrast, the addition of 64-fold excess of unlabeled Mab2 (Mab2′) that binds a different epitope did not affect assay performance (Fig. 3).

To optimize assay performance, a range of concentrations (1–50 nM) of both donor and acceptor were examined using a fixed concentration of PA (10 ng/ml) to determine the optimal concentration of the donor and acceptor for the assay (data not shown). The concentration of 5 nM for both Abs was selected, in a ratio of 1:1 of acceptor to donor. To examine the performance of the assay for the detection of wide range of PA concentrations, a dose response assay was done using increasing amounts of PA (0–10,000 ng/ml) diluted in PBS. Figure 4a shows the standard curve demonstrating an increase of FRET signal as a function to the amount of PA in the mixture of the direct assay. The dose response curve had a wide linear dynamic range of more than 3 orders of magnitude ranging from the LOD of 2 ng/ml to 2,500 ng/ml PA. The LOD was determined as 3 standard deviations above the control background without PA in the samples. Above 5,000 ng/ml PA the ΔF values decreased, leading to a phenomenon called "prozone"—typical effect of homogenous assays, where high excess of analyte induces a decrease in assay signals [24, 25]. The minimal incubation time required for stable assay signals was only 15 min (data not shown), demonstrating a simple and fast assay for the detection of PA.

In an attempt to improve the FRET signal and to increase the sensitivity of the assay, an indirect format was also tested using Mab3 covalently bound to four biotin molecules (in average), which each of them binds streptavidin molecule containing an average of three A647 molecules (see Materials and Methods). In this indirect format, each acceptor has total of 12 molecules of A647 compared to six A647 molecules in the directly conjugated format, and therefore anticipated to induce stronger FRET signals. Moreover, the use of an Avidin-biotin linkage of the Ab to the acceptors might enable more common use of the method due to the vast availability of these reagents. As shown in Fig. 4b such indirect configuration resulted in similar LOD of 2 ng/ml, however the dynamic range of the assay was only 2 orders of magnitude, reaching plateau at 300 ng/ml (Fig. 4b). This reduction in the signal could be due to the increase in the distance between the donor and the acceptor presented by the biotin-streptavidin complex leading to a detrimental effect on the FRET efficiency. It was therefore decided to continue with the direct format in the rest of the study.

Detection of PA in Serum Samples

The amount of PA in the serum of *B. anthracis*-infected hosts is in high correlation with bacteremia, making it an appropriate diagnostic marker in serum samples for monitoring disease progression. However, high excess of proteins and immunoglobulins in the serum might interfere with the pair binding to PA, leading to reduction in FRET signals and assay performance. To evaluate the efficacy of the HTRF assay for the detection of PA in serum samples, PA was spiked (0–5,000 ng/ml) in a pool of naïve normal rabbit serum (NRS) and was examined in the direct HTRF assay format. The results of the HTRF assay in NRS are presented in Fig. 5 in comparison to PA spiked in PBS. As can be seen, the sensitivity of PA detection in serum is similar to PA in PBS, suggesting that the HTRF assay can be used for the examination of complex serum samples and is not affected by quenching or blocking effects of components in the serum. Moreover, the assay was not influenced by the autofluorescence background of the serum components due to the time-resolved delay effect of the EuK that enabled the energy transfer to the acceptor at the time that the autofluorescence of the background was faded.

Next, the detection of PA in serum samples derived from *B. anthracis*-infected rabbits were tested. Six animals were inoculated by intranasal instillation with *B. anthracis* spores and their sera were collected in different time points within 3 days post inoculation, as was previously described [3]. To evaluate the validity of the HTRF assay for the detection of PA in serum, we compared the results of this method with the long (4 h.) ELISA method as was previously reported [3]. The amount of PA in the serum of the infected rabbits was calculated using the calibration curve for PA spiked in NRS using the linear equation of the graph (data not shown). As one can observe, there is high correlation between the HTRF to the ELISA results, exhibiting a coefficient correlation value (r^2) of 0.9984 (Fig. 6). Moreover, the calculated PA concentrations,

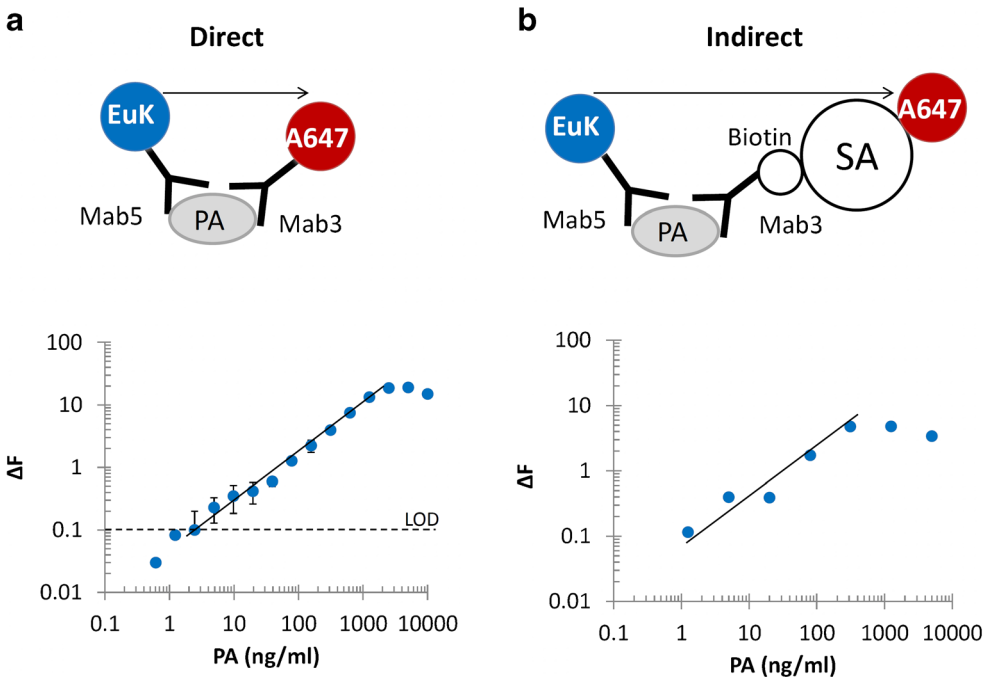

Fig. 4 **a** Direct assay: the donor (EuK) and the acceptor (A647) were conjugated directly to Mab5 and Mab3 respectively (top schematic illustration), or **b** Indirect assay: the acceptor was conjugated to biotin and streptavidin (SA)-A647 was added to form biotin-streptavidin complex (top schematic illustration). Dose response tests of both assays are presented in logarithmic scale of ΔF values as a function of PA concentrations (*bottom* graphs). The black dashed line refers to the LOD of PA (2 ng/ml)

which ranged between 3 and 10,000 ng/ml were highly correlated to the different stages of the disease. These results demonstrate that the HTRF assay is sensitive and accurate as the previously reported ELISA method. However the HTRF assay is more rapid, giving results in 15 min compared to few hours using ELISA, thus can be beneficial in case that rapid result is needed to allow medical treatment for multiple exposed patients.

HTRF Assay for the Detection of *B. anthracis* Spores

Since *B. anthracis* spores are very stable and persist for long time, they are in a high risk to be used as bioterror agents. Therefore, it is essential to develop such sensitive and fast method for the bacterial spores to avoid possible exposure to the population. In contrast to proteins that are singled-epitopes, bacteria have multiple epitopes with high repetition. In order to achieve a valid assay, the abundance of the epitopes should allow donor—acceptor distance of up to 10 nm, otherwise the FRET process would not be efficient. The donor and acceptor Abs chosen for the assay need to recognize two near epitopes on the bacterial surface, making it hard to find using monoclonal Abs. To increase the probability of binding in close proximity of the donor and the acceptor, we used polyclonal rabbit anti-spore Abs that recognize more than 10^5 different epitopes on the spore surface [7]. The anti-spore Abs were conjugated directly to the EuK donor and A647 acceptor and were used in a direct assay as was described above for PA. To optimize assay performance, a range of concentrations (1–

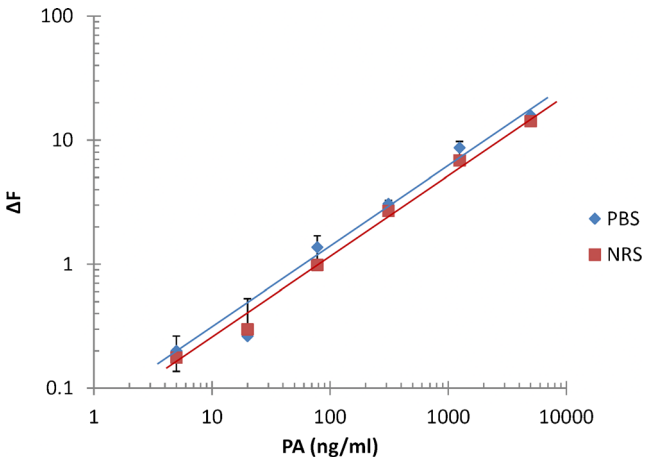

Fig. 5 HTRF assay for the detection of PA spiked in Normal Rabbit Serum (NRS) (*red/square*) compared to PBS (*blue/diamond*) presented in logarithmic scale of ΔF values as a function of PA concentrations

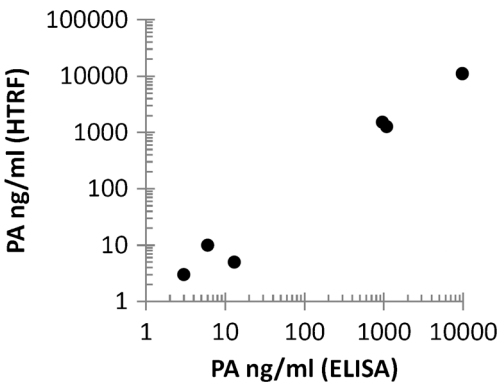

Fig. 6 Correlation dot plot: Serum samples collected from six *Bacillus anthracis*-infected rabbits were examined for PA amount using the HTRF assay compared to ELISA and ECLI methods. The results were examined for correlation and were plotted in the graph. R^2=0.9984 and p<0.0001

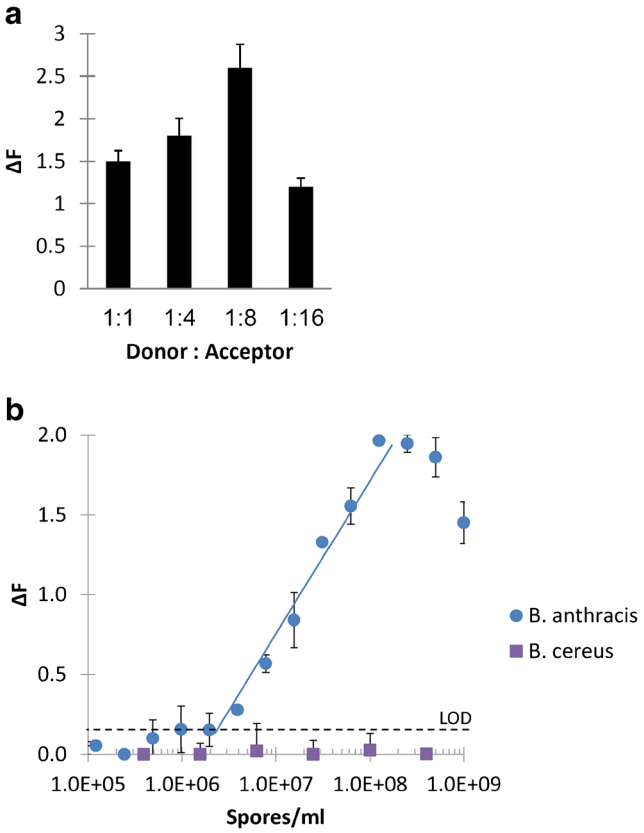

Fig. 7 **a** HTRF assay results (ΔF) for the detection of *Bacillus anthracis* (10^8 spores/ml) using different donor-acceptor ratios (1:1-1:16). **b** Dose response of HTRF assay for the detection of *Bacillus anthracis* (*(blue/circle)*) or *Bacillus cereus* (*(purple/square)*) presented in logarithmic scale of ΔF values as a function of PA concentrations. The LOD is presented in *black dashed line* (2×10^6 spores/ml)

50nM) of both donor and acceptor Abs was examined using a fixed concentration of spores (10^7spores/ml). It was found that, in contrast to PA, here a ratio of 8-fold more acceptors over donors produced the best assay performance (Fig. 7a). The use of excess of acceptors here consists with our previous observation using the homogeneous LOCI method [11], suggesting that the formation of clusters of acceptors around donors increases the proximity of donor-acceptor interactions on the surface of the bacteria.

To examine the use of the HTRF assay for the detection of *B. anthracis* spores in the contaminated area, swabs were spiked with elevated concentrations of spores (0–10^9 spores/ml). Figure 7b presents the dose response curve of a HTRF assay for the detection of *B. anthracis* spores; the LOD was 2×10^6 spores/ml and the dynamic range of the assay was 2 orders of magnitude. The minimal incubation time for optimal assay performance was 30 min (data not shown).

The overriding concern for *B. anthracis* identification in environmental samples is the presence of *B. cereus*—a non-virulent closely related philogenetically to anthrax that can mistakenly be confused with the virulent strain of anthrax. Since we used polyclonal Abs in this assay that might be less selective compared to monoclonal Abs, there is a possibility of cross reactivity with the non-virulent strain. To examine the selectivity of the assay to anthrax spores, the assay was tested for the detection of *Bacillus cereus*. Figure 7b, shows no cross-reactivity, demonstrating that the assay is specific for *B. anthracis* spores.

Conclusions

In this study, we demonstrated the use of HTRF technology for the detection of anthrax spores in possible contaminated field and its protein marker—PA in sera of infected hosts. The HTRF assay combined two physical properties: time-resolved fluorescence of lanthanide cryptate and FRET, making it ideal for the detection of analytes in complex samples in a direct format. The developed assays were specific, sensitive and simple to operate requiring a single incubation and a direct read using a standard fluorescence reader, and not unique or expensive equipment. The detection of PA in the serum was rapid giving results in 15 min with LOD of 2 ng/ml, allowing the detection of the disease in early stage. These results are comparable to few hours in the classic ELISA sandwich assays. Additionally, this study demonstrates the detection of the bacterial spores in high specificity, opening new directions for further use of the HTRF assay for the detection of multi-repetitive-epitope particulate antigens.

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