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A quantitative electrochemiluminescence assay for *Clostridium perfringens* alpha toxin☆, ☆☆

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Abstract

Described is a rapid direct sandwich format electrochemiluminescence assay for identifying and assaying *Clostridium perfringens* alpha toxin. Biotinylated antibodies to *C. perfringens* alpha toxin bound to streptavidin paramagnetic beads specifically immunoadsorbed soluble sample alpha toxin which subsequently selectively immunoadsorbed ruthenium (Ru)-labeled detection antibodies. The ruthenium chelate of detection antibodies chemically reacted in the presence of tripropylamine and upon electronic stimulation emitted photons (electrochemiluminescence) that were detected by the photodiode of the detector. Elevated toxin concentrations increased toxin immunoadsorption and the specific immunoadsorption of Ru-labeled antibodies to alpha toxin, which resulted in increased dose-dependent electrochemiluminescent signals. The standardized assay was rapid (single 2.5-h coincubation of all reagents), required no wash steps, and had a sensitivity of about 1 ng/ml of toxin. The assay had excellent accuracy and precision and was validated in buffer, serum, and urine with no apparent matrix effects.

Keywords: Electrochemiluminescence (ECL); Immunoassay; *Clostridium perfringens*; Alpha toxin; Gangrene; PLC

The gram-positive spore-forming obligate anaerobic bacillus *Clostridium perfringens* is the predominant etiological agent responsible for gas gangrene [1], although other *Clostridium* species including *C. novyi*, *C. bifermentans*, and *C. septicum* can also result in gangrenous infections [1]. Although notorious as a major complication of battlefield injuries, recent wars have seen a great reduction of the incidence of gangrene [1]. Gas gangrene remains a potential problematic disease after traumatic injury such as burns and automobile accidents and after natural disasters such as the recent tsunamis and hurricanes. The potential for use of *Clostridium* spores or toxins in biowarfare and bioterrorism also exists [2]. The potential severity of gangrenous infections warrants development of a rapid assay for detecting the specific toxins as a means to rapidly identify the *Clostridium* bacteria involved. Monitoring toxin levels in various tissues and body fluids should also prove useful for evaluating the effectiveness of therapeutics.

Clostridium perfringens is a bacterium associated with three disease syndromes: classic gas gangrene (a muscle tissue infection resulting in muscle necrosis), enteritis necroticans (necrosis of intestinal tissues), and toxin-dependent food poisoning [1,2]. Each of these syndromes requires a different inoculum and/or route of entry. In today's political environment, biowarfare/bioterrorism against either human or livestock targets are of potential concern, although realistic scenarios using either *C. perfringens* spores or vegetative bacteria in mass-casualty biowarfare events are difficult to imagine [2]. However, the limited use of these agents or the toxins they produce are realistic possibilities [2,3]. Aerosol exposure to the alpha toxin of

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15. SUBJECT TERMS
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C. perfringens would likely be lethal [2,3]. In addition, *C. perfringens* type A food poisoning currently ranks as the third most commonly identified food-borne illness in the U.S. [4,5]. *C. perfringens* is important not only in human disease; it has also been responsible for fatal intestinal disease of domestic livestock and wildlife [2,6] and toxin release in animals has resulted in fatal food poisoning [6].

Although as many as 20 different toxins may be produced by *C. perfringens* [1,6], alpha, beta, epsilon, and iota toxins are used to distinguish five major varieties [3,6]. These toxins are responsible for tissue death, hemolysis, vasoconstriction, increased vascular permeability, and shock [3,7]. All five varieties of *C. perfringens* produce alpha toxin [3], a 370-amino acid necrotizing zinc metalloenzyme with phospholipase C (lecithinase, PLC)[1] activity [1,8–10]. Alpha toxin can also inhibit cardiac contractility, and induce production of endogenous mediators such as platelet activating factor and tumor necrosis factor, which may perpetuate vascular insufficiency and tissue destruction [7]. Although several toxins alone are capable of causing fatalities [3], much of the lethality of *C. perfringens* is believed to be the result of the effects of alpha toxin [1,7–9]. Alpha toxin has been implicated in human and other animal sudden infant death syndrome [8,11,12] and numerous human deaths associated with food poisoning by type A (alpha toxin secreting) *C. perfringens* occur annually [5,13,14].

Because early diagnosis and aggressive treatment are key to decreasing morbidity and mortality, development of a rapid yet sensitive assay for detecting and measuring *C. perfringens* alpha toxin is warranted. Culture of *C. perfringens* has shown peak alpha toxin concentrations in culture medium in late log phase [10], suggesting the possibility that detection of soluble toxin might precede symptom-dependent or bacterial culture diagnosis of gangrene. Although the epsilon toxin is the only *C. perfringens* select agent ($LD_{50} < 100$ ng/kg), the *C. perfringens* toxins are considered to be moderately to highly toxic, with LD_{50} concentrations ranging from 0.1 to 5 μg/kg [15].

Several methods are currently available for detection and quantification of *C. perfringens* alpha toxin including enzyme-linked immunosorbent assay (ELISA) [16,17], PLC enzymatic activity [18], and mouse lethality assays [19,20]. An ELISA developed in 1997 was able to detect only to the 25 ng/ml toxin level [16]. An improved ELISA published 2 years later reported detection limits of 19 ng/ml [17], demonstrating considerable increase in detection sensitivity as compared to enzymatic activity [18] (detection limits of 130 ng/ml) or mouse lethality assays [19,20] (detection limits above 1 μg/mouse). Although polymerase chain reaction assays [21] are available to detect the gene for alpha toxin in bacteria with sensitivities of as low as 10 colony forming units, these methods are not capable of detecting systemic toxin or purified toxin resulting from a bioterrorist attack. Thus, a rapid assay with a limit of detection in the low-ng/ml range would prove useful in early diagnosis of *C. perfringens* toxemia. We have successfully implemented direct capture sandwich assays [22–25] and electrochemiluminescence (ECL) assays for other toxins [26,27]. We report here a rapid ECL capture immunoassay capable of detecting *C. perfringens* alpha toxin in a direct and dose-dependent manner in buffer, serum, and urine at a sensitivity of 1.1 ng/ml toxin.

Methods and materials

Antibodies

Antiserum was obtained by immunizing goats repeatedly with purified recombinant *C. perfringens* alpha toxin kindly provided by Dr. R. Titball [19]. Polyclonal antibodies to *C. perfringens* alpha toxin were obtained by ammonium sulfate fractionation of serum followed by affinity purification on a column of alpha toxin immobilized to cyanogen-bromide-activated Sepharose 6MB (Pharmacia Fine Chemicals, Piscataway, NJ). Affinity-purified antibodies were eluted by adding 0.1 M glycine, pH 3.0, and immediately neutralized with 1 M dibasic sodium phosphate. Neutralized purified antibodies were dialyzed against purified water and lyophilized.

Biotinylation of antibodies

Affinity-purified goat antibodies to *C. perfringens* alpha toxin (5 mg) were dissolved in 10 mM phosphate-buffered saline (PBS), pH 7.4. They were then reacted with a twofold molar excess of EZ-Link Sulfo-NHS-biotin (Pierce, Rockford, IL; Product No. 21217) for 2 h at room temperature followed by dialysis (Snakeskin 10,000 MWCO, Pierce, Product No. 68100) against PBS to remove unreacted biotinylation reagent. Biotinylated antibody (2.5 mg) was added to 25 mg of M280 paramagnetic streptavidin Dynabeads (Dynal Biotech) and PBS was added to a total reaction volume of 4 ml. This reaction mixture was incubated for 1 h at room temperature on an end-over-end rotator. The beads were then allowed to settle by gravity and the supernatant was removed and discarded. The antibody-coated beads were washed three times in this manner with approximately 10 ml of PBS per wash and suspended in 2.5 ml of assay buffer (6 mM PBS with 0.1% v/v Tween 20 and 0.2% w/v bovine serum albumin) to achieve a streptavidin bead concentration of 10 mg/ml. This stable capture antibody-bead reagent (capture Ab-beads) was stored at 4 °C and used in all experiments.

Ruthenylation of antibodies

Affinity-purified goat antibodies to *C. perfringens* alpha toxin (5 mg in PBS, pH 7.4) were reacted with a twofold molar excess of ruthenium(II) Tris–bipyridine NHS ester

[1] *Abbreviations used:* PLC, phospholipase C; ELISA, enzyme-linked immunosorbent assay; ECL, electrochemiluminescence; PBS, phosphate-buffered saline.

(BV-TAG, BioVeris, Gaithersburg, MD) in a 2-ml total reaction volume for 1 h at room temperature on an end-over-end rotator. The ruthenium–labeled antibodies (Ru–Ab) were separated from unreacted BV-TAG by size exclusion chromatography using a 5 ml D-salt Excellulose column (Pierce, Product No. 20449) with a 5000-MW exclusion limit The protein concentration of each eluted 1 ml fraction was determined by a micro bicinchoninic acid (micro-BCA) assay (Pierce, Product No. 23235) and the two sequential major protein-containing fractions were pooled.

Assay

The assay components were sequentially added to the wells of 96-well polypropylene round-bottomed microtiter plates (Costar, Cambridge, MA). The standardized assay in a total volume per well of 220 µl consisted of 45 µl of assay buffer, 50 µl of Ru–Ab, 25 µl of capture Ab-beads, and 100 µl of either sample or toxin. All assays were performed in triplicate. The assay was optimized with respect to capture Ab-bead concentration (assay concentration range 2.3–56.8 µg/ml), Ru–Ab concentration (assay range 1.42–5.68 µg/ml), and incubation time (30, 70, and 150 min). Alpha toxin (8.4 mg/ml) was initially diluted to 1 µg/ml and stored at 4 °C. Standard curves were prepared for each assay by serial dilution of 1 µg/ml stock to prepare additional concentrations for assay addition. Microtiter plates were incubated uncovered at room temperature with vigorous shaking by a tabletop shaker (Model 4626, Lab-Line Instruments Inc., Melrose Park, IL) to keep beads suspended. Fig. 1 depicts the initial assay conditions achieved by addition of Ru–Ab, capture Ab-beads, and toxin (Panel 1). Panel 2 depicts the formation of toxin-dependent immunocomplexes between capture Ab-beads and Ru–Ab. Panel 3 depicts washing of the immunocomplexes while attracted to a capture magnet in the M8 tricorder (BioVeris) flow cell. The resulting Ru-dependent ECL signal (Panel 4) was measured using a BioVeris M-Series M8 analyzer (M8). Three assay matrices were evaluated: assay buffer, human serum (bioWhittaker, Catalog No. 14-402E, Cambrex Bio-Science Walkersville Inc., Walkersville MD), and human urine (Accumark urine control, level 2, Sigma Diagnostics, Inc. St. Louis, MO).

Statistical analyses

Assays were evaluated for slope and coefficient of regression (R^2) of standard curves based on means of triplicate determinations. Assay sensitivity was defined as the minimum toxin concentration producing a mean ECL signal above that produced in the absence of toxin. Each validation assay included determination of precision and accuracy for three samples prepared at concentrations of 5, 20,

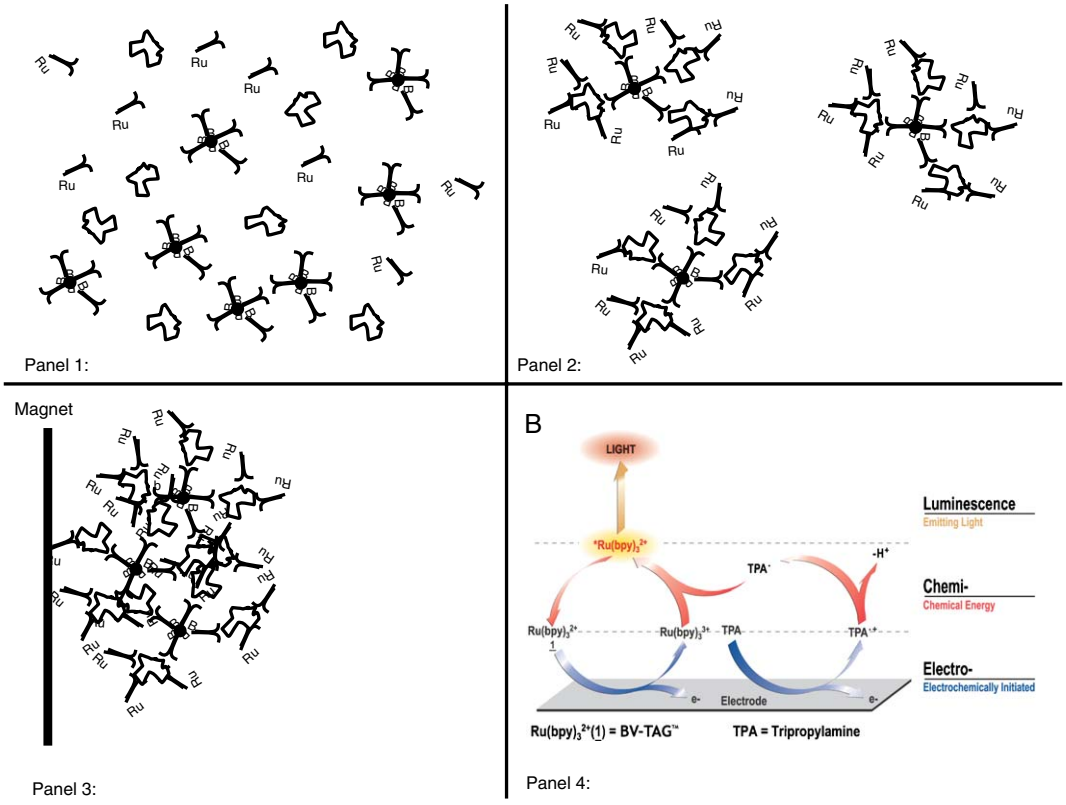

Fig. 1. ECL Assay design. (Panel 1) Coincubation of alpha toxin, Ru–Ab, and preformed capture Ab-beads. (Panel 2) Formation of alpha-toxin-dependent immunological sandwich complex. (Panel 3) Attraction of immunocomplex to magnetic field due to paramagnetic properties of M280 beads. Free reagents (not bound to beads) are removed by washing. (Panel 4): BioVeris technology electrochemiluminescence detection process.

and 40 ng/ml in each matrix (corresponding to low, middle, and high assay concentrations of 2.27, 9.1, and 18.2 ng/ml, respectively). All samples were prepared on one occasion and stored frozen (−20 °C) in small aliquots adequate for a single assay. Each result consisted of the mean of eight replicates. Analysis of these eight replicates of each quality control sample on each microtiter plate allowed determination of the intraassay coefficient of variation for each region of the assay standard curve. Three assays were performed during a single day using independently prepared reagents to assess within-day variation. Assays were also performed on each of 3 days to assess repeatability. All validation assays were performed by one individual.

Results

Assay optimization

Capture Ab-beads were diluted into assay buffer to 500, 250, 100, and 20 μg/ml. When 50 μl of each concentration was added to microtiter wells containing other assay reagents (for a total incubation volume of 220 μl) assay concentrations of 113.6, 56.8, 22.7, and 4.5 μg/ml were achieved. Total ECL signal increased with higher concentrations of capture Ab-beads (Fig. 2). Slight increases in nonspecific signal were also observed with higher bead concentrations (data not shown). Although addition of capture Ab-bead concentrations of 500 and 250 μg/ml resulted in stronger ECL signals, the signal produced by use of capture Ab-bead working concentrations of 100 μg/ml was adequate (4–6000 ECL units and signal/noise ratio > 7). However, a further five fold reduction of capture Ab-bead concentration produced insufficient signal. To ensure a strong ECL signal while minimizing assay costs and assay artifacts potentially arising from high magnetic bead concentrations, an 11.4 μg/ml final concentration of capture Ab-beads was selected for subsequent optimization since this Ab-bead concentration was predicted (by extrapolation from the data of Fig. 2) to meet selection criteria (4–6000 ECL units and signal/noise ratio > 7).

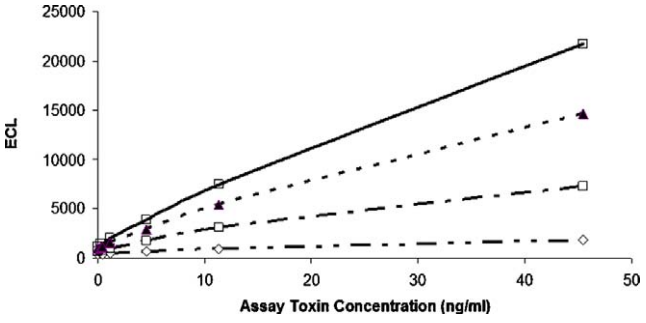

Fig. 2. Effect of capture Ab-bead concentration on ECL assay for *C. perfringens* alpha toxin. Plots of ECL signals for four capture Ab-bead concentrations as a function of assay toxin concentration. Assay components included toxin (100 μl well) at stock concentrations of 100, 25, 10, 2.5, 1, 0.5, and 0 ng/ml (correlating to assay concentrations of 0–45.4 ng/ml), 25 μl Ru–Ab (assay concentration 0.91 μg/ml), capture Ab-beads (50 μl at concentrations of 500, 250, 100, and 20 μg/ml correlating to assay concentrations of 113.6 (□), 56.8 (▲), 22.7 (■), and 4.5 (◇) μg/ml, respectively), and 45 μl assay buffer. Assay was incubated for 70 min at room temperature with vigorous shaking. Plotted values are means of triplicate determinations ± SE (SE bars not visible are minimal with respect to symbol size).

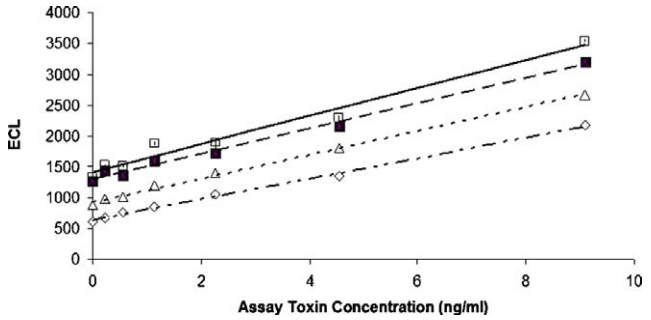

Fig. 3. Effect of Ru–Ab concentration on ECL assay for *C. perfringens* alpha toxin. Linear regression of ECL signals for four Ru–Ab concentrations as a function of assay toxin concentration are shown. Assay components included 25 μl/well of capture Ab-beads (11.4 μg/ml assay concentration), toxin (100 μl/well) at concentrations ranging from 0 to 20 ng/ml (assay concentrations 0–9.1 ng/ml), Ru–Ab (50 μl/well) diluted in assay buffer to concentrations of 25, 15, 10, and 6.25 μg/ml, corresponding to assay concentrations of 5.67 (□) 3.41 (■), 2.27 (Δ), and 1.42 (◇) μg/ml, respectively, and 45 μl assay buffer. Assay was incubated for 70 min at room temperature with vigorous shaking. Plotted values are means of triplicate determinations ± SE (SE bars not visible are minimal with respect to symbol size).

The effect of varying concentrations of Ru–Ab was evaluated by using working concentrations of 25, 15, 10, and 6.25 μg/ml (50 μl/well, 220 μl total volume) to achieve assay concentrations of 5.67, 3.41, 2.27, and 1.42 μg/ml. Although 5.67 and 3.41 μg/ml produced elevated ECL signals (Fig. 3), nonspecific signals also increased proportionately. Thus, signal/noise ratio was not increased (data not shown). A Ru–Ab assay concentration of 2.27 μg/ml resulted in the best combination of signal strength and signal/noise ratio and was therefore selected for subsequent use.

Performance of the assay with incubation times of 30, 70, and 150 min was also evaluated (Fig. 4). Increased incubation time resulted in increased slopes of the standard curves (30.2, 62.4, and 125.3, respectively) and increased ECL signal intensity. Comparison of ECL signals resulting from incubations of 30 and 150 min indicates that the increased incubation time resulted in approximately twice the nonspecific signal but more than three times the total signal. Thus, incubation time was standardized to 150 min for all subsequent assays.

Performance of optimized assay

The standardized assay conditions selected after optimization included a total volume of 220 μl consisting of assay buffer, 11.3 μg/ml of capture Ab-beads, 2.27 μg/ml of Ru–Ab, 100 μl of toxin standard or quality control sample, and an incubation time at room temperature of 150 min. The standard curves were linear over the entire range (1.1 to 45.4 ng/ml assay toxin concentrations, plus a 0 ng/ml toxin

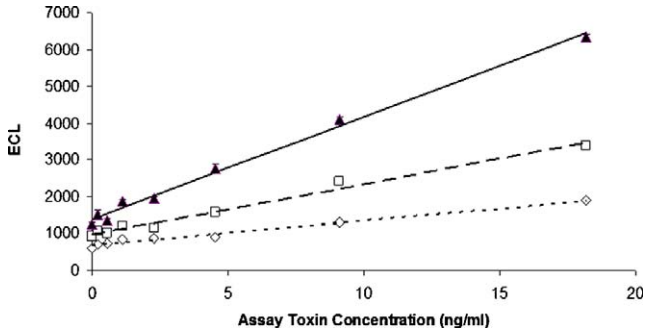

Fig. 4. Effect of incubation time on ECL assay for *C. perfringens* alpha toxin. Shown are the plots of ECL signals resulting from three different incubation times as a function of assay toxin concentration. Assay components included capture Ab-beads (11.4 μg/ml assay concentration), toxin (100 μl/well) at concentrations ranging from 0 to 40 ng/ml (0–18.2 ng/ml assay concentration), Ru–Ab (0.91 μl/ml assay concentration), and assay buffer. Assays were incubated for 30 min (◇), 70 min (■), and 150 min (▲) at room temperature with vigorous shaking. Plotted are linear regressions of means of triplicate determinations ± SE (SE bars not visible are minimal with respect to symbol size).

buffer control) with R^2 values of all standard curves used in the validation exceeding 0.98.

No significant matrix effects were observed, as the target values for the high (18.2 ng/ml), middle (9.1 ng/ml), and low (2.3 ng/ml) quality control samples were closely approximated in all three test matrices (Tables 1 and 2).

Table 1
Intraassay variability

Matrix	Target values of quality control samples		
	Low (2.3 ng/ml)	Mid (9.1 ng/ml)	High (18.2 ng/ml)
Assay Buffer	2.77 ± 0.54	10.32 ± 0.77	19.09 ± 1.59
Urine	1.95 ± 0.45	11.27 ± 0.64	18.68 ± 0.77
Serum	2.32 ± 0.59	10.54 ± 0.68	17.50 ± 2.41

Variability of repeated measurements of low, middle, and high alpha toxin concentration in assay buffer, human urine, and human serum was assessed in ($n = 8$ for each quality control value in each matrix a single assay) by one individual. Values shown are means ± standard deviations. Assay conditions were 150 min incubation time with a total volume of 220 μl containing assay buffer, 11.4 μg/ml capture Ab-beads, 100 μl toxin (1.1–45.4 ng/ml assay concentrations) or quality control sample, and 2.3 μg/ml Ru–Ab.

Table 2
Interassay variability

Matrix	Target values of quality control samples		
	Low (2.3 ng/ml)	Mid (9.1 ng/ml)	High (18.2 ng/ml)
Assay Buffer	3.14 ± 0.50	11.86 ± 0.68	18.64 ± 1.45
Urine	2.77 ± 0.73	11.27 ± 0.73	21.18 ± 1.27
Serum	2.41 ± 0.68	11.54 ± 0.68	17.27 ± 0.91

Variability of repeated measurements of low, middle, and high alpha toxin concentration in assay buffer, human urine, and human serum was assessed in a single assay ($n = 8$ for each quality control value in each matrix). Values shown are means ± standard deviations. Assay conditions were 150 min incubation time with a total volume of 220 μl containing assay buffer, 11.4 μg/ml capture Ab-beads, 100 μl toxin (1.1–45.4 ng/ml assay concentrations) or quality control sample, and 2.3 μg/ml Ru–Ab.

There were no consistent trends in deviations from the target values of the quality control samples with regard to any of the three matrices. The standard deviations of the means reflecting both intra- and interassay variation were minimal. The mean intraassay coefficients of variation ((standard deviation/mean)*100) including samples prepared in all three matrices were 22.8, 6.5, and 8.7% for the low, middle, and high quality control samples, respectively, with no significant differences among matrices at any control level. Mean interassay coefficients of variation for the low, middle, and high control samples were respectively 15.9, 5.7, and 7.8% for assay buffer, 26.2, 6.5, and 6.0% for urine, and 28.3, 5.9, and 5.3% for serum. Interassay coefficients of variation for all three matrices combined over three assays were similar with values of 23.5, 6.0, and 6.4%, respectively, for the low, middle, and high quality control samples with no significant differences between matrices at any of the three control levels. Assay sensitivity (lowest detectable toxin concentration) was defined as the lowest toxin concentration with a mean ECL (±SD) which does not overlap the range (mean ECL ± SD) of the 0 toxin control. The optimized assay was able to detect an assay toxin concentration of 1.1 ng/ml but not an assay concentration of 0.45 ng/ml.

Discussion

The goal of this research was to establish a rapid and sensitive assay for detecting and measuring *C. perfringens* alpha toxin in clinical matrices. Because all five subtypes of *C. perfringens* produce the alpha toxin, this toxin was the obvious target for assay development for clinical use. Potential use of the toxin as a weapon of bioterror further justified development of a rapid and sensitive assay for early detection.

The assay was designed as a direct assay using capture antibody immobilized to paramagnetic beads. The immobilization of the capture antibody was achieved by incubating biotinylated antibody with streptavidin paramagnetic beads. The avidity of the biotin-streptavidin interaction ensured very rapid and effective conjugation. In this format, the degree of saturation of beads with capture antibody will influence the sensitivity of the assay, because reduced capture potential will reduce the detection signal. To maximize the ECL signal, the magnetic beads were completely saturated by incubation with excess biotinylated antibody.

The total assay time was short as it required only one 2.5-h incubation period and no time-consuming wash steps prior to sample analysis. Shorter incubation periods resulted in a less-sensitive assay. Longer incubations were not evaluated as adequate sensitivity was obtained from nondiluted samples without the need for lengthening the incubation period.

The same affinity-purified antibodies were both biotinylated for use in toxin capture and ruthenylated for toxin detection. It was therefore critical to optimize these

reagents. Effective assay concentrations of these reagents were 11.4 μg/ml capture Ab-beads and 2.3 μg/ml Ru–Ab.

The ECL assay reported here had reasonable toxin sensitivity (lowest detectable concentration) of 1.1 ng/ml—suitable for detection of toxin in the upper LD_{50} toxin ranges. This sensitivity might allow detection of the toxin in clinical samples of infected patients and allow earlier treatment of infections. This sensitivity could also allow detection of toxin in the environment after a bioterrorism attack. The ECL assay described has a more than 7.5-fold increased sensitivity compared to the previously reported ELISA assay [17] which had been reported [17] to exceed the sensitivity for detection of alpha toxin by PLC activity or mouse lethality assay. It can also detect the toxin alone (i.e., does not require the presence of toxin-producing bacteria) as is required for PCR-based assays. This assay also eliminates the need for live animals required for lethality assays.

This assay was both accurate and reproducible throughout the selected assay range (1.1–45.4 ng/ml assay toxin concentrations). Accuracy was evidenced by the close approximation of the determined values for the three quality control samples and the target values in spiked samples of each matrix fluid (Tables 1 and 2). Reproducibility of the assay was demonstrated by the small standard deviations of the measurements of the quality control samples in the same assay (intraassay variation) and between assays on different days (interassay variation). The interassay variation between assays conducted on the same day and the variation between assays conducted on different days was not significantly different (data not shown). The coefficient of variation was somewhat greater for the low control than for the middle and high controls. This result was anticipated because direct assays inherently have more variability as the lower limits of detection are approached. Note that none of the three matrices tested here (buffer, human serum, and human urine) had significant matrix effects on the assay as the determined values of the three quality control samples in the three matrices were not significantly different from one another either in the same assay (Table 1) or in different assays (Table 2).

Conclusion

The goal of this investigation was thus achieved. A simple, rapid, and sensitive direct immunoassay with ECL detection that was both accurate and reproducible was developed. The assay is robust and was not subject to significant matrix effects by any of the matrices tested (buffer, serum, and urine). The assay shows potential for detecting and monitoring all five subtypes of *C. perfringens* infections via the organism's secretion of the alpha toxin virulence factor and would prove useful for monitoring environmental samples for *C. perfringens* toxin.

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