Application note

Increasing assay specificity of an existing clinical ELISA kit using Affimers as a capture surface for *Clostridium difficile* Toxin B

Summary

- Affimer reagents entirely specific to the virulent endotoxin form, toxin B, of C. difficile were identified.
- Substitution of Affimer reagents into a commercial diagnostic ELISA improved the specificity and sensitivity of the assay.
- Affimer binders used as diagnostics reagents can increase or confer assay specificity, sensitivity and stability.

Introduction

Clostridium difficile currently represents the most common cause of nosocomial infection in the US, accounting for up to 25% of all cases of antibiotic-associated diarrhoea.[1-3] Over the past twenty years an increase in the incidence and severity of *C. difficile* cases has been reported across Western countries, associated with increasing healthcare costs.[2,3] Recent estimates put the cost of hospital-management of this infection at $6.3 billion annually within the US alone.[4]

The main virulence factors responsible for *C. difficile* associated disease are two endotoxins: toxin A and toxin B. These toxins are proinflammatory and cytotoxic, disrupting the actin cytoskeleton and tight junctions of the intestinal epithelial cells and ultimately leading to cell death and loss of the intestinal barrier.[2,5,6] Most pathogenic strains of *C. difficile* produce both toxins (A^+B^+), yet analysis of variant strains of *C. difficile* carrying endotoxin mutations indicate that toxin B plays a major role in the pathogenesis of *C. difficile* infection. Consequently, toxin A^-B^+ strains of *C. difficile* are able to maintain virulence, but the reverse A^+B^- strains cannot.[2, 7]

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Clinical guidance for the diagnosis of *C. difficile* recommends a combination of two tests, one of which must include a sensitive toxin enzyme immunoassay.[8] The current commercially available toxin immunodiagnostic assays for the detection and diagnosis of *C. difficile* infection show limitations in terms of sensitivity and a high degree of cross-reactivity between the two endotoxins.[8] As much as a ten-fold greater sensitivity for toxin A over the primary virulence factor toxin B is exhibited by these assays (Fig. 1).

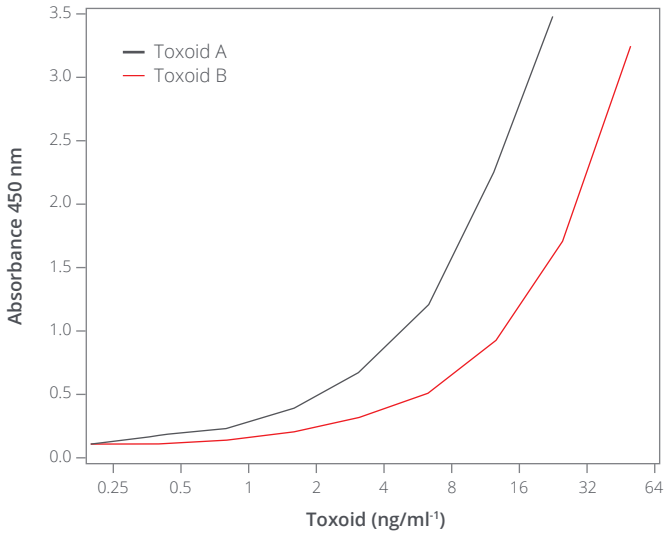

Figure 1: *Current diagnostic ELISA kits show a high degree of cross-reactivity between the two C. difficile endotoxins, with less sensitivity exhibited for the more virulent toxoid B, increasing the risk of misdiagnosis of this infection.*

As *C. difficile* toxin B is known to drive pathogenicity, detection and diagnosis should be focused on the more virulent of the two endotoxins.[2] Increased sensitivity for toxin A over toxin B within a diagnostic assay raises the potential for inaccurate diagnosis, delaying treatment and increasing patient suffering. A more sensitive and specific diagnostic assay is required, targeted to the virulence factor toxin B. This would allow for a more rapid and accurate diagnosis and treatment of *C. difficile* infection, reducing healthcare costs.

Within this study we aimed to develop a rapid ELISA-based assay for the specific detection of *C. difficile* toxin B using an endotoxin-capturing Affimer-coated surface to increase specificity whilst maintaining assay sensitivity.

Methods

Affimer reagents are recombinant protein binders that act as antibody mimetics. Binders are selected according to their desired characteristics from libraries of 10^{10} phage and have been shown to be highly target specific, functional when attached to solid support, highly thermally stable, and resistant to a wide range of solvents and pH conditions. Consequently, Affimer technology offers an ideal platform for the development of highly specific and stable reagents for use in rapid diagnostic assays to increase the potential specificity and sensitivity of these tests.

The Affimer screening process was employed to identify a binder specific for *C. difficile* toxin B that did not interact with toxin A. This constituted three successive phage panning rounds using toxin B as the target protein. The phage output was subsequently characterised via our rapid binding screening assay, where binding to toxin B, toxin A and both attenuated toxoid forms of the proteins, was assessed. Of the ten identified clones exhibiting the desired binding properties, the lead candidate Affimer binder was selected for further analysis and assay development.

Results

To establish the performance of the anti-toxin B Affimer reagent in comparison to a leading commercially available diagnostic ELISA assay for *C. difficile* toxin A/B, we developed a hybrid assay using the anti-toxin B Affimer binder as a capture reagent. Immunoassay plates were coated with the anti-toxin B Affimer reagent at a concentration of 2.5 µg/mL and exposed to the attenuated form of toxin B, toxoid B, over the concentration range 0.195-50 ng/mL. Following sample incubation, the HRP-conjugated detection antibody from the commercially available *C. difficile* toxin A/B diagnostic ELISA was added to each sample well and absorbance measured at 450 nm.

The Affimer reagent capture surface was entirely specific for the more virulent toxoid B, showing no interaction with toxoid A (Fig. 2). A side-by-side analysis of both the commercial polyclonal capture surface and the Affimer capture surface for both native toxin B and toxoid B yielded comparable sensitivity for both target proteins. Thus, the inert toxoid B form was used for further experiments.

Comparing the toxin B Affimer capture surface with the manufacturer polyclonal anti-toxin A/B antibody surface showed an improved performance of the Affimer surface over the optimised antibody surface in terms of sensitivity for toxin B (Fig. 3). This was achieved with no deviation from the manufacturer's protocol, allowing the Affimer surface to be directly incorporated into the assay for increased assay specificity and sensitivity, with no change to the workflow. Use of the manufacturer's ELISA kit reagents and protocol enabled detection of *C. difficile* toxoid A to a lower limit of approximately 100 pg/mL and toxoid B to a lower limit of approximately 200 pg/mL. Substituting the Affimer reagent as a capture surface within this assay improved the limit of detection to 73 ± 6 pg/mL for toxoid B, demonstrating the high specificity and increased sensitivity towards the more virulent endotoxin form.

The thermal stability of the anti-toxin B Affimer binder was determined by monitoring both the intrinsic fluorescence and extrinsic fluorescence (with SYPRO Orange dye) during a thermal ramp in the Optim platform (Fig 4). Neither measure displayed an unfolding transition over the temperature range studied, indicating a T_m value for the Affimer of greater than 90 °C. Static light scattering (SLS) measurements, that monitor aggregation state during the thermal ramp, detected no aggregation of the Affimer proteins under these conditions. The high stability demonstrated ensures robust performance of Affimer reagents incorporated into diagnostic assays across a range of assay conditions.

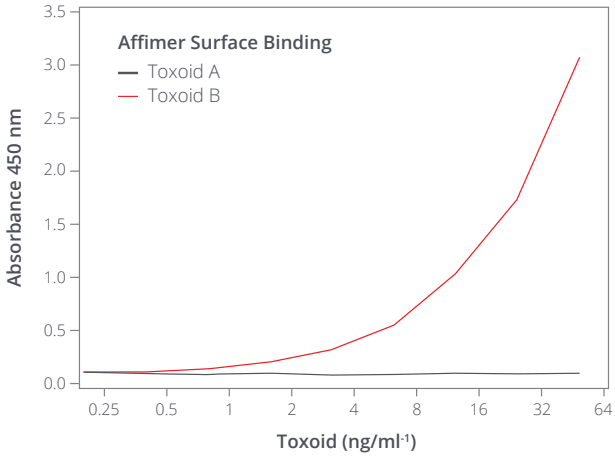

Figure 2: *The selected Affimer reagent is specific for C. difficile toxoid B and shows no interaction with the less virulent toxoid A form. An Affimer coated surface was used as a capture reagent for the bacterial toxoids, in conjunction with the detection antibody from a commercially available diagnostic ELISA.*

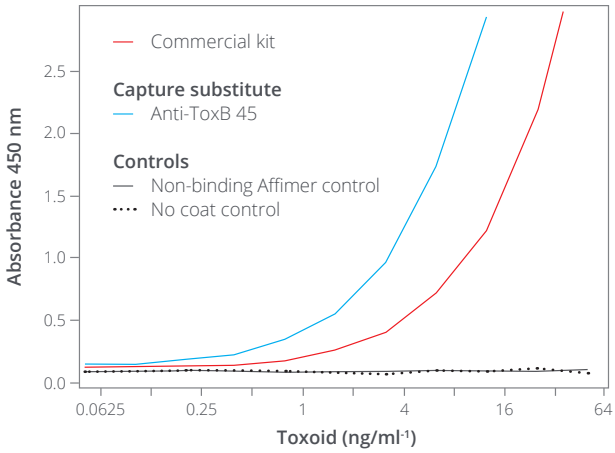

Figure 3: *Substituting the toxinA/B-capturing polycolonal antibody within a commercially available ELISA with a toxin B-specific Affimer improves assay sensitivity while introducing specificity towards toxin B (Fig. 3).*

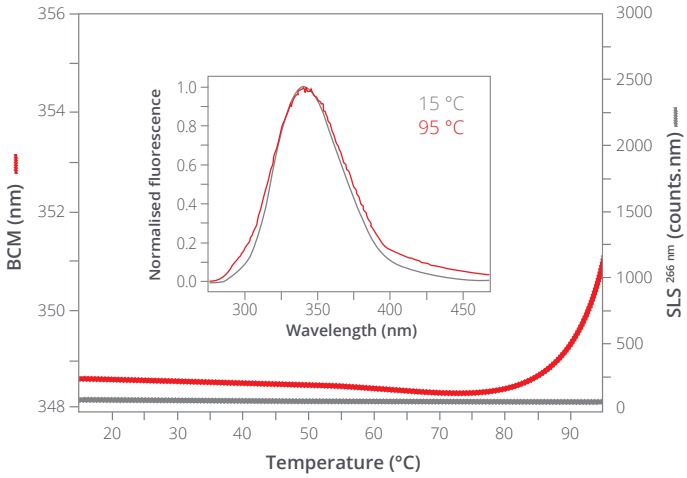

Figure 4: *The anti-toxin B Affimer binder displays high thermal stability. No complete transitions were observed in simultaneous measurements of intrinsic fluorescence (red line and example spectra) and SLS (grey) over the temperature range studied.*

Conclusion

Within this study we were able to develop highly specific binders to *C. difficile* toxin B that showed no interaction with the commonly cross-reactive toxin A. Using the selected Affimer binder we introduced toxin B specificity while maintaining the sensitivity of a leading, commercially available diagnostic ELISA test for *C. difficile* infection. Cross-reactivity between the two endotoxin forms within current commercially available ELISA assays prevents accurate diagnosis of *C. difficile* infection. While the majority of infectious *C. difficile* strains produce both endotoxins, only toxin B is able to maintain the virulence of infection.[2, 7] Using Affimer reagents to accurately distinguish between the different strains of infectious bacteria could allow for more rapid and effective treatment of patients and reduce healthcare costs, particularly within the context of increasing antibiotic resistance.

Affimer binders as capture reagents can increase or confer specificity to existing assays. This offers potential performance improvements to many research and diagnostic assays.

Affimer reagents are ideally suited for incorporation into diagnostic assays, as they exhibit high stability and robustness to a range of experimental conditions, including temperature and pH. These characteristics ensure their assay function without the need for complex storage conditions. Additionally, as Affimer reagents comprise of recombinant technology, with no batch-to-batch variability, the long-term supply of any selected Affimer reagent is assured, reducing the cost and timescales associated with standardising new reagent lots for inclusion in any assay.

References

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