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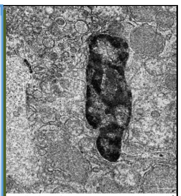

TEM liver image of *Puntius javanicus* exposed to 5 p.p.m. Cu

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Determination of the Detection Limit using the Four-Parameter Logistic Model for The Double-Antibody Sandwich ELISA for the Rapid Detection of *Bacillus cereus* in Food

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ABSTRACT

Bacillus cereus is increasingly recognized as one of the major causes of food poisoning in the industrialized world. In a previous publication, a sandwich ELISA is developed for the detection of *B. cereus* whole cells. The curve showed a sigmoidal calibration curve, but the model used was linear. This study remodels the sigmoidal data obtained using a four-parameter logistic (4PL) equation that can cover the whole range of data obtained. The sigmoidal ELISA calibration curve was efficiently fit by the 4-PL equation. A good correlation coefficient value of 0.996 was obtained indicating good fitting. The LOD value was 5.36 x10^4 CFU/mL with the 95% confidence interval from 1.07x10^4 to 9.64x10^4 CFU/mL, which is about one order of magnitude less sensitive that the previously claimed LOD. The modelling exercise shows that the 4PL equation can fit the ELISA curve very well.

INTRODUCTION

Bacillus cereus is a gram-positive, aerobic-to-facultative, spore-forming, rod-shaped bacterium and has been indicated as the causative agent of food poisoning [1]. Although it has been estimated that the minimal level of the bacterium that can cause illness related to food poisoning is about 10^5 colony-forming units (CFU)/g of food, there are reports of emetic syndrome caused by a concentration of as low as 10^3 CFU/g of food [2], which prompted for the development of an even more sensitive detection methods.

One such method is the Enzyme-linked immunosorbent assay (ELISA), which is a specific, sensitive method and has been conveniently used to routinely detect *B. cereus*. To date, commercial kits are not yet available to detect whole cells of the bacterium [3].

In a previous publication, the *B. cereus* whole cells were utilized as immunogens to generate rabbit polyclonal and mouse monoclonal antibodies against *B. cereus*. These antibodies were then used to develop a sandwich ELISA for fast, direct and convenient measurement of *B. cereus* contamination of food. The curve showed a sigmoidal calibration curve but was not modelled according to any of the sigmoidal models available [3]. The objective of this study is the remodel the ELISA data using the standard 4-PL model and to determine the Limits of Detection (LOD) based on the standard method.

MATERIALS AND METHODS

Acquisition of Data

Data from the works of Zhu et al. [3] from figure 6 showing a double-antibody sandwich ELISA for the determination of *B. cereus* using monoclonal antibody 2D2 as the detecting antibody and rabbit polyclonal antibody as the capturing antibody. The data were processed using the software Webplotdigitizer 2.5 [4] which digitizes the scanned figure into a comma separated data and has been utilized by many researchers and acknowledged for its reliability [5,6].

Four parameter logistics modelling

A non-linear regression using four-parameter logistic equations based on least square fitting [7] was utilized to fit the curve as follows;

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - x)*\text{Hillslope}}}$$

where y is the absorbance obtained (nm), x is the concentration of *B. cereus* (log unit), a and d are the maximum and minimum responses (nm), respectively, Log EC_{50} is the value that produces a 50% signal response, and Hillslope is the slope-like parameter (Hill coefficient). Regression analysis using the four-parameter logistics model was calculated using the PRISM software (v 5.0) available from www.graphpad.com.

The limit of Detection (LOD) was calculated based on the value of three times the standard deviation of the blank value or the lowest concentration of *B. cereus* utilized. These values were then interpolated from the sigmoidal dose-response 4-PL equation, and the corresponding concentration of *B. cereus* value was then calculated including the confidence interval.

RESULTS AND DISCUSSION

ELISA-based standard curves are generally nonlinear and sigmoidal in property, and the best way to fit this kind of curve is to use a standard four-parameter logistic (4-PL) or the rarely used five parameter logistic (5-PL) models [8]. The raw data should then be fitted to the 4-PL curve through a modification of the curve model's parameters to achieve an ideal fitting between experimental and calculated data; the latter is often represented by a line running through the experimental data [9]. Although a patently sigmoidal profile was obtained by Zhu et al. [3], the authors resorted to the use of a linear regression model which resulted in the linear equation y = 0.612x - 1.698, giving a correlation coefficient or R^2 value of > 0.99. They reported a detection limit of 0.9×10^3 cells/mL.

The result in **Fig. 1**. shows a typical sigmoidal curve for the ELISA calibration curve based on the 4-PL equation. A typical sigmoidal profile was obtained. A good correlation coefficient value of 0.996 was obtained indicating good fitting.

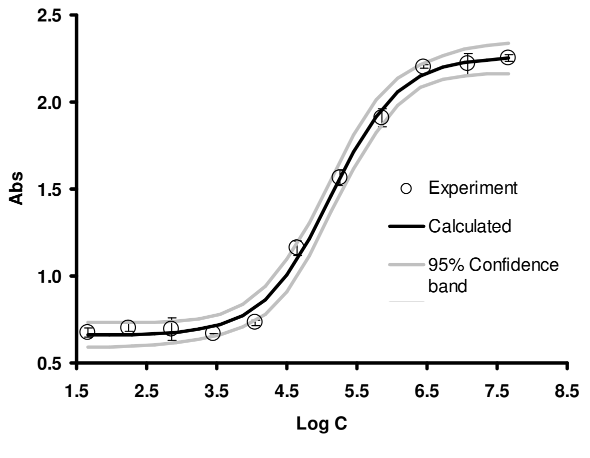

Fig. 1. A double-antibody sandwich ELISA for the determination of *B. cereus* modelled according to the four-parameter logistic equation, and its 95% confidence band.

The LOD value was 5.36 $\times 10^4$ CFU/mL with the 95% confidence interval from 1.07×10^4 to 9.64×10^4 CFU/mL. This a bit higher than the LOD approximated in the works of Zhu et al. [3] where they reported a LOD value of 0.9×10^3 cells/mL without mentioning the confidence interval of the LOD value, which is very important for comparative purpose. The value of the curve parameters is shown in the form of the four-parameter logistic equation as follows;

$$y = 0.6604 + \frac{1.599}{1 + 10^{(5.134 - x)*0.8904}}$$

The LOD value obtained through the 4PL modelling exercise shows that the developed method is one order of magnitude less sensitive than the reported LOD based on linear regression. As LOD values are advised to be calculated based on the 4-PL method in the event the curve shows a patently sigmoidal profile, the LOD value obtained through the 4PL modelling method should be used to report the LOD value.

CONCLUSION

In conclusion, in the event where an ELISA calibration curve exhibits a sigmoidal profile, the 4PL model should be used to fit the data instead of a linear model and the LOD value calculated using the 4PL model. In this study, the use of the 4Pl model was a success and was able to model the whole date curve instead of only the linear portion of the curve. The linear portion is important as a convenient and rapid method to assess the sensitivity of a developed ELISA method and is usually a more valuable method in field application where a fast and simple assessment is needed. However, the 4Pl model should not be abandoned as it is able to accurately report the LOD value and its 95% confidence interval for any developed method.

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