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Detection Limit Determination using the Four-Parameter Logistic Model for the Ultrasensitive Detection of *Vibrio cholerae* DNA with Polystyrene-coacrylic Acid Composite Nanospheres

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ABSTRACT

Standard laboratory techniques for isolating and identifying *V. cholerae* can take up to three days and need a well-equipped laboratory with highly experienced personnel. If cholera epidemics go unnoticed for a lengthy period of time, inadequate public health measures, disease spread, and increased death and morbidity are all possible. Cholera cases must be detected and confirmed as soon as feasible using sustainable and precise approaches in order to provide necessary support. Biochemical diagnostic techniques for this pathogen, such as antigen binding, rely on biomolecular interactions as its diagnostic modality, resulting in more complex calibration curves. Additionally, these tests frequently employ sigmoidal curves. When there is asymmetry, a logistic (5PL) or logistic (4PL) curve may be the best representation of a separate sigmoidal relationship. It is possible that linearizing an otherwise nonlinear connection by log transformation disrupts the curve's error structure, having the opposite effect of reducing or even eliminating error in the relationship. The aim of this study is the remodel the data from a calibration curve for the detection of *Vibrio cholerae* DNA by means of polystyrene-coacrylic acid composite nanospheres using the standard 4-PL model and to determine the Limits of Detection (LOD) based on two methods; the classical definition of limits of detection method and the method based on pooled standard deviation (PSD). The LOD value obtained through the 4PL modelling exercise based on the classical method was 0.255 pM (95% confidence interval of 0.167 to 0.379) while the PSD method yielded an LOD value of 0.035 pM (95% confidence interval of 0.011 to 0.067), which indicates that the PSD method was superior. The classical method was higher than the rough estimation employed originally with an LOD value of 0.125 pM. The use of the 4PL method based on the PSD method was more reliable and robust in estimating the LOD values.

INTRODUCTION

A significant limitation of the current literature on bioassay creation is the absence of statistically rigorous methods for determining the limit of detection. As an alternative, researchers frequently employ crude detection-limit methods that are just approximate representations of the true detection limit. We can only presume that this is due to a practical requirement for streamlined techniques, as well as the fact that the theoretical limit of detection for bioassays has already been lowered in practise [1–3]. The current body of knowledge on bioassay development does not incorporate statistically robust methodologies for assessing a test's limit of detection, which is a serious drawback. Rather than that, researchers frequently employ fundamental techniques that provide an approximation of the detection limit, sometimes without providing any indication of confidence in the estimate. This dearth of robust techniques is likely due to both a practical desire for simple and accessible procedures and a dearth of such ways that have reduced the concepts of limit of detection theory to practise for bioassays [4].

Cervical cancer is an international public health problem that affects women of all ages. The illness is the second most common cancer in women globally, and it is the fourth greatest

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cause of cancer-related deaths among women [5,6]. FIGO reports that cervical cancer has a 5-year recurrence rate of 28 percent and a 5-year total death rate of 27.8 percent, respectively, among women who have had the disease for five years. According to Muoz et al., squamous cell carcinoma (SCC) is the most frequent histological form of cervical cancer, accounting for 60–80 percent of all cases [5]. It was Kato and Torigoe that discovered Squamous cell carcinoma antigen (SCCa), a glycoprotein with isoforms varying in size from 45 to 55 kDa, and identified it as a tumor-associated antigen for the first time[7,8]. Since its discovery, SCCa has been discovered to be raised in a variety of SCCs, including those of the uterine cervix, lung, oral cavity, skin, oesophagus, head and neck, oesophagus, anal canal, and vulva [9,10].

Patients with SCC of the uterine cervix are increasingly using SCCa as a particular tumour marker for immunostimulatory diagnosis, predictive risk appraisal, treatment monitoring, and follow-up of recurrence in order to improve their overall survival [11]. Nevertheless, the clinical application of SCCa as a diagnostic marker for cervical cancer initial detection until care is seriously constrained by the huge disparity in detectability, which varies from 28 percent to 88 percent as per various studies, based on a variety of inclusion criteria, inconsistent cut-off values, and methodological defects [12].

Previously, an electrochemical biosensor for the detection of the determination of pathogenic *Vibrio cholerae* (V. cholerae) DNA using nanoparticle technology was developed. However, based on the calibration curve, it conforms to the majority on sigmoidal shape curve for biological receptor-type sensing system. The resultant curve showed a sigmoidal calibration curve but was not modelled according to any of the sigmoidal models available [13]. The objective of this study is the remodel the data using the standard 4-PL model and to determine the Limits of Detection (LOD) based on the two methods; the classical definition of limits of detection method and the method based on pooled standard deviation (PSD).

METHODS

Acquisition of Data

Data from a published work [13] from figure 6b showing *Vibrio cholerae* DNA biosensor calibration curve. The data were processed using the software Webplotdigitizer 2.5 [14] which digitizes the scanned figure into a comma separated value [15].

Four parameter logistics modelling

In the assay, the calibration curves will be fitted with a non-linear regression using a four-parameter dose-response equation [16,17] as following:

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

Where y signifies the response signal (optical density), x signifies the DNA log concentration, a and d signify the maximum and minimum signal response of the calibration curve, correspondingly, b is the Hill coefficient which represents the slope-like parameter and c represents the DNA log concentration producing a 50% signal response (EC_{50}) value. Both the classical three times the standard deviation of the blank and another statistically robust technique for estimating the analytical LOD of a classic sigmoidal correlation based on the pooled standard deviation of datapoints will be utilized [2]. In the traditional LOD determination, a single test concentration which is usually taken from the lowest tested concentration or control will be utilized to obtain the standard deviation of the blank.

Most assays are carried out using fewer than 10 replicates per concentration with the norm is three replicates per concentration level. Anything less than 10 replicates per concentration have been shown to give a less accurate representation of the population variance and recommendation is to pool the standard deviation for all test samples [2].

To account for the variability in variance when there are less than 10 replicates per concentration level, the data points can be weighted by inverse variance. Due to the fact that this strategy is not suggested when there are fewer than 10 replicates per concentration level, an unweighted fit was utilised in this particular instance instead. In addition, it should be emphasised that the unweighted fit is consistent with the assumption of homoscedasticity that was established for the pooled estimate of variance that was originally applied. The limit of detection (LOD) will be determined by calculating the mean value of absorbance at a blank concentration of DNA log concentration at three successive PSDs. For the purpose of calculating the LOD and performing regression analysis, the four-parameter logistics model and non-linear regression analysis software will be used in conjunction (PRISM, v 5.1) from www.graphpad.com.

RESULT AND DISCUSSION

Cholera continues to be a severe public health issue in a number of countries worldwide. 1 In 2017, 34 countries reported a total of 1,227,391 cases and 5654 deaths, yielding an overall worldwide mortality rate of 0.5% [1. Other notable epidemiological occurrences in the history of cholera include the first outbreak of cholera in Latin America in more than a century [2, 3], the first appearance of Vibrio cholerae O139 in Asian countries [3, 4], and the first appearance of hybrid El Tor strains in Asia and Africa [3, 4].

Haiti's cholera pandemic began in October 2010 and ended in May 2017, with 8,09,000 cases and 9670 deaths [5] recorded throughout that time period. Approximately 1700 people died from cholera in Yemen between April and July 2017, making it the world's second-largest cholera pandemic in history [6] after the Haitian cholera epidemic. Hence, there needs a sensitive detection method for this disease and the use of biosensor based on nucleic acid continues to be an emerging sensitive, rapid and cost-efficient technique.

ELISA-based and biological receptors-based or DNA-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 [18]. 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 [19]. Although a patently sigmoidal profile was obtained by [13], the authors reported that the detection limits (LODs), calculated as the lowest amount of DNA that is able to produce a signal distinguished from the blanks were between 110 to 460 mg/g (0.011–0.046%).

The result in **Fig. 1**. shows a typical sigmoidal curve for the calibration curve based on the 4-PL equation. A typical sigmoidal profile was obtained. A good correlation coefficient value of 0.965 was obtained indicating good fitting.

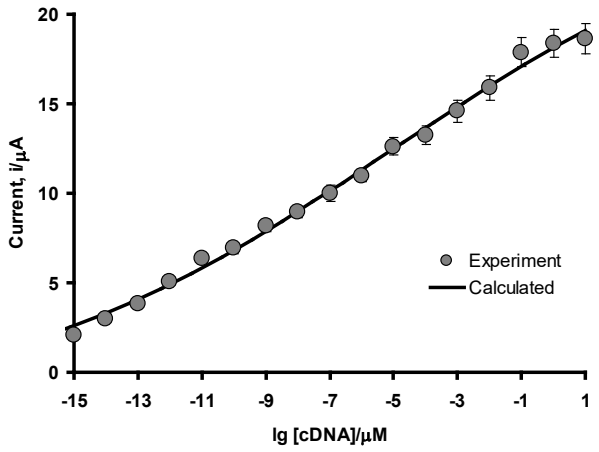
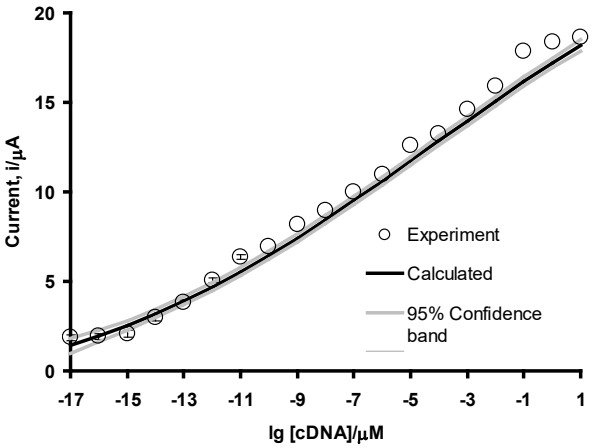


Fig. 1. The calibration curve for the detection of GMO using the Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs.



Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs calibration curve, and its 95% confidence band.

The value of the curve parameters is shown in the form of the four-parameter logistic equation as follows;

$$y = -2.428 + \frac{29.83}{1 + 10^{(-4.993-x)*0.0.07}}$$

The LOD value obtained through the 4PL modelling exercise based on the classical method was 0.255 pM (95% confidence interval of 0.167 to 0.379) while the PSD method yielded an LOD value of 0.035 pM (95% confidence interval of 0.011 to 0.067), which indicates that the PSD method was superior. The classical method was higher than the rough estimation employed by [13] with an LOD value of 0.125 pM. Because it is recommended that LOD values be computed using the 4-PL technique in the case that the curve has an obviously sigmoidal profile, the LOD value derived using the 4PL modelling approach should be utilised to report the LOD value in this situation.

Last but not least, in the event that the calibration curve has a sigmoidal profile, the 4PL model should be used to fit the data rather than a linear model, and the LOD value should be derived using the 4PL model rather than a linear model. According to the findings of this study, the adoption of the 4Pl model proved successful, as it was able to represent the entire date curve rather than just a linear section of it. When developing an ELISA technique, the linear part is crucial since it is a handy and speedy approach for determining the sensitivity of the method. It is also typically a more beneficial method in field applications when a quick and easy assessment is required. The 4Pl model, on the other hand, should not be abandoned because it is capable of reporting the LOD value and its 95 percent confidence range for any created technique with high accuracy.

CONCLUSION

Standard laboratory procedures to isolate and identify *V. cholerae* can take up to three days and require a well-equipped laboratory and highly trained staff. Cholera cases must be identified as soon as possible and confirmed in order to perform the appropriate assistance. PCR-based biosensor technique is amongst the most sensitive and accurate method for detection of this pathogen. Biosensor works for this pathogen shows a sigmoidal profile, but nonlinear regression was not utilized to estimate the LOD values. The LOD value obtained through the 4PL modelling exercise based on the classical method was less sensitive compared to the PSD method. It is recommended that LOD values be computed using the 4-PL technique in the case that the curve has an obviously sigmoidal profile, the LOD value derived using the 4PL modelling approach should be utilised to report the LOD value in this situation.

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