

Detection of *Pyricularia oryzae* Based on Indirect ELISA Using Germinating Conidia as the Antigen Preparation

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ABSTRACT

One of the most destructive fungal diseases in rice crop in Malaysia is the Rice blast disease or Penyakit Karah, which is caused by *Pyricularia oryzae* Cav. (previously known as *Magnaporthe grisea*). The disease can cause a loss of yield of more than 50 percent. In this study a sensitive indirect ELISA method was developed for the detection of *Pyricularia oryzae* through an indirect ELISA method using rabbit polyclonal antibody against different concentrations of *Pyricularia oryzae* conidia to give seven calibration standard points ranging from 1×10^1 to 1×10^9 conidia/mL. The calibration curve shows a typical sigmoidal curve and the fitting of the curve was based on the four-parameter logistic equation. The calculated LOD value was 21.60 conidia/mL (95% confidence interval, 11.63 to 31.57 conidia/mL) with a good correlation coefficient value of 0.999 indicating the model fit the experimental data in an excellent manner. The sensitivity was comparable to another published work which claimed to be the most sensitive to date. This indicate that the current developed method can be utilized in the future as a sensitive method for the rapid and simple detection of this pathogen.

INTRODUCTION

Rice diseases are probably the most important restricting elements which affect rice manufacturing, leading to yearly yield loss conservatively believed at 5%. Greater than Seventy diseases brought on by fungi, bacteria, viruses or nematodes have already been documented on rice amongst which rice blast is regarded as the severe of disease impacting rice production. Rice blast disease or Penyakit Karah, is caused by *Pyricularia oryzae* Cav. (previously known as *Magnaporthe grisea*). It is the most destructive of fungal diseases in rice crop and has caused significant yield losses worldwide including Malaysia with potential yield losses of more than 50 percent.

Pyricularia oryzae Cav. (previously known as *Magnaporthe grisea*) is the cause of rice blast disease or Penyakit Karah; the most destructive disease of fungal origin to rice crop. Significant yield losses of more than 50 percent has been reported worldwide including Malaysia [1]. The cycle of this fungal disease

commences whenever a blast infects and generates a lesion on all the parts of the shoot, in addition to stem rot and panicle blight. The infection concludes when the fungus sporulates and produces fresh air-borne spores. Rice blast is classed into leaf blast, seedling blast, neck blast, rice node blast, and corn blast based on the areas of the afflicted sites [2]. Movements of this disease from diseased to healthy plants occur through the airborne transfer of spores to nearby fields. A visible evaluation is not adequate to differentiate between the pathogenic and non-pathogenic spores [3]. At the moment, pathogenic spores are only able to be verified in a the least of between five and six days after the progression of lesion on the rice crops. As soon as noticeable lesions are observed on rice crops, it might be far too late to eliminate rice blast through the use of fungicide creating the devastation of most or huge area of the whole crop [4].

These days, spraying fungicide is among the most favored ways of controlling rice blast. Nevertheless, rigorous spraying demands a lot of fungicide which usually raises price,

contaminates the surroundings and results in the introduction of multi-resistant fungi strains [5]. In certain nations around the world spore traps are utilized to forecast rice blast outbreaks. Nonetheless, the accessible traps are costly and predictive data depending on the number of spores caught can be applied to the immediate area merely [1].

Consequently, precise and fast detection of rice blast disease is important for efficient disease management. It allows a more knowledgeable judgement to be made about cultivar selection and just how and whenever fungicide may be used most efficiently to manage disease outbreaks. Conventional methods to rice blast detection usually involve the decryption of visible symptoms of the disease [6]. This can be accompanied by research laboratory recognition, comprising identifying from the diseased tissue the fungus or from trapped spores, culminating in the culturing of the fungus to produce spores and eventually inoculating rice plants that are predisposed to the disease. However, this conventional methods are not only difficult, laborious, and required highly trained personnel [1–3,7–9]. To overcome this issue, molecular identification methods such as polymerase chain reaction (PCR) and microscopic observation have been developed. Nonetheless, this technique also suffers a major disadvantages including time-consuming, costly and prone to cross contamination [1].

Taking into account of all of these hurdles, rapid diagnostic tests based on immunoassay are needed. However, work in this area is very rare with one of the few developed methods such as a previous work using a monoclonal approach was successful in detecting as low as 10 to 20 conidia [8]. Monoclonal antibody is expensive and difficult to be produced at a large scale and from other works on bacteria detection have shown that a polyclonal preparation can be as sensitive or more sensitive than a monoclonal preparation [10]. We have prepared a polyclonal preparation for this pathogen previously [3] but have not resort to construct a calibration curve for this pathogen. The aim of this study is hence to construct a calibration curve for this pathogen using the popular four-parameter logistic model (4PL) and determine the limits of detection (LOD) together with its 95% confidence interval.

MATERIALS AND METHODS

Preparation of polyclonal antibody against *Pyricularia oryzae*
Polyclonal antibody against *Pyricularia oryzae* was produced using the conidia as the antigen [3].

Indirect ELISA

Growth and maintenance of *Pyricularia oryzae* and indirect ELISA was carried out as before [3]. Microtiter plate wells was coated with 100 μ L of *Pyricularia oryzae* conidia at various concentrations in 100 mM carbonate buffer at pH 9.6. The plate was incubated at 4 $^{\circ}$ C for 24 h. The microplate washed with 10 mM PBS, pH 7.4, with 0.05% Tween 20 buffer (PBS-T) three times. A 1% solution of BSA diluted in PBS (200 μ L per well) was utilized to block unoccupied sites and incubation was carried out for 2 h at 37 $^{\circ}$ C. A 1:1000 diluent of Polyclonal antibody (100 μ L) in 10 mM PBS, pH 7.4, containing 1% BSA and 0.05% Tween 20 buffer were added per well and further incubated for 2 h at 37 $^{\circ}$ C.

The microplate was washed three times after the incubation period had elapsed. A 1:8,000 dilution of Goat-anti rabbit IgG-AKP (alkaline phosphatase) that forms the secondary antibody conjugate and Goat-anti mouse IgG-AKP, a conjugate that is specific to mouse monoclonal antibody were added into the wells

at 50 μ L each and incubated for 2 h at 37 $^{\circ}$ C. The microplate was washed three times after the incubation period had elapsed. p-nitrophenyl phosphate (pNPP) a substrate of AKP was added into each well at 100 μ L and then incubated in the dark for 30 min at room temperature. Then, 1 M NaOH (50 μ L per well) was added to stop the reaction. The formation of the yellow product was determined at 405 nm (BioRad 680 microplate reader, BioRad, USA). The experiments were in triplicates.

Limit of detection (LOD) calculation for ELISA method

A non-linear regression using four-parameter logistic equations based on least square fitting [11] was utilized to fit the curve as follows;

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

where y is the absorbance obtained, x is the concentration of conidia (log unit), a and d are the maximum and minimum responses, respectively. Log EC_{50} is the value that produces a 50% signal response and Hillslope is the slope-like parameter (Hill coefficient). the mean value of absorbance at a blank concentration of bacteria at three standard deviations (SD) was utilized to calculate the limit of detection (LOD). Regression analysis using the four-parameter logistics model was calculated using the PRISM software (v 5.0) available from www.graphpad.com.

RESULTS

The construction of standard curve for the detection of *Pyricularia oryzae* through indirect ELISA method was carried out using the rabbit polyclonal antibody against different concentrations of *Pyricularia oryzae* conidia to give seven calibration standard points ranging from 1×10^1 to 1×10^9 conidia/mL. The result in **Fig. 1** shows a typical sigmoidal curve based on the four-parameter logistic equation. There was no increase in binding response observed at conidia concentrations from 1×10^0 to 1×10^2 conidia/mL. A typical sigmoidal profile was obtained. The calculated LOD value was 21.60 conidia/mL (95% confidence interval, 11.63 to 31.57 conidia/mL) with a good correlation coefficient value of 0.999. The final four-parameter logistic equation is as follows;

$$y = 0.1334 + \frac{3.555}{1 + 10^{(4.05 - x) \cdot 0.998}}$$

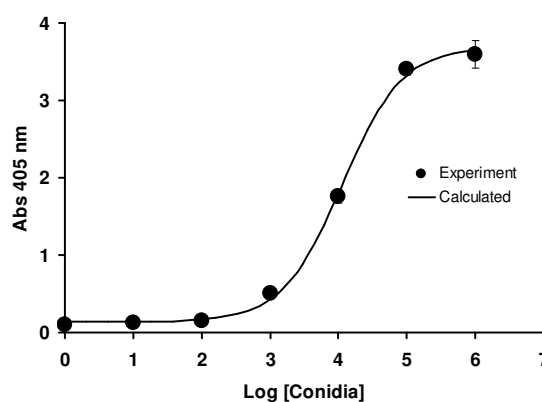


Fig. 1. Standard curve constructed for the detection of *Pyricularia oryzae* through an indirect ELISA method. Error bars represent the average \pm standard deviation of triplicates.

DISCUSSION

Currently, detection for this pathogen in the field involved visual examination, which is not sufficient to discriminate between the pathogenic and non-pathogenic spores. Presently, pathogenic spores can only be confirmed after a minimum of five to six days later by the development of lesion on the rice plants. By the time visible lesions have developed on the rice plants, it may be too late to eradicate rice blast through the application of fungicide resulting in the destruction of all or large portion of the entire crop. Therefore, the development of a simple, inexpensive and accurate enzyme immunoassay screening kit for detecting *P. oryzae* in paddy field is highly desirable in deciding upon appropriate control for this fungus. In this work we have prepared germinating conidial suspension at concentration of 10^8 conidia/mL as an antigen for immunization in rabbits for the production of polyclonal antibody.

The application of the indirect ELISA assay provides several positive aspects for example; i) a heightened in level of sensitivity because of the use of multiple labelled antibody per primary antibody, ii) the assay provides versatility because numerous diverse primary detection antibodies and secondary labelled antibody may be used, and iii) overall economy, as much less labelled antibodies will have to be used.

The result obtained in this study is comparable to a previous work on the detection of *Pyricularia grisea* using a monoclonal preparation with a successful detection of as low as from 10 to 20 conidia [8]. Even though the utilization of monoclonal as the capturing antibody is generally a lot more specific and provides greater binding affinity as compared to polyclonal normally, the usage of monoclonal has its own disadvantages as not merely it can be pricey, it can occasionally provide bad effectiveness in comparison to a polyclonal antibody. For instance, a monoclonal preparation for the detection of the pathogen *Francisella tularensis* in the indirect format demonstrated a big difference in absorbance of 0.15 between the lowest and highest determination range while the polyclonal preparation offered a difference in absorbance of 0.45, with the limit of detection of 5.4×10^6 CFU mL^{-1} for polyclonal antibodies and 6.9×10^6 CFU mL^{-1} for monoclonal antibodies [12]. In addition, the detection of the food pathogen *C. jejuni* also shows a much better sensitivity when a polyclonal preparation is used while the commercially available monoclonal preparation performs poorly [10]. Hence, the development of a simple detection method for this pathogen based on an indirect ELISA is hoped to improve the time and sensitivity of detection of this pathogen.

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