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Lateral Flow Immunoassay Detection of *Ralstonia syzygii* subsp. *celebensis* Causing Banana Blood Disease in Symptomatic and Asymptomatic Banana Plants

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ABSTRACT

Banana blood disease caused by *Ralstonia syzgii* subsp. *celebensis* (*Rsc*) is one of the most well-known and most critical phytopathogenic bacteria which causes severe bacterial wilt in banana and considerable economic losses to the growers. Unlike common bacterial wilt caused by *R. solanacearum*, *Rsc* did not cause infection in solanaceous plant. The banana blood disease caused by the bacteria is fatal to the banana industry in Malaysia and other parts of the world. In an effort to better control the disease, a swift, sensitive, and user-friendly method for routine diagnostics is essential. This study aimed to raise polyclonal antibodies (pAb) from rabbit against whole cells of *Rsc* and to develop a lateral flow immunoassay (LFIA) strip utilising the purified IgG to detect the pathogen. We have developed the *Rsc* LFIA strips and obtained the sensitivity limit of 10^4 CFU/mL *Rsc*. The strip performance in real samples were performed using samples from symptomatic and asymptomatic banana plants consisting of samples from the pseudostems, petioles and leaves. The LFIA results were validated using polymerase chain reaction (PCR) with 100% correlation. The results showed that the strips were able to detect *Rsc* in all symptomatic samples. Additionally, positive result was obtained from the pseudostem of the asymptomatic samples. This study indicated the capability of the LFIA strips to detect *Rsc* not only in symptomatic plants but also before visible symptom appearance was observed which offered the potential for the LFIA strip to be used as a handy screening tool to detect *Rsc*.

Keywords: *Ralstonia syzygii* subsp. *celebensis*; immunochromatographic; *Musa* sp.; plant disease.

INTRODUCTION

In terms of production and trade volume, bananas (*Musa* sp.) are the most important fruit crop in the world (FAOSTAT, 2014). Banana blood disease (BDB) or blood disease bacterium of banana as the preferred common name, has caused a severe threat to the banana industry in Malaysia and other parts of the world. In the early 1900s, the possible danger of blood disease to bananas was recognised when it caused the production of dessert banana plantations in the Salayar Islands to be abandoned. The disease has been widely spread following its discovery in Java in the late 1980s and later was recorded from North Sumatra in the west to 3500 km distance in Irian Jaya, east of Indonesia. The mechanism for such rapid dissemination is unclear, but a combination of insect transmission and human activity appears likely. The disease was first

identified in Peninsular Malaysia in Perak and later in Selangor (Heng, 2012). Four years later, the disease was reported in Sabah between 2012 and 2013 (IPPC, 2016).

Ralstonia solanacearum is the pathogenic bacterium responsible for causing plant bacterial wilt worldwide due to its diverse and broad range of hosts (Fegan and Prior, 2006; Prior et al., 2016). It infects over 200 plant species including solanaceous crops causing visible symptoms and latent infection (Aliye et al., 2015). The bacterium is one of the well-known and most critical phytopathogenic bacteria causing bacterial wilt in banana. BDB was reported to be caused by *R. solanacearum* phylotype IV or recently named as *R. syzgii* subsp. *celebensis* (*Rsc*) (Bakar et al., 2018). The difference between this phylotype IV with other phylotypes is that this species does not cause infection in solanaceous plant. The slow-growing bacterium forms an irregular round, creamy colonies with red centres when cultured on Kelman's tetrazolium chloride (TZC) medium (Thomas and Upreti, 2014).

Due to the laborious time in detecting the bacteria by conventional culturing and other methods, (She et al., 2018) we aimed to develop a fast and user-friendly detection method through lateral flow immunoassay (LFIA) against *Rsc*. Previously, Suriyadi (2009) raised antibody against *R. solanacearum* isolated from tomato, potato and ground nut using dot-blot enzyme-linked immunosorbent assay (ELISA) method for the detection. In our study, we intended to produce antibody against *R. syzgii* subsp. *celebensis* that was isolated from banana using lateral flow immunoassay technology for the detection method. In this study, random symptomatic and asymptomatic banana plants at the Malaysian Agricultural Research and Development Institute (MARDI) plantations were tested to assess the detection ability and performance of the LFIA strips in the actual banana samples.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *Rsc* isolate obtained from MARDI culture depository (A2 HR-MARDI) was cultured in TZC broth and incubated at 28^{o}C for 72 h (Kelman, 1954; Pontes et al., 2017). The bacterial suspension was centrifuged at 5,000 rpm for 15 min and rinsed three times with PBS containing 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. Serial dilution of the bacterial suspension was made and 100 µL of the dilution was spread on TZC agar plates and incubated for 48 h for colony counting. After the concentration of the bacterial suspension was calculated, the bacterial concentration was adjusted to 10^8 CFU/mL and was used as an immunogen and positive control to test the LFIA strip. For specificity tests in LFIA and PCR, *Pantoea amanatis*, *Pseudomonas* spp., *Erwinia mallotivora* and *Fusarium* spp. were cultured in nutrient broth for 24 to 48 h. Bacterial suspension was centrifuged and serially-diluted as above but plated onto nutrient agar for colony counting.

Immunisation in rabbit and production of polyclonal antibodies

The antibody production and animal handling protocol were reviewed and approved by the Animal Ethics Committee of Malaysian Agricultural Research and Development Institute, Malaysia (Approval number: 20170717/R/MAEC00015). Immunisation was done using whole cells of 10^8 CFU/mL *Rsc* suspension in PBS emulsified in Freund's complete adjuvant. The immunisation was done every 4 weeks interval as primary and booster injections by subcutaneous injection in New Zealand White rabbit consisting of six booster cycles. The rabbit's blood was taken 15 mL through central ear artery or marginal ear vein every 2 weeks following each booster session. The total period from the first injection of antigen and the final bleed was 31 weeks. The blood collected from each bleed was purified by Protein A affinity chromatography using the ÄKTAprime fast protein liquid chromatography system (GE, USA). The highest titre of the bleed was determined by indirect ELISA method (not shown in this study). Anti-*Rsc* IgG polyclonal antibody (pAb) that had the highest titre was used for conjugation with gold nanoparticles to be used in the development of LFIA. The anti-*Rsc* IgG was quantified using Bicinchoninic acid (BCA) assay (Olson and

Markwell, 2007). Analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with 0.15% Coomassie Brilliant blue was performed to determine the IgG purity and molecular weight as well as investigate the subunit compositions (Gallagher, 2012). The image was captured using AlphaImager™ (Alpha Innotech, USA) gel documentation system.

Optimisation of anti-Rsc concentrations for conjugation with gold nanoparticles (GNP)

A series of anti-*Rsc* IgG concentration of 0 to 20 µg/mL, in the increment of 2 µg/mL each, were added to 500 µL of 40 nm colloidal gold nanoparticles at pH 8.0 and vortexed vigorously for 15 s for conjugation to take place. Then, 100 µL of 10% (w/v) sodium chloride was added to challenge the anti-*Rsc* IgG-GNP conjugates stability. After leaving the reaction for 2 min, optical density (OD) for the 600 µL conjugates was measured at 540 nm using spectrophotometer. The results were plotted to determine the optimal stabilising concentration of anti-*Rsc* IgG with GNPs. The required volume to attain the optimum anti-*Rsc* IgG concentration was then up-scaled for conjugation with 10 mL GNPs, without the sodium chloride challenge. The resulting anti-*Rsc* IgG-GNPs conjugates was measured at 540 nm and final conjugates was adjusted to 10 OD and added with final 1% bovine serum albumin (BSA) and 5% sucrose as the conjugates reagent to be sprayed onto the conjugate pad.

LFIA development

The schematic diagram of the components that made up the LFIA strip was as shown in Figure 1. The LFIA strip utilised the polyclonal antibodies raised in New Zealand White rabbit which was immunised against the *Rsc* (anti-*Rsc* pAb), then conjugated with 40 nm gold nanoparticles and used as the capture reagent component in the LFIA system. The detection component of the test line and control lines consisted of anti-*Rsc* pAb and goat anti-rabbit pAb, respectively. The conjugates were sprayed at 5 µL/cm dispensing speed by dispensing platform (BIODOT, XYZ3060™, USA) to the fibre glass conjugate pad (10 mm x 300 mm) to serve as the detection reagent in the LFIA system. Goat anti-rabbit IgG (1 mg/mL) manufactured by Thermo Fisher Scientific and anti-*Rsc* IgG (1 mg/mL) were striped as control line and test line, respectively, at 1 µL/cm dispensing speed onto nitrocellulose membrane (25 mm x 300 mm) using the dispensing platform to serve as the capture zone (Figure 1). After conjugate spraying and control and test line striping, the conjugate pad and the nitrocellulose membrane were dried at 37°C for 1 h in oven (Memmert, Germany) and left to cure overnight at < 20% relative humidity using a dry box (Eureka, Taiwan). The conjugate pad, nitrocellulose membrane, cellulose fibre sample pad and absorbent pad were assembled with 2 mm overlap between each other and cut to 3.5 mm in width for each strip using a guillotine cutter CM5000™ (BIODOT, USA) to produce individual LFIA strip for subsequent testing. BSA at 1% (w/v) was used as the blocking agent for the conjugates.

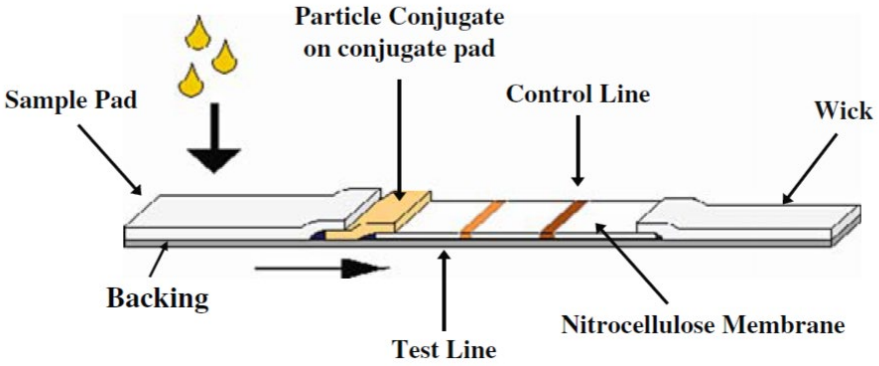

Figure 1. Schematic diagram of LFIA strip

Sensitivity and specificity test of LFIA strips

The individual LFIA strips with the size of 3.5 mm x 60 mm were tested with serially-diluted *Rsc* cultures in PBS with concentrations ranging from 10^3 to 10^8 CFU/mL to determine the sensitivity limit of the strips. Ten microlitre of each *Rsc* concentration was mixed with 90 µL of lysis buffer (PBS pH 7.4, 1% Tween 20). One hundred microlitres of the mixture was then dropped onto the sample pad of the LFIA strip. To observe cross-reactivity of the LFIA strips, specificity test was performed by testing the LFIA strip with 10 µL of 10^8 CFU/mL cultures of *Pantoea amanatis*, *Pseudomonas* spp., *E. mallotivora* and *Fusarium* spp. respectively, mixed with 90 µL of lysis buffer. One hundred microlitre of the mixture was then dropped onto the sample pad of the LFIA strip. The results were photographed after 15 min.

PCR test of symptomatic and asymptomatic banana plants

DNA extraction was performed using Wizard Genomic DNA extraction kit (Promega, USA) according to the manufacturer's protocol. PCR conditions were based on primers sequences and protocols adapted from Ito et al. (1998) with slight modification. Reaction volume of 25 µL of PCR mixture using 1X PCR master mix (containing 2 mM $MgCl_2$, 0.025 U/µL *Taq* DNA polymerase and 0.2 mM of each dNTP), 0.5 µM of each forward (GTCGCCGTCAACTC ACTTTCC) and reverse primer (GTCGCCGTCAGCAA TGCGGAATCG), 2 µL of DNA template and nuclease-free water adjusted to a total volume of 25 µL. PCR reaction was performed out in a thermocycler (DNA Dyad, BioRad, USA). The thermocyler was programmed by preheating for 2 min at 94ºC, followed by 30 cycles of 15 s at 94ºC, 15 s at 60ºC, 15 s at 72ºC and final extension for 5 min at 72ºC. A 5 µL of PCR product was analysed by electrophoresis on 2% agarose gel and stained with ethidium bromide to visualise the amplicons under UV light.

Testing of LFIA strips

The LFIA strips were tested with negative control (PBS), healthy banana plant sample, *Rsc* cultures as positive control and field samples from the visually symptomatic and asymptomatic banana plants (Figure 2) with two sample replicates. For positive control testing, 10 µL of 10^8 CFU/mL of *Rsc* cultures were mixed with 90 µL of lysis buffer. One hundred microlitres of the mixture was dropped onto the sample pad of the LFIA strip. Healthy plant and field samples consisting of the pseudostems, petioles and leaves of banana plants were collected and 0.5 g of each sample was weighed and transferred into 1.5 mL microfuge tubes. Lysis buffer (200 µL) was added to the sample and the mixture was vortexed for 30 s and left for 5 min. The mixture (100 µL) was then dropped onto the sample pad of the LFIA strip. Pictures of the strips were taken 15 min after the sample mixtures were dropped onto the sample pad.

RESULTS AND DISCUSSION

Morphometrics and physiological attributes of fruits and seeds

The *Rsc* concentration of the broth culture obtained was 3 x 10^8 CFU/mL. The bacterial suspension in PBS was used as positive control as well as for immunisation in the New Zealand White Rabbit to elicit immune response against the whole bacterial cells. The highest titre of the purified anti-*Rsc* IgG was Bleed 4 and Bleed 5 with 12.55 mg/mL and 10.5 mg/mL, respectively. Bleed 4 of the anti-*Rsc* IgG was selected for conjugation due to the highest purified IgG content and diluted to 1 mg/mL in PBS. The anti-*Rsc* IgG was used as test lines striping in the development of LFIA and conjugated with 40 nm gold nanoparticles to serve as conjugates in the detection system.

Figure 2. Appearance of symptomatic banana plant (left) and asymptomatic banana plant (right)

After injections of the mixture of antigen/adjuvant into the animal of choice to initiate an amplified immune response, the antibody was purified from the extracted blood (Figure 3). The chromatogram of the antibody fraction eluted from Protein A affinity chromatography column showed two peaks of protein elution curve. The first peak (peak 1) eluted when binding buffer was added, which promoted the binding of IgG to the Protein A ligands and the elution represented protein contaminants in the serum sample loaded into the column under conditions that allowed maximum binding of IgG onto the affinity ligand and weak binding of the non-IgG proteins. The antibody binds to the affinity ligand through mostly non-covalent contacts between reactive groups in the receptors and ligands (Wood and Wright, 2019) such as hydrogen bonds, and ionic and hydrophobic interactions (Firer, 2001). The second peak (peak 2) which showed the highest absorbance was obtained after the desorption step took place where the elution buffer caused a weakening to the non-covalent forces holding the antibody to the affinity ligand, which subsequently dislodged the antibody from the affinity ligand to the elution. These also proved that the binding and eluted buffer used in this experiment were suitable to purify the antibody as investigated previously by Kirley and Norman (2018) in pre-electrophoretic sample preparation protocols.

The purified anti-*Rsc* IgG analysed using SDS-PAGE showed two bands representing the subunits consisting of heavy chain and light chain of the IgG with the approximate sizes of 50 kDA and 25 kDA, respectively (Figure 4). This characteristic indicated that the IgG was successfully purified by the Protein A affinity-column chromatography method.

Based on the trend in Figure 5, the minimum anti-*Rsc* IgG concentration that coated the surface of GNPs by passive adsorption was found to be at 4 μg/mL, and 10 μg/mL was determined as the optimal stabilising anti-*Rsc* IgG concentration with gold nanoparticles, in which the GNPs to be coated with IgG was in excess amount to reduce or eliminate any remaining uncoated surface that can cause unspecific binding with the antigen or other materials (Wong and Tse, 2009). This unspecific binding could lead to false-positive result in LFIA.

Passive adsorption is formed between the negatively charged GNPs and positively charged IgG in which the IgG is positioned in non-directional manner to the GNPs surface (Nadezhda et al., 2017). On the other hand, the insufficient IgG coating could not protect the GNPs from aggregation (0 and 2 µg/mL) and causing the ionic charge from sodium chloride solution to destabilise the exposed GNPs surface causing it to aggregate. Lower OD 540 nm obtained when using 0 and 2 µg/mL of anti-*Rsc* IgG indicating this aggregation phenomenon. The 10 OD of final conjugates was added with 1% BSA as blocking agent to fill-up any non-conjugated GNPs surface. Sucrose was added as stabilisers for the dried conjugates to preserve its biological elements and activity once liquid form of the antibody was changed to the dried form (Mensink et al., 2017).

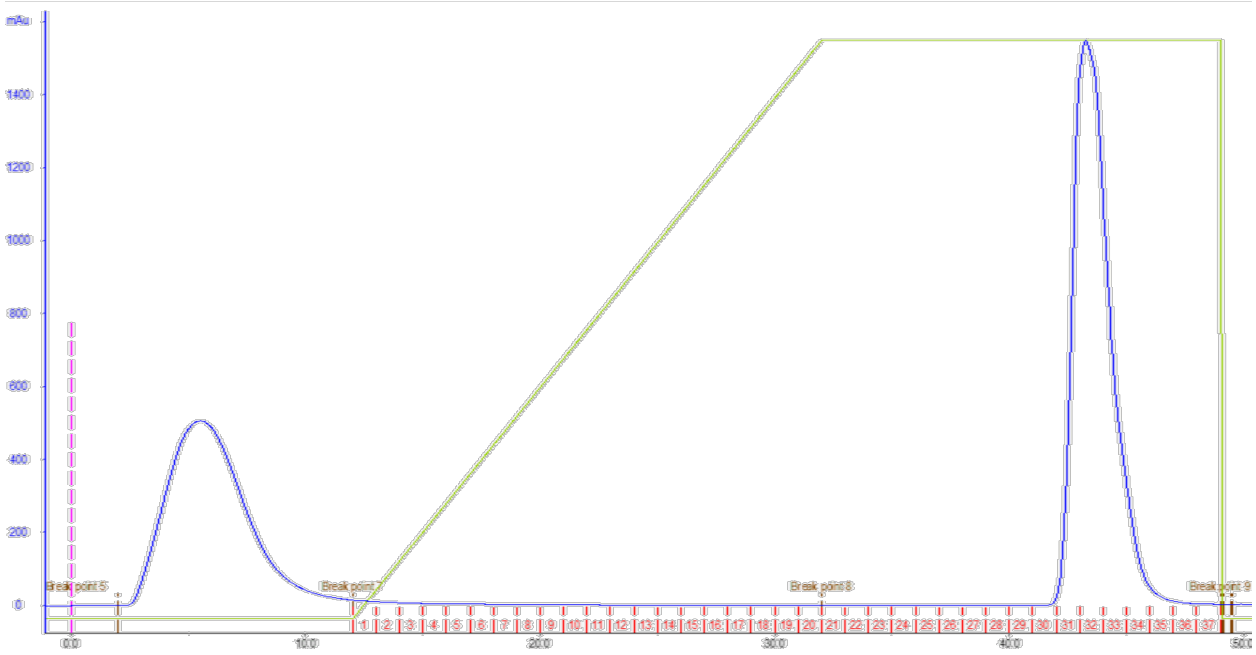

Figure 3. Chromatogram of antibody purification using Protein A affinity column

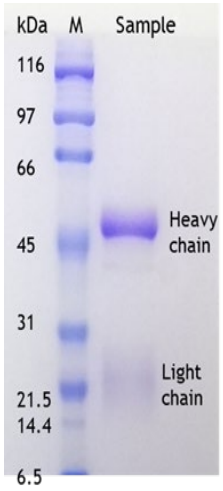

Figure 4. The purified anti-*Rsc* IgG analysed using SDS-PAGE. Lane M: Broad-range protein marker; Lane sample: Column-purified anti-*Rsc* IgG sample.

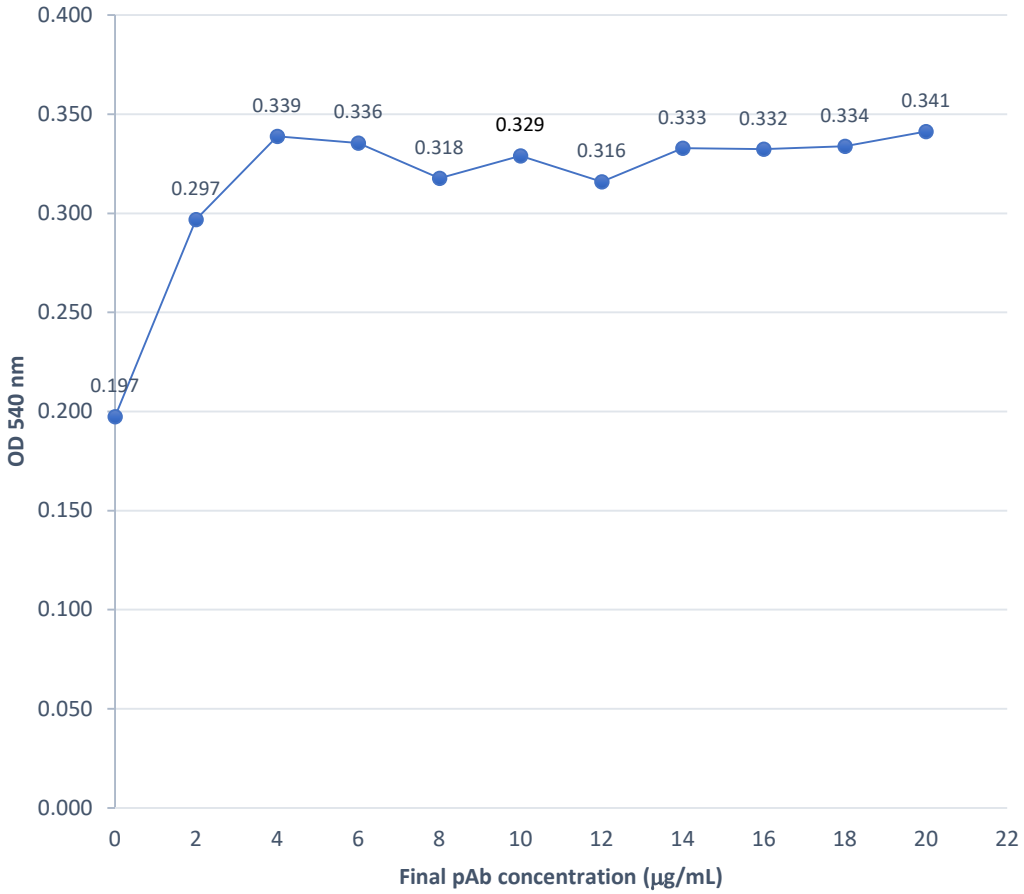

Figure 5. Optimal stabilising of anti-*Rsc* IgG concentrations with gold nanoparticles when challenged with 10% sodium chloride

The conjugate pad, nitrocellulose membrane, sample pad and absorbent pad were assembled with 2 mm overlap to ensure that sufficient capillary action of the lateral flow can be achieved in the assembled strip. The sensitivity of the LFIA strips was tested using 10^3 to 10^8 CFU/mL *Rsc* cultures as shown in Figure 6. The strips produced test line signals proportional to the *Rsc* concentration tested from the highest to the lowest concentration amount. However, at 10^3 CFU/mL concentration, no visible test line signal was seen on the LFIA strips, which meant that the sensitivity limit of the LFIA strips to detect *Rsc* was 10^4 CFU/mL. To date, there is no literature reporting the detection of *Rsc* by the LFIA method. However, LFIA sensitivity to detect *R. solanacearum* reported by Panferov et al. (2016) was similar with the results obtained in our study, in which the lowest visible detection was 1.5 x 10^4 CFU/mL of the bacteria concentration which was detected in both PBS and potato tuber extracts before enhancement by silver was performed. In addition, the result obtained in our study was more sensitive by 10-fold compared to the study conducted by Hodgetts et al. (2015) where their LFIA was able to detect 10^3 CFU/mL of *Xanthomonas campestris* pv. *musacearum* NCPPB2251. The specificity test using cultures of *Pantoea amanatis*, *Pseudomonas* spp., *E. mallotivora* and *Fusarium* spp. showed that there was no cross reactivity (Figure 7).

The performance of the strips was tested with negative control of PBS buffer and also healthy banana plant sample, positive control and samples from symptomatic and asymptomatic banana plants. In this study, symptomatic plant was judged based on the wilted leaves and the discolouration of pseudostem, while asymptomatic plant was judged based on healthy, green leaves and no discolouration of pseudostem (Figure 2).

Six samples from banana plants were tested using the LFIA strips (Figure 8). Positive results were judged by the visible control and test lines, whereas negative results were judged by the visible control line only. Negative control and positive controls (both PBS and healthy samples) yielded the result of control lines only, as expected. However, plant samples gave different results in each part where the samples were taken from. Negative signal was observed for the petiole of the symptomatic plant whereas positive signals were seen when tested with leaf and pseudostem with pseudostem giving higher test lines intensity than the leaf which appeared as very faint. The brownish background colour of the strip tested with the symptomatic leaf was evident as the samples appeared wilted. This was in contrast with the strip background of the healthy leaf which appeared as light greenish colour. The appearance of healthy/asymptomatic plant samples correlated with the negative strip results of the petiole and leaf tested. However, positive signal was seen when the asymptomatic pseudostem was tested. All samples tested using the LFIA strips had been confirmed with PCR technique showing 100% correlation (Figure 9). Although the asymptomatic pseudostem had no sign of discolouration which often a sign of severe *Rsc* infection, it could be that *Rsc* had already infected the stem and was able to be detected by the LFIA strips, but the physical appearance was not at severe stage to cause discolouration of the vascular system, indicating severe infection. There is a possibility that the bacteria have infected the pseudostem and have not reached to the petiole and the leaf section (Fegan and Prior, 2005), based on the fact that *Ralstonica* species is a soil-borne bacterium (Nguyen and Ranamukhaarachchi, 2010). This phenomenon explained the negative signals of the asymptomatic petiole and leaf obtained in this study.

LFIA is a membrane or paper-based platform famously used as a rapid screening tool to help user perform rapid screening of the targeted pathogen. The results can be obtained in as early as 5 to 15 min. Such rapid test results together with the versatility of formats, biorecognition elements, labels and detection systems make the platform favourable to the developer and overall users (Lazka et al., 2007, Wang et al., 2013). The relatively low development cost, low sample volume, long shelf life with no energy consumption and requiring no expert personnel to operate, make the LFIA as the ideal and popular kit for detection of many analytes (Sajid et al., 2014). Early and accurate detection of infection specific for *Rsc* is essential in the effective disease management (Safenkoya et al., 2017) and will help stakeholders to make an informed decision once infection has occurred to better control the outbreak in the banana plantation.

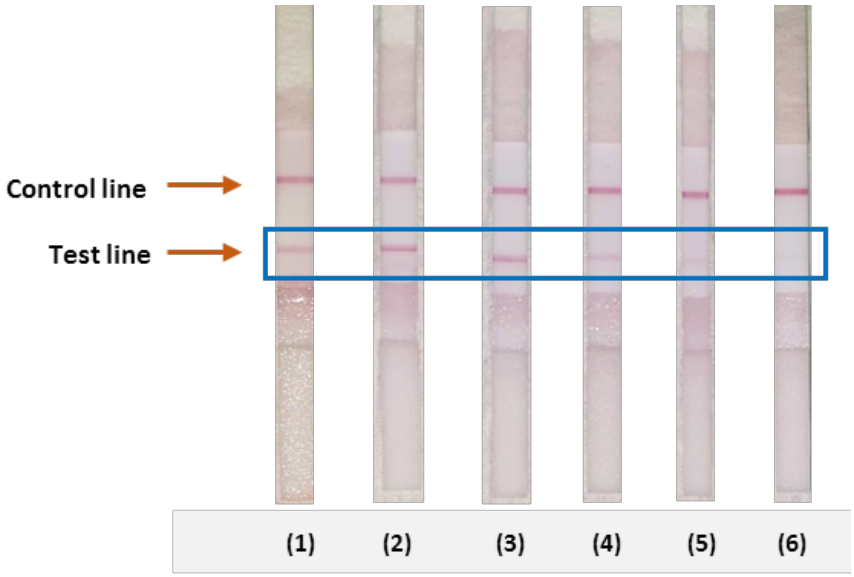

Figure 6. Sensitivity results of LFIA strips tested with serially-diluted *Rsc* in PBS. *Rsc* at 10^8 CFU/mL (1), 10^7 CFU/mL (2), 10^6 CFU/mL (3), 10^5 CFU/mL (4), 10^4 CFU/mL (5) and 10^3 CFU/mL (6).

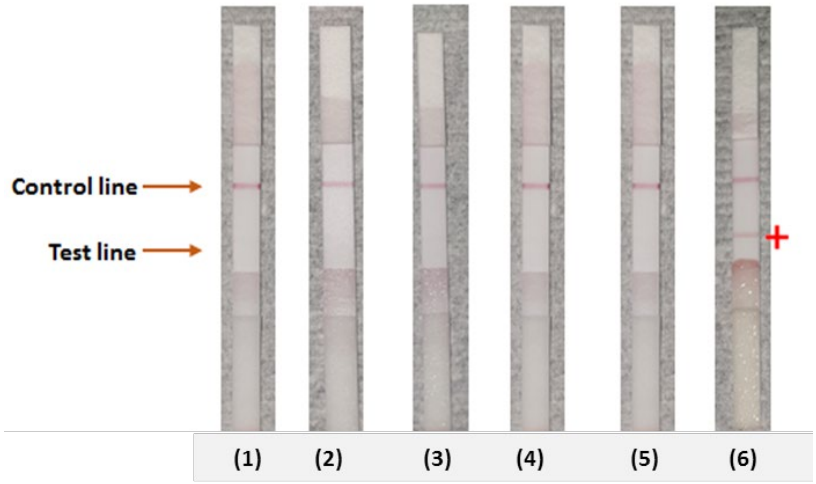

Figure 7. Specificity results of LFIA strips tested with control (negative), photographed 15 min after sample drop. Negative control (1), *Pantoea amanatis*, (2), *Pseudomonas* spp., (3), *Erwinia mallotivora* (4), *Fusarium* spp. (5) and *Rsc* (6).

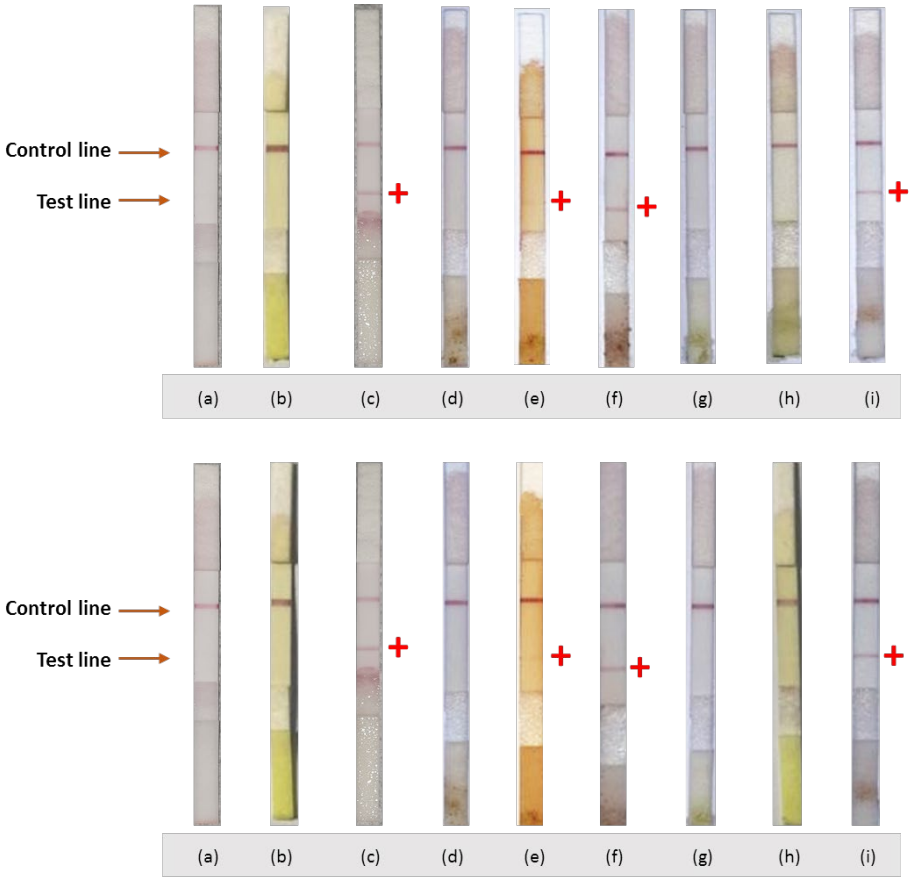

Figure 8. LFIA strip results tested with symptomatic and asymptomatic samples of banana plants with two replicates, photographed at 15 min after sample drop. Negative control (a), positive control, *Rsc* (b), healthy plant (c), symptomatic plant petiole (d), leaf (e), pseudostem (f), and asymptomatic plant petiole (g), leaf (h), pseudostem (i).

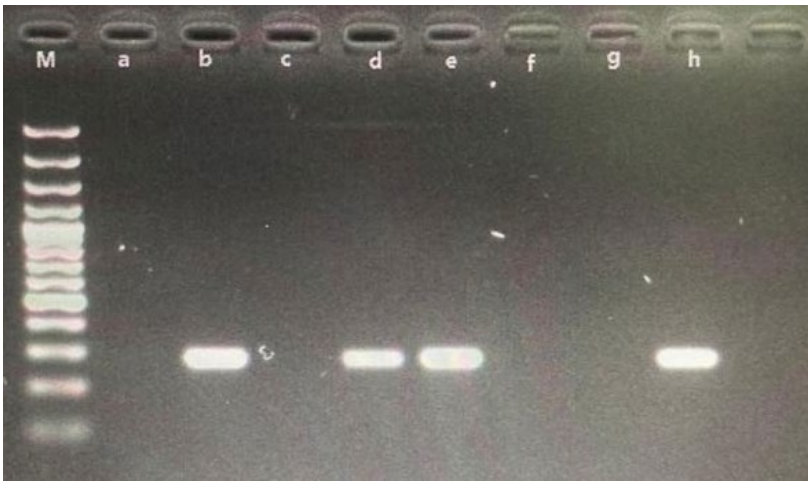

Figure 9. PCR results of symptomatic and asymptomatic samples of banana plants. M: 100 bp plus DNA marker, negative control (a), positive control, *Rsc* (b), symptomatic plant petiole (c), leaf (d), pseudostem (e), and asymptomatic plant, petiole (f), leaf (g) and pseudostem (h).

CONCLUSIONS

The LFIA strip showed a promising result for the rapid detection of *Rsc* infection in banana plants as opposed to the laborious technique of conventional culturing, PCR or as well as the traditional phytopathology diagnosis based on visual assessment. The sensitivity limit of the technique for detecting 10^4 CFU/mL *Rsc* was a convenient assay for on-site or laboratory testing in a relatively fast assay of 15 min. The strips could be potentially used for screening of *Rsc*-infected banana plant as well as *Rsc*-free banana planting material to curb the disease in a simple and user-friendly approach without the need of specialised equipment. Further work is ongoing to improve the signal intensity of the LFIA strips and to be tested with samples from *Rsc* hotspot area to gather more conclusive findings.

AUTHORS CONTRIBUTION

MAAT and AAAK conceived and designed the work. MAAT, NFNA, KAS, NSJ, SNB, FFH, ZM and AAAK performed the analysis. MAAT wrote the paper, and AAAK checked and approved the submission.

CONFLICT OF INTEREST

The authors declare no competing interests in the work submitted.

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REFERENCES

Aliye, N., Dilbo C. and Pillay, M. (2015). Understanding reaction of potato (*Solanum tuberosum*) to *Ralstonia solanacearum* and relationship of wilt incidence to latent Infection. *Journal of Phytopathology*, 163, 444-455.

Bakar, R. A. H., Badrun, R., Ahmad, K. and Abu Bakar, N. (2018). Symptomatology and range of the blood disease bacterium A2 HR MARDI strain (*Ralstonia syzygii* subsp. *celebensis*) on selected hosts. *IOSR Journal of Agriculture and Veterinary Science*, 11(9), 71-77.

FAOSTAT. (2014). *Banana and plantain surface and production in 2013.* Available at: http://faostat.fao.org/site/339/default.aspx [Accessed on 10th June 2021].

Fegan, M. and Prior P. (2005). How complex is the "*Ralstonia solanacearum* species complex." In: Allen, C., Prior, P. and Hayward, A. C. (Eds.) Bacterial wilt: the disease and the *Ralstonia solanacearum* species complex. Minnesota, USA, APS Press, pp. 449-461.

Fegan, M. and Prior, P. (2006). Diverse members of the *Ralstonia solanacearum* species complex cause bacterial wilts of banana. *Australasian Plant Pathology*, 35, 93-101.

Firer, M. A. (2001). Efficient elution of functional proteins in affinity chromatography. *Journal of Biochemical and Biophysical Methods*, 49(1-3), 433-442.

Gallagher, S.R. (2012). SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). *Current Protocols Essential Laboratory Techniques,* 6(1), 7.3.11-7.3.28.

Heng, S. B. (2012). Blood Disease, Banana: Malaysia. ProMed.

Hodgetts, J., Karamura, G., Johnson, G., Hall, J., Perkins, K., Beed, F., Nakato, V., Grant, M., Studholme D. J. and Smith, J. (2015). Development of a lateral flow device for in-field detection and evaluation of PCR-based diagnostic methods for *Xanthomonas campestris* pv. *musacearum*, the causal agent of banana *Xanthomonas* wilt. *Plant Pathology*, 64, 559-567.

IPPC (2016). Occurrence of blood disease of banana (*Ralstonia solanacearum* species complex) in Malaysia. *IPPC Official Pest Report*, No. MYS-01/2. Rome, Italy: FAO. Available at: https://www.ippc.int/ [Accessed on 10th June 2021].

Ito, S., Ushijima, Y., Fujii, T., Tanaka, S., Kameya-Iwaki, M., Yoshiwara, S. and Kishi F. (1998). Detection of viable cells of *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique. *Journal of Phytopathology*, 146, 379-384.

Kelman, A. (1954). The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology*, 44, 693-695.

Kirley, T. L. and Norman, A. B. (2018). Unfolding of IgG domains detected by non-reducing SDS-PAGE. *Biochemical and Biophysical Research Communications*, 503(2), 944-949.

Lazcka, O., Del Campo, F. J., & Muñoz, F. X. (2007). Pathogen detection: a perspective of traditional methods and biosensors. *Biosensors & Bioelectronics*, 22(7), 1205-1217.

Mensink, M. A., Frijlink, H. W., Maarschalk, K. V. and Hinrichs, W. L. J. (2017). How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. *European Journal of Pharmaceutics and Biopharmaceutics*, 114, 288-295.

Nguyen, M. and Ranamukhaarachchi, S. (2010). Soil-borne antagonists for biological control of bacterial wilt disease caused by *Ralstonia solanacearum* in tomato and pepper. *Journal of Plant Pathology*, 92(2), 395-405.

Olson, B. J. S. C., and Markwell, J. (2007). Assays for determination of protein concentration. *Current Protocols in Protein Science*, 48(1), 1-29.

Panferov, V. G., Safenkova, I. V., Varitsev, Y. A., Drenova, N. V., Kornev, K. P., Zherdev, A. V. and Dzantiev, B. B. (2016). Development of the sensitive lateral flow immunoassay with silver enhancement for the detection of *Ralstonia solanacearum* in potato tubers. *Talanta*, 152, 521–530.

Pontes, N. C., Fujinawa, M. F. and Oliveira, J. R. (2017). Selective media for detection and quantification of Brazilian *Ralstonia solanacearum* isolates in soil. *Horticultura Brasileira*, 35(1), 41-47.

Prior, P., Ailloud, F., Dalsing, B. L., Remenant, B., Sanchez, B. and Allen, C. (2016). Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. *BMC Genomics*, 17(1), 1-11.

Safenkova, I.V., Zaitsev, I.A., Varitsev, Y.A., Byzova, N. A., Drenova, N.V., Zherdhev A. V. and Dzantiev B. B. (2016). Development of a lateral flow immunoassay for rapid diagnosis of potato blackleg caused by *Dickeya* species. *Analytical and Bioanalytical Chemistry*, 409, 1915-1927.

Sajid, M., Kawde, A. and Daud, M. (2015). Designs, formats and applications of lateral flow assay: A literature review. *Journal of Saudi Chemical Society*, 19(6), 689-705.

She, X., Yu L., Lan, G., Tang, Y. and He, Z. (2017). Identification and genetic characterization of *Ralstonia solanacearum* species complex isolates from *Cucurbita maxima* in China. *Frontiers in Plant Science*, 8(1794), 1-11.

Suryadi, Y. (2009). Development of antibody to *Ralstonia solanacearum* and its application for detection of bacterial wilt. *Biotropa,* 16(2), 79-87.

Thomas, P., and Upreti, R. (2014). Significant Effects Due to Peptone in Kelman Medium on Colony Characteristics and Virulence of *Ralstonia solanacearum* in Tomato. *The Open Microbiology Journal*, 8, 95-114.

Wang, Y. K., Shi, Y. B., Zou, Q., Sun, J. H., Chen, Z. F. and Wang, H. (2013). Development of a rapid and simultaneous immunochromatographic assay for the determination of zearalenone and fumonisin B1 in corn, wheat and feedstuff samples. *Food Control* 31, 180-188.

Wong, R. and Tse, H. (2009). *Lateral Flow Immunoassay*. Totowa, NJ: Humana Press.

Wood, L. and Wright, G. J. (2019). Approaches to identify extracellular receptor–ligand interactions, *Current Opinion in Structural Biology*, 56, 28-36.