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DNA Probe as Biosensor Candidate for *Clavibacter michiganensis* subsp. *michiganensis* on Tomato Plants

Probe DNA sebagai Kandidat Biosensor *Clavibacter michiganensis* subsp. *michiganensis* pada Tanaman Tomat

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ABSTRACT

The study was conducted to evaluate gene detection technique using a specific DNA probe to detect *tomA* gene in *Clavibacter michiganensis* subsp. *michiganensis*, a bacteria causing cancer in tomatoes. The probe was designed using Primer3Plus program, labeled with the non-radioactive molecule digoxigenin (DIG) and used in the hybridization method with the dot blot technique. The test samples consisted of two types, i.e. genomic DNA samples from pure bacterial cultures and from artificially infected tomato seeds with *C. michiganensis* subsp. *michiganensis*. Samples derived from pure bacterial cultures showed positive hybridization results at all levels of DNA concentration; while samples from tomato seeds only showed positive reactions at concentrations of 10, 8, 6, and 4 g L^{-1}. This study concludes that the designed probe has the potential to be used in the development of biosensor-based detection methods for *C. michiganensis* subsp. *michiganensis* in tomato seeds and is quite specific because there is no cross-reaction with non-target bacterial groups.

Keywords: digoxigenin, dot blot, hybridization, non-radioactive, *tomA* gene

ABSTRAK

Penelitian dilakukan untuk menguji teknik deteksi gen menggunakan DNA probe spesifik untuk mendeteksi gen *tomA* pada *Clavibacter michiganensis* subsp. *michiganensis*, bakteri penyebab kanker pada tomat. Probe dirancang menggunakan program Primer3Plus, dilabel dengan molekul nonradioaktif digoxigenin (DIG) dan digunakan pada metode hibridisasi dengan teknik dot blot. Sampel uji terdiri atas dua jenis, yaitu sampel DNA genom yang berasal dari kultur bakteri murni dan dari benih tomat yang diinfeksi buatan dengan *C. michiganensis* subsp. *michiganensis*. Sampel yang berasal dari kultur murni bakteri menunjukkan hasil hibridisasi positif pada semua tingkat konsentrasi DNA; sedangkan sampel yang berasal dari biji tomat hanya menunjukkan reaksi positif pada konsentrasi 10, 8, 6, dan 4 µg μL^{-1}. Studi ini menyimpulkan bahwa probe yang dirancang berpotensi untuk digunakan dalam pengembangan metode deteksi *C. michiganensis* subsp. *michiganensis* pada biji tomat berbasis biosensor dan bersifat cukup spesifik karena tidak ada reaksi silang dengan kelompok bakteri bukan target.

Kata kunci: digoxigenin, dot blot, hibridisasi, gen *tomA*, non radioaktif

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INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* is known as the causal agent of bacterial canker in tomato plants. This bacterium is a seed-borne pathogen and has the potential to cause economic harm because it may cause fatal damage on infected plant. For this reason, *C. michiganensis* subsp. *michiganensis* is included in the quarantine (regulated) pests in various countries. Therefore, a fast and accurate detection method is necessary in order to monitor the incidence and distribution of this pathogen.

Molecular detection has been used widely in recent years. Nucleic acid-based detection is particularly favored because it provides a fast and accurate detection method. A specific gene can be selected as the target and used as biosensor (Teles and Fonseca 2008; Nandi *et al.* 2018; Mohan and Ananda 2019). This method is suitable to be applied for the detection of plant diseases, including *C. michiganensis* subsp. *michiganensis* (Huang *et al.* 2015).

The use of DNA probes has been applied for detection of pathogens in different hosts. Nursista (2018) used DNA probe to detect *Salmonella* spp. from various commercial sushi products. Subspecies-specific primers based on endoglucanase genes was designed and used by Trianom *et al.* (2018) for detection of *Ralstonia syzygii* subsp. *syzygii*. Hartati *et al.* (2016) developed DNA biosensing technology for the electrochemical detection of pathogenic *Mycobacterium tuberculosis* using a modified electrode.

The use of nucleic acids has great potential as molecular probes because they are easily synthesized and modified for various purposes. Their high base-pair specificity and predictability for intermolecular or intramolecular interactions are also unquestionable. Unlike classical microbiological methods, the technique is quite simple and fast which will reduce the time needed for pathogen identification. Therefore, DNA probes become more attractive for application in diagnostic method (Koopaee *et al.* 2020).

This article discusses the use of nonradioactive DIG (digoxigenin) labeled DNA probe for detection of *C. michiganensis* subsp. *michiganensis*. A specific gene known as *tomA* was selected for developing the DNA probes. This gene was involved in the regulation of *C. michiganensis* subsp. *michiganensis* virulence against tomato plants (Albuquerque *et al.* 2017).

MATERIALS AND METHODS

Probes Design

Probe design was initiated by tracing database sequence of *tomA* gene presents in *Clavibacter michiganensis* subsp. *michiganensis*, *Agrobacterium fabrum* and *Streptomyces scabiei*. Six *tomA* gene sequences were found from the GenBank National Center for Biotechnology Information (NCBI), i.e. KJ724007.1, KJ724008.1, KJ724009.1, KJ724010.1, KJ724011.1, and KJ724012.1. These sequences were downloaded and then aligned using BioEdit program to determine the level of conservation. The conserved region obtained from the alignment process was then further analyzed as the candidate for probe templates.

Selection of potential probe templates was conducted using Primer3Plus software based on several parameters, such as the number of nucleotides, the order of the codons, length of the amplicon, the composition of guanine and cytosine base (GC content), and melting temperature. A series of analyzes were also carried out using a DNA calculator olygoanalyzer to identify potential dimers, hairpins and self-annealing. For final analysis, the potential probes were checked for its complementary to the target species but not the non-targeted one by running BLAST program from GenBank and FastPCR. Once the design of the probes were confirmed, the probe synthesis was carried out with the help of Integrated DNA Technology in Singapore.

Probes Labelling

DNA labelling was conducted following the protocol provided by DIG-High Prime

DNA Labeling and Detection Starter Kit I (Roche, Switzerland). For the complete DNA denaturation step, 1 µg of the synthesized probe was added to 15 µL of sterile deionized water. Denaturation was done at 99 ℃ for 10 minutes, followed by cooling on ice. The denatured probe then entered the labeling stage with the addition of 4 µL DIG-High Prime and was briefly centrifuged. Optimizing the yield of DIG-labeled DNA was done by incubating the samples overnight at 37 ℃. After incubation, the reaction was stopped by adding 2 µL of 0.2 M EDTA and heating for 10 minutes at 65 ℃.

Determination of Labeling Efficiency

A labeling efficiency test was carried out for the labeled control DNA and the labeled probe. The first dilution step was carried out on labeled control DNA that was available in the DNA labeling kit from the supplier (from a concentration of 5 ng μL^{-1} to 1 ng μL^{-1}). Then, a series of dilutions were performed, including the following concentrations: 0.01 pg μL^{-1}, 0.1 pg μL^{-1}, 1 pg μL^{-1}, 10 pg μL^{-1}, and 1000 pg μL^{-1} (the labeled probe was diluted from 100 ng μL^{-1} as an assumption of theoretical yield). Next, each sample concentration was transmitted into a positively charged nylon membrane (Roche, Switzerland) with a volume of 1 µL. Fixing membranes for DIG-labeled control DNA and labeled probes was performed with an oven (temperature 120 ℃ for 30 minutes).

The membrane was then incubated using 2 mL of maleic acid buffer 1×, which was performed in a container for 2 minutes. The membrane was then transferred to 10 mL of 1× blocking solution to be incubated for 30 minutes, soaked in 10 mL of antibody solution (30 minutes), and washed twice with 10 mL of 1× washing buffer solution for 15 minutes. The next step was set for 5 minutes of 1× detection buffer in the 10 mL volume, and all incubation steps were conducted on a rotary shaker at 100 rpm at 25 ℃.

Detection buffer 1× with 2 mL in volume was added into 40 µL of the NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) immunodetection color substrate solution in a lightproof container. The membrane was left in this mixture under stationary conditions for 10 hours and washed with sterile deionized water (5 minutes) to stop the reaction. The intensity of the spot formed was then analyzed.

Hybridization of DIG-Labeled Probes with DNA Target

Pure isolates of *C. michiganensis* subsp. *michiganensis* were extracted using the QIAmp DNA Minikit (Qiagen). Artificially infected tomato seeds by *C. michiganensis* subsp. *michiganensis* were extracted using the Plant Genomic DNA Mini Kit (Geneaid). Both genomic DNA obtained from this phase was denatured at 99 ℃ for 10 minutes and used as DNA targets. As much as 1 µg from each genomic DNA (10, 8, 6, 4, 2, and 1 µg μL^{-1} concentrations) was spotted on a nylon membrane with a positive charge. The exact working procedure was applied to genomic DNA derived from pure bacterial cultures. This genomic DNA was designated as positive control while distilled water was a negative control.

The nylon membrane was then soaked in a solution of 10× SSC and dried in an oven at 120 ℃ for 30 minutes. This stage was accommodated to ensure the success of the binding process between DNA and the membrane. The membrane was then rinsed with sterile deionized water and air-dried. The next step was incubating the membrane in 10 mL of DIG Easy Hyb solution, which had been preheated (37 ℃). This phase was conducted in an incubator at 37 ℃ for 30 minutes. Then, in a hybridization chamber, 1 µL of labeled DNA probe was denatured at 99 ℃ for 10 minutes and put into a volume of 3 mL of a heated (42 ℃) DIG Easy Hyb solution.

Visible spots formed as a sign that the genomic DNA was fully bound to the membrane. Next, the membrane was inserted into a chamber containing the hybridization solution. The hybridization process was carried out for 24 hours at 42 ℃. After the

hybridization, the membrane was washed twice with the post-hybridization solution. The immunological detection procedure was finished by incubation of NBT/BCIP. To ensure that the probe does not cross-react with another genome of bacteria, additional testing was carried out on the genomic DNA from bacterial colony of *Xanthomonas campestris, Ralstonia solanacearum,* and *Fusarium oxysporum.*

RESULTS

Homology analysis of the *tomA* gene in *C. michiganensis* subsp. *michiganensis* with the same gene of other bacteria revealed a conserved region with a length of 15 bp at the base sequence numbers 285–299 (Figure 1). Furthermore, *tomA* gene sequence alignment using Bioedit program resulted in a conserved region with a length of 456 bp at the base sequence numbers 54–509 (Figure 2). A total of five specific probe candidates were obtained using Primer3Plus program and all probe candidates has been further analysed using DNA calculator olygoanalyzer as shown in Table 1. *In silico* detection performed by FastPCR software on the DNA probe candidates indicated that all the probe candidates could detect 100% of *C. michiganensis* subsp. *michiganensis* strain VL527 complete genome (GenBank code: CP047054.1) (Table 2). The BLAST analysis confirmed that the top ten results were 100% specific for *C. michiganensis* subsp. *michiganensis* (Table 3).

Probe no. 2 with sequence order 5'-TGAAGTGCTCTGTCATCGCCGCCC GCATCT -3' was selected as the best probe candidate for further synthesis. This probe composed of 30 nucleotides in length, had a melting temperature of 68.3 ℃ and contained 60% GC. Based on analysis by the DNA calculator olygoanalyzer, this probe did not have the potential for the hairpin, run, repeat, 3'complementarity and self-annealing.

The efficiency of the DIG-labeled probe was accomplished by dot blot hybridization. Purple dots on the nylon membrane indicated that the labeled probe matched with the control probe (Figure 3). Different concentration of genomic DNA target from a pure culture of *C. michiganensis* subsp. *michiganensis* gave positive reaction to the probes with similar color intensity. Different

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BioEdit version 7.2.5 (12/11/2013)
Conserved region search
Alignment file: Untitled1
4/19/2021 12:45:31 PM

Minimum segment length (actual for each sequence): 15
Maximum average entropy: 0.2
Maximum entropy per position: 0.2
Gaps limited to 2 per segment
Contiguous gaps limited to 1 in any segment

1 conserved regions found

Position 54 to 509
Consensus:
54
GGAAGGCGTCAGCGATGTACCCGGGACCGAGGACCTTAAAGAAGGTGGTGCTTTGAAGC
GTCGTACCTTGCGACTCGAGCGCTTCAGAGACAACGTCCCACCGATCGATCCTTCCGTC
GTAACGCTCCATCACAGTGGTGAAGTGCTCTGTCATCGCCGCCCGCATCTCGCCGGCAT
CCGTGATCGAGGTCACGTACTCCGGATCGCAGCAGCTGGAGATCAGACCATGGCCCTTT
ACCGCCATGTCGTGCTCTTCGGCGAAGGCGACCAGTCGGTCAAGCCCAGCGAAGTCGTA
CACACCCTCTTCGGGTTGCGTGAAGGCCCATTTCATCTCGTTCTCGGGAGAAAGGCCAT
TGAACTGTTCGGCGAGGGTGTCGGCGAACCGCGGATCGGTCAGGTAGTTCGACGGGCGG
TCGTCCTTCGTCCACTCCGCGGCCTTGATCGAGCCGGATCCGA 509

Segment Length: 456
Average entropy (Hx): 0.0000
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Figure 2 Conserved region obtained from six *tomA* gene sequences from *Clavibacter michiganensis* subsp. *michiganensis.*

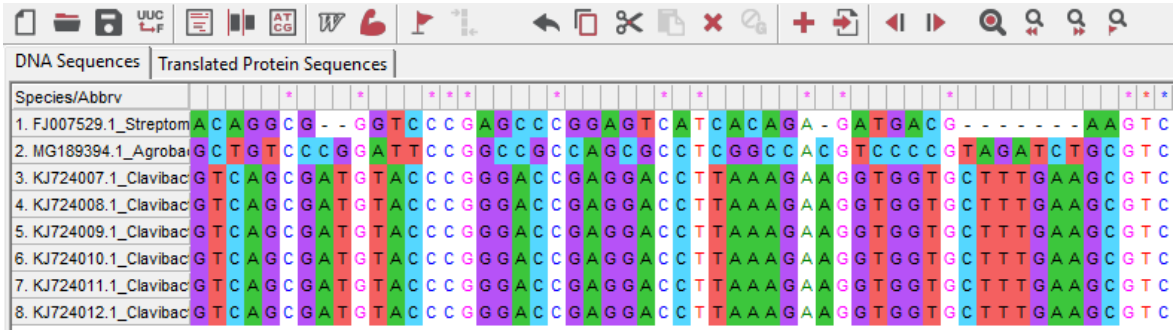

Figure 1 Alignment of *tomA* gene sequences present in *Clavibacter michiganensis* subsp. *michiganensis* (KJ724007.1, KJ724008.1, KJ724009.1, KJ724010.1, KJ724011.1, KJ724012.1, *Agrobacterium fabrum* (MG189394.1) and *Streptomyces scabiei* (FJ007529.1).

Table 1 Criteria analysis of the candidate probes designed by Primer3Plus software

Probe number	Sequence (5' to 3')	Length (bp)	% GC	Tm (°C)	Dimer	Hairpin
Probe 1	CCCGCATCTCGCCGGCATCCGT-GATCGA	28	67.9	69.2	Yes	No
Probe 2	TGAAGTGCTCTGTCATCGCCGCCC-GCATCT	30	60.0	68.3	No	No
Probe 3	TGTCATCGCCGCCCGCATCTCGCCG-GCA	28	72.0	69.1	No	Yes
Probe 4	CGCAGCAGCTGGAGATCAGAC-CATGGCCCT	30	63.3	68.3	Yes	No
Probe 5	CGAACCGCGGATCGGTCAGG-TAGTTCGA	28	70.0	60.0	Yes	Yes

Table 2 *In silico* analysis performed by FastPCR software

Probe number	Target	Compatibility (%)	Tm (°C)
Probe 1	*C. michiganensis* subsp. *michiganensis* strain VL527 complete genome	100	77.4
Probe 2	*C. michiganensis* subsp. *michiganensis* strain VL527 complete genome	100	75.6
Probe 3	*C. michiganensis* subsp. *michiganensis* strain VL527 complete genome	100	76.2
Probe 4	*C. michiganensis* subsp. *michiganensis* strain VL527 complete genome	100	75.4
Probe 5	*C. michiganensis* subsp. *michiganensis* strain VL527 complete genome	100	71.3

Table 3 BLAST analysis for the best probe candidate

Description*	Query Cover (%)	E Value	Identity (%)	Accession Length	GenBank Accession No.
Cmm strain VL 527 chromosome, complete genome	100	4e-06	100	3321579	CP047054.1
Cmm strain MSF322 chromosome, complete genome	100	4e-06	100	3284014	CP047051.1
Cmm strain UF1 chromosome, complete genome	100	4e-06	100	3271848	CP033724.1
Cmm strain 0690 tomatinase (tomA) gene, partial cds	100	4e-06	100	509	KJ723988.1
Cmm strain 0651 tomatinase (tomA) gene, partial cds	100	4e-06	100	509	KJ723984.1
Cmm strain 0572 tomatinase (tomA) gene, partial cds	100	4e-06	100	509	KJ723979.1
Cmm strain 04100 tomatinase (tomA) gene, partial cds	100	4e-06	100	509	KJ723973.1
Cmm strain 0310 tomatinase (tomA) gene, partial cds	100	4e-06	100	509	KJ723968.1
Cmm strain OP7 tomatinase (tomA) gene, partial cds	100	4e-06	100	497	MZ356312.1
Cmm strain OP3 tomatinase (tomA) gene, partial cds	100	4e-06	100	497	MZ356311.1

**Cmm, Clavibacter michiganensis* subsp. *michiganensis*

response was observed when using samples from tomato seed artificially infected by this bacteria. Positive reaction was observed at the concentration 10 µg µL^{-1}, 8 µg µL^{-1}, 6 µg µL^{-1}, and 4 µg µL^{-1}, but the reaction was found negative at 2 µg µL^{-1}, and 1 µg µL^{-1} (Figure 4).

Additional test was performed to confirm probe specificity, i.e. using samples from different pathogen species. No hybridization of the samples of *X. campestris, R. solanacearum,* and *F. oxysporum* to the probes (Figure 5). According to this data, the DNA probe has a detection limit of up to 4 µg µL^{-1} to detect the presence of *C. michiganensis* subsp. *michiganensis* in tomato seeds. Additional tests carried out using different species of pathogen proved that the probe synthesized in this study was specific for *C. michiganensis* subsp. *michiganensis* and no cross-reaction with other target bacteria that may infect tomato plants.

DISCUSSIONS

DNA probes design is now becoming easier and faster which cannot be separated from the increasing public interest in bioinformatics and computational biology (Vizzini *et al.* 2017). DNA probes have been widely applied in several fields, including the detection of plant pathogens due its quantitative natures. This technique is especially preferred over other

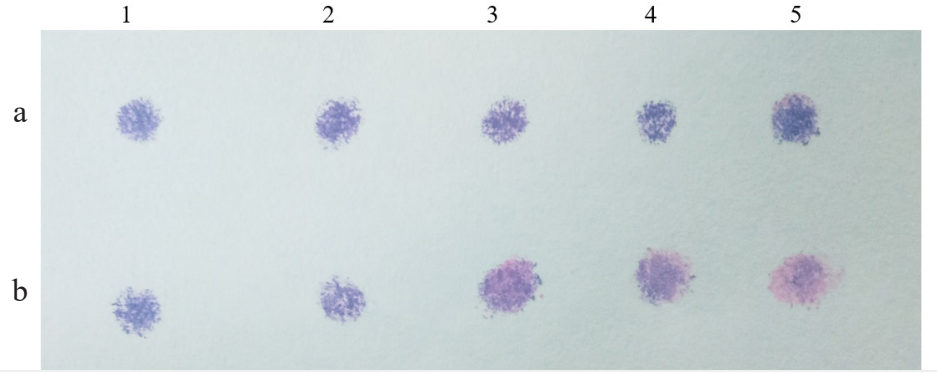

Figure 3 Determination of labeling efficiency of DIG-labeled probes. a, DIG-labeled control DNA (supplied from DNA labeling kit); b, DIG labeled probes. Samples are genomic DNA of *Clavibacter michiganensis* subsp. *michiganensis* that has been diluted. 1, 1000 pg µL^{-1}; 2, 10 pg µL^{-1}; 3, 1 pg µL^{-1}; 4, 0.1 pg µL^{-1}; and 5, 0.01 pg µL^{-1}.

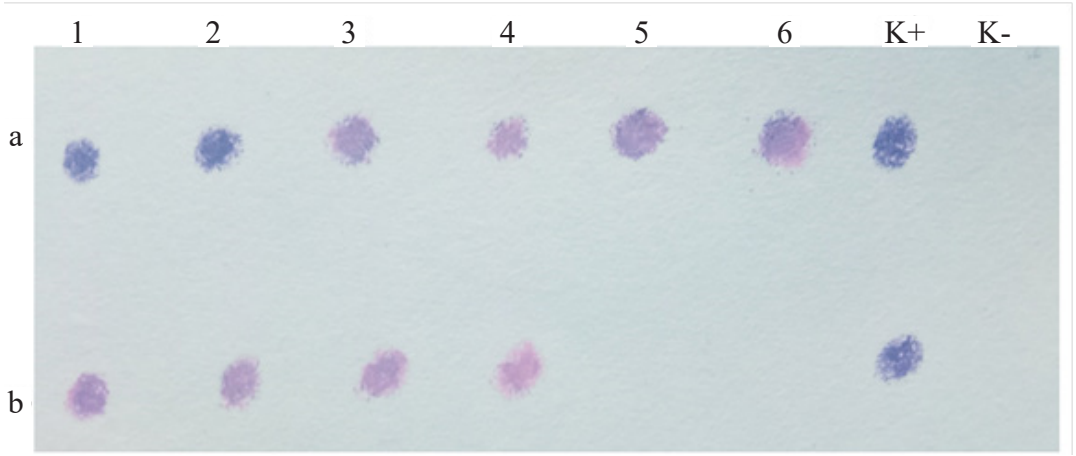

Figure 4 Hybridization of DIG-labeled probes with DNA target. a, genomic DNA from *Clavibacter michiganensis* subsp. *michiganensis* pure bacterial culture; and b, genomic DNA from tomato seed artificially infected by *C. michiganensis* subsp. *michiganensis*. Genomic DNA samples has been diluted at 10 µg µL^{-1}; 8 µg µL^{-1}; 6 µg µL^{-1}; 4 µg µL^{-1}; 2 µg µL^{-1}; and 1 µg µL^{-1}, respectively in colomn 1 to 6; K+, positive control; and K-, negative control.

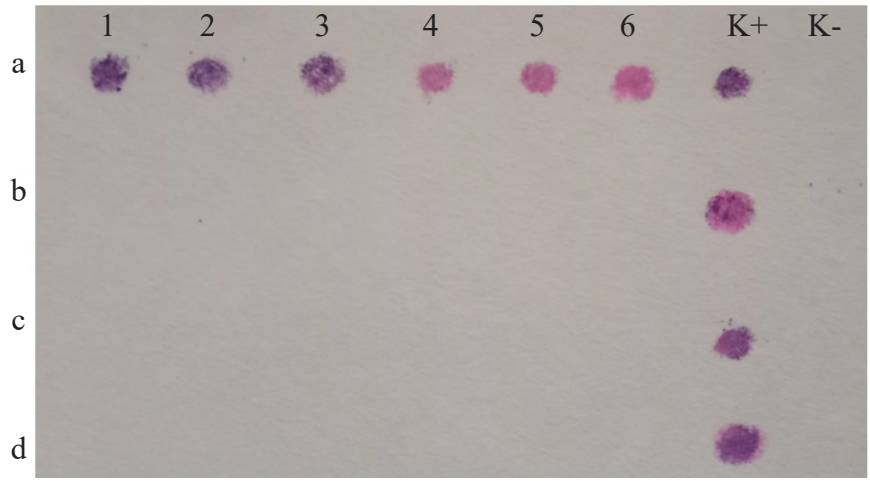

Figure 5 Hybridization of DIG-labeled probes with other target of bacteria. a, genomic DNA from *Clavibacter michiganensis* subsp. *michiganensis* pure bacterial culture; b, genomic DNA from *Xanthomonas campestris* bacterial culture; c, genomic DNA from *Ralstonia solanacearum* bacterial culture; and d, genomic DNA from *Fusarium oxysporum* bacterial culture. All genomic DNA samples has been diluted at 10 µg μL^{-1}; 8 µg μL^{-1}; 6 µg μL^{-1}; 4 µg μL^{-1}; 2 µg μL^{-1}; and 1 µg μL^{-1}, respectively, in colomn 1 to 6; K^+: positive control; K^-: negative control.

detection techniques because its accuracy and sensitivity, especially when the concentration of the target is low (Wu *et al.* 2019). Many experiments have been reported to improve the efficiency and accuracy of this method, including probe selection and the optimization of probe concentrations (Wu *et al.* 2016).

In our study, we selected *tomA* gene as the candidate for detection of *C. michiganensis* subsp. *michiganensis.* Although *tomA* gene was found in many microorganisms, but its homology level is considered low among them. This indicates that the *tomA* gene can be used as a specific molecular marker. Once the gene target has been selected as candidate for DNA probe, analysis of specific sequences within the gene is important.

Some characteristics of the sequences that should be evaluated are the potential location of mismatch and the possible formation of secondary structures, such as hairpins and dimers patterns. Evaluating the specificity of the probe sequence through testing the DNA of various non-target organisms was also necessary. Finally, it is essential to optimize the hybridization conditions (Hendling and Barisic 2019).

The length of the probe may affect hybridization result. In general, probes are designed with a maximum length of 30 bases, as well to the results of this study. The longer the probe size, the longer it will take to hybridize with the target. On the other hand, the specificity and sensitivity of the DNA probe are not affected by the length of the base used. Specific DNA probes with different base lengths produced the same level of specificity and sensitivity. The use of DNA probes with shorter nucleotide base sequences also provides an advantage in terms of saving the use of DIG-dioxygenin labeling (Nuhantoro *et al.* 2018).

The GC content was the next criterion to get a good probe design. The qualification of GC content for the excellent probe was 35–65%. GC content outside the criteria range can lead to probing hybridization instability in the target area. The melting temperature must be considered to prevent probe degradation, thereby reducing efficiency (Rahmaryani *et al.* 2017).

Probe analysis was then carried out on the possibility of the run emergence and repeat criteria. As a qualification of an excellent probe, the number of runs and repeats that appear should not exceed at four. Consecutive repetitions for three or more base sequences should be avoided, exclusively for guanine. Three or more successive guanine bases

can make the template fold into a tetraplex structure. This construction was durable enough and could not be identified by polymerase enzymes. Repetition can also affect the formation of secondary structures. Long dinucleotide repetition can produce complementary sequences to create hairpin structures. In addition, the number of runs and repeats that exceed in the criteria will also increase the time and cost of detection (Rahmaryani *et al.* 2017).

Detection of the presence of DNA probes will involves specific molecules in the labeling process (Lai *et al.* 2016). It has been widely reported that DIG-based dot blot hybridization has successfully applied in various methods with accurate, easy, and cost-effective results (Arlai *et al.* 2021). In this current condition, labeling with non-radioactive compounds is preferred because it is safer for the environment as well as the user (Mishra *et al.* 2018). Furthermore, digoxigenin (DIG)-labeled probes are considered superior compared to radioisotope-labeled probes (Viterbo *et al.* 2018). It is particularly true due to its ability to last longer, relatively cheaper production costs and safer to environment (Shanion and Basu 2009). Another advantage of using a DIG-dioxigenin-labeled DNA probe is the probe can be used repeatedly without losing its specificity and sensitivity. The use of DNA probe for four times with detection results at the same level of specificity and sensitivity has been reported for *Papaya ringspot virus* detection (Nuhantoro *et al.* 2018).

Based on the result discussed above it can be concluded that the probe that had been designed and synthesized are able to detect the presence of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds up to a concentration of 4 µg μL^{-1}, and potential to be used in further research, especially in the development of bacterial biosensors based method.

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