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The efficacy of a chicken antibody for the development of immunoassay-based rapid detection in sugarcane mosaic virus disease

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ABSTRACT Sugarcane Mosaic Virus (SCMV) infection is one of the most serious problems that can result in severe yield loss of sugarcane. Since the symptoms of SCMV infection are similar to other biotic and abiotic stress symptoms, the development of a rapid diagnostic with high precision is required. The use of laboratory animals such as rabbits is required for antibody production in immunoassay-based detection. However, due to its many advantages, specific chicken egg yolk immunoglobulin (IgY) has received considerable attention as an alternative antibody production in immunodiagnostics for infectious diseases. In this study, IgY antibody against SCMV recombinant coat protein (CP) was successfully obtained from chicken blood serum and tested to compare its efficacy against antibody from rabbit (IgG) using immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR). The result showed that IgY and IgG could detect 0.1 g SCMV infected leaves using 1000-times-diluted antibodies. The IgY antibody was also confirmed to be reproducible and potentially applicable in plant disease diagnostics using an antibody-based detection.

KEYWORDS IgG antibody; IgY antibody; Immunoassay; Immunocapture Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR); Sugarcane Mosaic Virus (SCMV)

1. Introduction

The virus which causes mosaic disease in sugarcane is the most widespread viral disease, particularly in the East Java region, Indonesia, and it can significantly lead to a decrease in the productivity of sugarcane (Addy et al. 2017). Sugarcane mosaic virus (SCMV) belongs to the Potyvirus subgroup, and these capsids are filled with single-stranded RNA viruses with an open reading frame. The capsid protein has an approximately 10 kb genome encoding ten functional proteins for transmission through aphids, cell-to-cell virus movement, the formation of virus envelopes, and the viral replication (Reddy et al. 2011; Apriasti et al. 2018). It has been reported that the gene coding for coat protein (CP) is able to differentiate among potyviruses strains (Khanal and Ali 2021). SCMV causes interveinal chlorotic streaks or stripes on sugarcane leaves (Astuti et al. 2019). Therefore, it is difficult to determine whether the symptoms are affected by SCMV or environmental conditions such as abiotic stress since there is no report regarding rapid and reliable diagnosis for sugarcane mosaic disease in Indonesia. Developing virus-free planting material is one of the control management methods in vegetatively propagated sugarcane. To monitor the virus-free in quarantined sugarcane germplasm, the disease surveillance tools become an essential concern for early plant virus detection (Rubio et al. 2020).

An antibody-based detection is widely used in a serological test for plant virus disease diagnostic. The polyclonal antibodies are generated from recombinant virus-specific antisera expressed in the *Escherichia coli* system (Lima et al. 2012; Cerovska et al. 2012). In general, antibody production requires a substantial number of animals, such as rabbits, rodents, and large mammalian species (Stills 2012). However, animal welfare concern has been raised since the method that causes animal pain and dis-

tress while producing antibodies has become an issue for the scientific community. Therefore, it has been suggested to utilize the chicken egg yolk (immunoglobulin Y, IgY) technique for antibody production. It is considered a refinement method since no bleeding comes out from the chicken during the preparation.

The IgM, IgY, and IgA are blood circulating antibodies in chicken, thus, immunized hens transported immunoglobulins to their offspring by a transplacental passage in egg yolk (Narat 2003). The major blood antibody in chickens is IgY, while in mammals is IgG, that both IgY and IgG have similar character in biological functions. However, there are several benefits to using the IgY technique beyond the animal welfare concern, such as less expensive, easy to extract, and a higher yield that can be generated compared to mammals (Li et al. 2015; Júnior et al. 2018).

In the present study, we compared the production of polyclonal antibodies from the chicken blood serum (IgY) and rabbit (IgG) to analyze the efficacy of both antibodies against CP-SCMV. The technique of immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) was developed using IgY antibody for sensitive and specific detection of SCMV in symptomatic sugarcane leaves. This research will give an information of potential large-scale antibody production and provide a direct use of rapid diagnostic tool for SCMV using IgY from chicken egg yolk.

2. Materials and Methods

2.1. Immunization of rabbit and chicken

The production of CP-SCMV polyclonal antibodies was conducted by vaccinating antigens in experimental animals. The antigen for antibody induction was a recombinant capsid protein (CP) SCMV Indonesia strain as previously described by (Darsono et al. 2018). The animals used in the experiment were a 4-month-old New Zealand White female rabbit (1.7 kg) and an 8-month-old local bred chicken hen (*Gallus gallus domesticus*), around 1.5 kg in weight. All procedures involving animal treatments were approved by the Ethics Committee of Faculty of Veterinary Medicine, University of Airlangga (no. 552-KE).

Purified and concentrated antigen proteins used in this study were obtained from recombinant Coat Protein Sugarcane Mosaic Virus (rCP-SCMV) harvested from *Escherichia coli* BL-21 with a concentration of 16.184 µg/µL after concentrated using electroelution (Astuti et al. 2019). The rabbit vaccination method was referred to the work of (Koohapitagtam and Nualsri 2013). The rabbit was injected with 500 µg rCP-SCMV antigen mixed with Freund's Complete Adjuvant (FCA) at a ratio of 1:1 in a 1 mL total volume subcutaneously. In a week after the first injection, repetition boosters were given three times in weekly intervals with mixed antigen and Freund's Incomplete Adjuvant (FIA) at the ratio of 1:1.

The chicken vaccination method was referred to Amro et al. (2018). An antigen dose of 80 µg was mixed with FCA at a ratio of 1:1 until a total volume of 0.6 mL, then injected subcutaneously. The repetition booster was performed a week after the first vaccination and repeated three times at weekly intervals. Booster solution was made from an antigen dose of 60 µg mixed with FIA at 1: 1 ratio (total volume 0.4 mL).

2.2. Animal blood harvesting of crude antibodies

Negative antibodies were obtained from animals' blood one day before the first vaccination. After the vaccination, the antibodies were then collected every week after the second booster injection and thereafter. The rabbit blood collection was taken from auricularis veins at the ears as much as 3 mL, and the chicken blood was taken from pectoralis vein at the wings as much as 1 mL. Blood samples were incubated at room temperature for 1 hour, then centrifuged at 6,000 g for 15 min at room temperature. The supernatant containing crude antibodies was collected, labeled, and refrigerated at -20 °C prior to use.

2.3. Determination of antibody specificity using precipitation test

Agar gel immunodiffusion (AGID) test was carried out on the crude antibodies by using the method as described previously (Nemoto et al. 2018). It was performed to use the agar gel in four surrounding wells, and a well in the center containing the antigen. Precipitation of gel was made from 1% agarose with Ouchterlony buffer (15 mM NaN_3, 1 mM EDTA, 50 mM Tris-HCl pH 7.4, and 0.9% NaCl). Agarose gel was poured on the glass plate until solidified. Subsequently, a pattern consisting of four wells around a central was prepared using a cork borer. Each well was 7 mm in diameter and 5 mm apart and was added with 10 µL antigen (concentration of 15 µg/µL) in the center and 10 µL crude antibodies in surrounding wells. The solution was mixed manually for a few seconds or more and incubated at room temperature overnight. The direct agglutination was done by mixing of 10 µL crude antibodies with antigen rCP-SCMV (10 µL antigen, concentration of 15 µg/µL) at an object glass and observed the granulation formed after reaction using a stereo microscope.

2.4. Determination of antibody sensitivity using Western blot

SDS-PAGE and Western blot analysis were performed as previously described by Darsono et al. (2018). Four concentration (1 ng, 10 ng, 100 ng, and 1000 ng) of the antigen rCP-SCMV were used for sensitivity test of the antibody. The antigens were placed in each well of SDS-PAGE gel (separating gel 12% and stacking gel 3%). An electric current of 110 V was passed through the gel for 60 min. The separated proteins were electroblotted onto an Immobilon-P transfer membrane (Millipore) using a semi-dry transblotter (Bio-rad Richmond, CA) at a constant current of 250 mA for 60 min at room temperature. The membrane was removed from the transblotter and washed with Tris Buffer Saline buffer (TBS, consists of 25 mM Tris-HCl pH

7.5, 150 mM NaCl, 3 mM KCl) three times.

The membrane was blocked with 4% skim milk and incubated overnight at 4 °C for the binding process and to remove non-target protein. After washing step using TBST buffer (consists of TBS buffer and 0.1% Tween-20), the membrane was incubated with rabbit and chicken antibody diluted in TBS containing 0.5% skim milk (1:1000) for 2 h at room temperature with gentle agitation. The membrane was further washed three times, followed by incubation with the secondary antibody, goat anti-rabbit IgG alkaline phosphatase (AP)-conjugate (Bio-Rad) or rabbit anti chicken IgY alkaline phosphatase (AP)-conjugate (Bio-Rad), at 1:3,000 dilutions for 60 min at room temperature. The expected protein band was visualized by incubation in AP color developer (Alkaline Phosphatase conjugate substrate kit, #1706432, Bio-Rad) and AP buffer until the dark blue color appeared on the desired protein band size.

2.5. The efficacy of antibodies using sugarcane leaves as antigen for Western blot

The samples used for Western blot test were sugarcane leaves with mosaic symptoms and healthy leaves. The method was modified from Darsono et al. (2018). The amount of 0.1 g of fresh leaves were grounded in liquid nitrogen and mixed with extraction buffer (50 mM MOPS + NaOH, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5, 4 °C) at a ratio of 1:3. The leaf extract was centrifuged at 16,000 g for 10 min for protein separation. According to Apriasti et al. (2018) it has been reported that CP-SCMV was expressed in insoluble fraction, thus pellets as insoluble fraction were solubilized in the solubilization buffer (50 mM Tris base; 1 mM EDTA, 2% SDS; 30% sucrose; pH 8.5; room temperature) at ratio 1:3 (labeled as pellet 1). The supernatant as a soluble fraction (labeled as supernatant 1) was obtained after centrifugation using ultracentrifuge (Hitachi CS15NX) at 45,000 g for 1 h at 4 °C. Pellets from this process were solubilized in solubilization buffer as above (labeled as pellet 2). Supernatant from this process was labeled as supernatant 2. The supernatant and pellet proteins were separated with SDS-PAGE and subjected to Western blot analysis using rabbit and chicken antibodies.

2.6. The efficacy of antibodies using sugarcane leaves as antigen for IC-RT-PCR

Sugarcane leaves with mosaic symptoms, and healthy leaves (0.1 g) were grounded in liquid nitrogen and mixed with Tris-HCl buffer (0.01 M Na_2SO_3, 2% PVP, 3 mM $NaNO_3$, 140 mM NaCl, 0.05% Tween-20, pH 8.3) at a ratio of 1:3. Crude leaf extract was centrifuged to separate protein at 12,000 rpm for 10 min. The supernatant was used as the sample for the immunocapture assay.

Immunocapture steps began with coating the antibodies to PCR tubes. Fifty microliters of 500× and 1,000× diluted antibodies in carbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 0.02% sodium azide, 0.5 g PVP, pH 9.6) were incubated for 2 h at 37 °C and washed sufficiently. After being blocked with 50 µL blocking solution (4% skim milk in PBS buffer), the PCR tubes incubated for 1 h at 37 °C. The PCR tubes were further washed with Phosphate Buffer Saline Tween (PBST) washing buffer (135 mM NaCl, 1.5 mM KH_2PO_4, 8 mM $NaHPO_4$, 3 mM KCl, 0.05% Tween-20, 3 mM NaN_3, pH 7.4) for three times. Supernatant of crude leaves extract were added to the tubes and incubated for 2 h at 37 °C. Coating step was ended with washing step using PBST washing buffer for three times and the final wash with Phosphate Buffer Saline (PBS, consists of 135 mM NaCl, 1.5 mM KH_2PO_4, 8 mM $NaHPO_4$, 3 mM KCl, 3 mM NaN_3, pH 7.4). The samples from this process were reverse transcribed using i-Script cDNA Synthesis Kit (#1708891, Bio-Rad) as manufacturer instruction.

Targeted coat protein cDNA was amplified using a pair of specific primer: F2: 5'-GCGGATCCGTCGATGCAGGTG-3' and R2: 5'-GTGCTCGAGCAGAGAGAGTGCAT-3' to produce a 924 bp DNA fragment. The PCR reaction consisted of one cycle pre-denaturation at 94 °C for 5 min, 35 cycles at 94 °C denaturation for 30 s, 56 °C for 30 s, 68 °C extension for 60 s, and the final extension step at 68 °C for 5 min. The amplified cDNA was visualized using 1% agarose gel electrophoresis (Astuti et al. 2019).

3. Results and Discussion

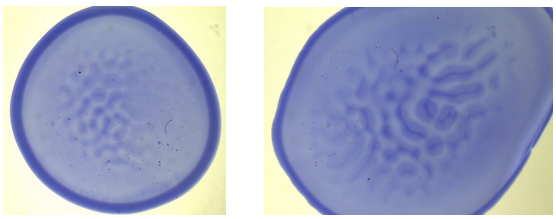
3.1. Determination of antibody specificity using precipitation test

A total of 10 µL serum from rabbits and chickens vaccinated with rCP-SCMV antigen were tested for the presence of specific polyclonal antibodies with the AGID test against rCP-SCMV antigen. According to the AGID test, the immunization treatment with rabbit and chicken antibodies showed the same specificity result. Transparent lines, which was precipitation line, appeared between the antigen well and antibodies well, except in pre- vaccination serum. The precipitation line was formed from the first harvesting period of serum collection until the fifth harvesting after the third booster, as seen in Table 1. These results indicated that immunization treatment given to rabbits and chickens could produce a specific polyclonal anti-

TABLE 1 Resume of AGID test from rabbit and chicken antibody.

Antibodies	Result of precipitation test					
	Week-0	1st harvest	2nd harvest	3rd harvest	4th harvest	5th harvest
Rabbit	-	+	+	+	+	+
Chicken	-	+	+	+	+	+

body rCP-SCMV from all periods of harvesting (Figure 1). According to Amro et al. (2018), immunization treatment given to chickens also showed the same result as rabbits. The antibody could be obtained from the first harvesting period of the serum collection and the following harvesting period. This method was proven to reduce stress in the animal because the injection treatment and blood serum collection were performed in different weeks. Stills (2012) stated that avoidance of stress in animals can be done by the use of adequate research facilities and appropriate immunization methods.



(a) (b)

FIGURE 1 Result of the direct agglutination test from (a) rabbit and (b) chicken.

3.2. *Determination of antibody sensitivity using Western blot*

The results of Western blot test showed that the molecular weight of CP-SCMV proteins was in the range of 40 kDa (Figure 2). It was similar to the statement from Astuti et al. (2019) that SCMV capsid protein had a molecular weight of about 40 kDa, and this size was comparable to the protein capsid of Papaya Ringspot Virus (Omar et al. 2011) and Grapevine Fanleaf Virus (Koolivand et al. 2016). Determination of sensitivity of antibody using Western blot method showed different antibodies response. To determine the antibodies sensitivity, recombinant antigens were used to detect in various concentrations of 1 ng, 10 ng, 100 ng, and 1,000 ng with 1,000× dilution. At 1,000× dilution, female rabbit antibodies were able to detect recombinant antigens up to 10 ng concentrations. Meanwhile, chicken antibodies were only able to detect 1,000 ng recombinant antigens (Figure 2). Thus, the sensitivity of the female rabbit antibodies was higher than chicken. Both antibodies were shown a protein band with molecular weight of 40 kDa specifically, whereas pre-vaccination serum showed no line of protein band. These findings indicated that the polyclonal antibody prepared in this study were highly specific for SCMV Indonesian isolate.

The similar results were also reported previously that female rabbit antibodies had the highest sensitivity that was able to detect recombinant antigens up to 1 ng levels (Hardjo 2014). There were many difficulties in producing polyclonal antibodies from animals besides the daily problem of handling the animals during the experiment. The slightest environment changed will generates the animal behavior changed. Therefore, producing high-titer virus-specific antiserum is one of the challanges since the animal behavior changed will affect the antibody production (Lima et al. 2012). According to Stills (2012), the use of female animals in the production of polyclonal antibodies was more advantageous since it was less aggressive and more adaptable in single cage handling. On the other hand, chicken's antibody can be produced at the early egg-laying period and it gives the benefit to be more adaptable in environmental changed.

3.3. *The efficacy of antibodies using sugarcane leaves as antigen for Western-blot*

The ability of polyclonal antibodies to detect SCMV on symptomatic sugarcane leaves was performed using 1,000× diluted antibodies. Using the rabbit antibody (IgG), the protein band appeared in line 3, 4, and 5, which means detectable in all fractions, with the protein band in line 5 (insoluble fractions) appearing thicker. In chicken antibody (IgY), the protein band was clearly visible only in line 5, which was insoluble fractions (Figure 3). It was confirrmed that SCMV could be found in all fractions, but the virions were more concentrated in the insoluble fraction. Therefore, only the IgG could detect the virus in soluble and insoluble fractions because the antibody's sensitivity of the female rabbit was higher than chicken.

We also suggested that a low virus concentration in soluble fractions might have caused detection problems. Besides, in Western blot using chicken antibodies, the background of the membrane turned to darker color during the staining process. Thus, it was challenging to observe the protein band clearly. This result showed that SCMV virions were concentrated in the insoluble fraction of symptomatic sugarcane leaves. In this insoluble fraction, both antibodies (female rabbit and chicken) could detect SCMV clearly. Similar results were presented on recombinant coat protein Potato Virus X (Mardanova and Ravin 2021) and on recombinant viral capsid proteins Sugarcane Streak Mosaic Virus (Hamdayanty et al. 2016). Both results confirmed that recombinant coat protein viruses were presented in the insoluble fraction.

3.4. *The efficacy of antibodies using sugarcane leaves as antigen for IC-RT-PCR*

We also developed an IC-RT-PCR procedure that offered sensitive, specific, and rapid detection of SCMV in field samples. This method can be applied for virus detection which has RNA for material genetic. Moreover, this method can eliminate the step of RNA extraction method and easier to carry out in invitro condition. The IC-RT-PCR methods have been developed to detect many other plant viruses, such as Lily Mottle Virus (Yoo and Jung 2014), and Sugarcane Streak Mosaic Virus (Viswanathan et al. 2013). We found that a single 924-bp band could be amplified from SCMV symptomatic leaf extract using IC-RT-PCR assay, whereas no DNA band was amplified from a healthy leaf (Figure 4). IgY against CP SCMV has been used successfully to detect SCMV infection through IC-RT-PCR. In Western blot, IgY detection showed a slightly thin protein band compared to IgG. However, in IC-RT-PCR showed a very clear cDNA band compared to IgG

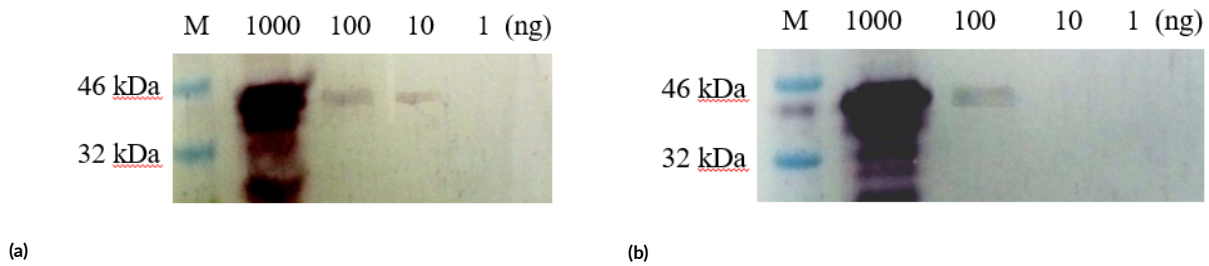


FIGURE 2 Result of sensitivity test from antibodies against rCP-SCMV by Western blot method. (a) Antibodies from a female rabbit. (b) Antibodies from chicken.

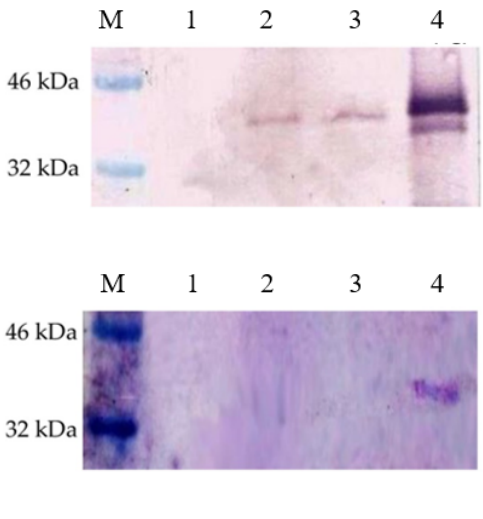


FIGURE 3 The result of polyclonal antibodies rCP-SCMV detection against an antigen from sugarcane leaves with the symptom of mosaic disease by Western b lot method. (a) Antibodies from female rabbits and (b) antibodies from chicken. M: protein marker; 1: supernatant 2 of symptomatic sugarcane leaves extract (soluble fraction 2); 2: pellet 2 of symptomatic sugarcane leaves extract (insoluble fraction 2); 3: supernatant 1 of symptomatic sugarcane leaves extract (soluble fraction 1); 4: pellet 1 of symptomatic sugarcane leaves extract (insoluble fraction 1).

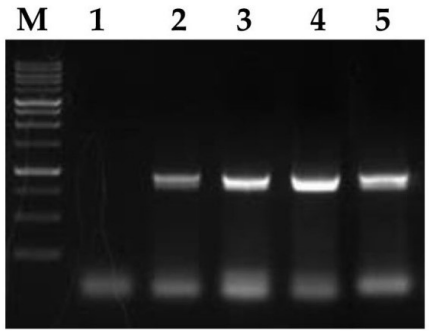


FIGURE 4 Result of IC-RT-PCR detection against an antigen from sugarcane leaves. A single band of 924 bp was observed. M: marker, 1: healthy leaves; 2: symptomatic leaves with 500× diluted rabbit antibody; 3: symptomatic leaves with 1,000× diluted rabbit antibody; 4: symptomatic leaves with 500× diluted chicken antibody; 5: symptomatic leaves with 1,000× diluted chicken antibody.

from the rabbit. These findings explained that IgY from chicken and IgG from rabbit has the same sensitivity for IC-RT-PCR detection.

4. Conclusions

Polyclonal antibodies against rCP-SCMV were able to be produced from rabbits and chicken hens. The sensitivity of the rabbit antibody (IgG) was higher than the chicken antibody (IgY) in Western blot. Both IgY and IgG successfully detected CP-SCMV in sugarcane leaves from field samples, indicating that both antibodies were highly specific for SCMV Indonesian isolate. In conclusion, we attained results that IgY is as effective as IgG and a potential source for antibody production for immunoassay-based rapid detection. IgY antibody production might offer a new large-scale source of low-cost antibodies. This method could be used for virus surveillance to maintain management control of pests and diseases in the nursery and commercial fields.

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Authors' contributions

ND and WDS : design the study and wrote the manuscript. RA, AHSW, PAS and VMP : carried out the laboratory works. ND, ST and WD : analyze the data.

Competing interests

The authors declare no conflict of interest.

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