Occurance of *Clavibacter michiganensis* subsp. *sepedonicus* on Potato in South Sulawesi

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Abstract. Bacterial ring rot caused by a gram-positive Coryneform bacterium *C.m* subsp. *sepedonicus* is an important disease in potato crops in the world. The disease is until now still belong to an A1 quarantine pathogen in Indonesia, although it was found in West Java since 2013. The objective of this study was to know the presence of bacterial ring rot in four potato district areas in South Sulawesi. Diseased samples were conducted from potato fields and storage warehouses in Enrekang, Gowa, Jeneponto and Bantaeng. Potato tuber samples were cutted and observed their vasiculer vessels and then isolated and grown the bacteria on NA and NBY media. Bacterial isolates were morphological and physiological characterized as well as pathogenicity on eggplant and PCR test using specific primer for Cms 50F and Cms 50 R. The results showed that Cms has become widespread in four districts in South Sulawesi. The disease incidence of bacterial ringrot in these districts reached above 30 %. All of 14 isolates were obtained from the results of a standard methods for identification (EPPO, 2006) showed that the presence of DNA band size of 224 bp, which indicated positively belong to *C.michiganensis* subsp. *sepedonicus*.

1. Introduction

Potato (*Solanum tuberosum* L.) is a crop of the world's major economic importance and number one non-grain food commodity [1]. Potatoes are also one of the types of vegetables that have priority to be developed in Indonesia. This can be seen from its consumption in fourth place after rice, wheat and corn. South Sulawesi Province is one of the producers of potatoes with an increased harvest area from 2009 to 2012, from 1,433 hectares to 1,816 hectares and potato production which has increased from 8.24 tons/ ha to 12.91 tons/ ha. However, there was a decrease in 2014 to 2016 production, which in 2014 amounted to 1,347,815 tons, in 2015 amounted to 1,219,269 tons and in 2016 amounted to
1,213,038 tons [2]. The attack of pests and diseases and the quality of seeds is one of the factors causing a decrease in potato crop production.

One of the obstacles to potato production is the presence of pests and diseases in potato plants, the use of non-certified seeds and changes in weather that occur causing the status of plant disturbing organisms to change and be difficult to predict. The provision of technical knowledge to control diseases as well as proper allocation of improved quality seed would help to increase profitability and productivity of potato [3,4]. One dangerous disease that attacks potato plants is ring rot caused by the bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Cms). One dangerous disease due to transmission of this disease can be through seeds (seedborne disescent) and agricultural tools, so that to control it chemically becomes ineffective [5]. This is in accordance with the opinion of one farmer as a resource person who stated that the control using a bactericide, dectoprima, was apparently unable to provide the expected impact to overcome wilt due to the attack of pathogenic bacteria. Some of the seeds used by farmers come from outside their area, which they obtain by buying seeds from West Java, while the indications of ring rot are found in West Java.

Risk Analysis of Plant Pest Organisms (RAPPO) is a scientific method in determining the status of an pest for importing countries as well as a tool to identify requirements and plant quarantine actions that must be carried out on the entry of agricultural commodities if they have a risk or potentially carry Quarantine Plant Pest Organisms. Based on the results of a survey conducted by the University of Padjadjaran team and the Indonesian Vegetable Research Institute in 2013 stated that the disease was spread in the centers of potato cultivation on Java Island even in South Sulawesi based on the results of the ELISA test.

*Polymerase chain Reaction* (PCR) is an in vitro reaction to double the number of DNA molecules in a particular target by synthesizing new DNA molecules that complement with target DNA molecules with the help of enzymes and Oligonucleotides as primers in a thermocycler (PCR machine) [6]. The PCR method has high sensitivity and can be done in a fast time. This method is used to detect bacteria that can be carried by the carrier media (eg seeds, cuttings, bulbs and others) and bacterial culture isolates on agar media. Some species of bacteria that infect plants that can be detected using this method include: *Acidovorax avenae* subsp. *avenae*, *Burkholderia andropogonis*, *Clavibacter michiganensis* subsp. *sepedonicus*, *Erwinia tracheiphila*, lethal yellowing phytoplasma. Based on the description above, it is necessary to further detect the presence of bacterial ring rot caused by the pathogen *Clavibacter michiganensis* subsp. *sepedonicus* in potato crops.

2. Materials and Methods

2.1. Sampling

Sampling was carried out in 4 districts. Each of the two sub-districts in each district. (Masalle and baroko sub-districts, in the district of Enrekang), (Tinggi Moncong and Tombolo Pao sub-district, in Gowa district) (Rumbia sub-districts and in Jeneponto district) and (Ulu Ere and Sinoa sub-districts, in Bantaeng district). Sampling was carried out by taking tubers in potato plantations. Randomly on farms. Each sampling point was taken as many potato tubers as plants so that the number of potato samples was 25 plants for 1 potato crop field.

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IS = (a:b) \times 100\%
\]  

| IS     | = Intensity of attack (%) |
|--------|---------------------------|
| a      | = Number of bulbs attacked |
| b      | = Number of tubes observed |
2.2. Isolation and Identification of Pathogenic Bacteria

Take symptomatic plant parts, then sterilize the surface by washing with 70% alcohol. Using a scalpel, then the symptomatic potato tuber is halved, the part that emits the liquid on the symptom is taken with the needle taken and then scratched in the NA medium, after which it is fertilized for 2-4 days. Then re-isolated for identification purposes. Furthermore, bacteria are grown on selective media, namely NBY media or NCP 88 media. Some simple tests to identify bacteria, include:

2.2.1. Gram Reaction. Bacterial colonies of pure culture are taken with ose needles and applied to the glass of the object that has been dripped with KOH. Colonies that appear slimy means a positive reaction (gram negative), whereas non-mucus means a negative reaction (gram positive).

2.2.2. Oxidase Test. Bacterial culture is taken with an ose needle and then applied to filtered paper that has been dripped with 1 percent tetramethyl paraphenildiamine and stirred in a circle. If within 10 seconds there is a change in color to purple then the reaction is positive.

2.2.3. Oxidative / fermentative growth test (Anaerob/Aerob). Fermentative oxidative testing is carried out to identify isolates of aerobic or anaerobic bacteria. Color changes in the Fermentative Oxidative media will determine the category of bacteria. If there is a change in color from blue to yellow on the tube indicates a positive for anerobic growth (fermentation occurs).

2.2.4. Test growth at 37° C atau 40°C. Inoculation of 5 - 10 ml of liquid media NBY, incubation one night at 25°C - 27°C using a shaker at a speed of 100 rpm. Take 50 ul culture using a micropipette, insert it into a test tube containing liquid NBY and incubate at 41°C with a shaker at 100 rpm. If a media change becomes cloudy, it means that bacteria grow well, so this can be an indication for the Acidovorax genus. However, if the media remains clear, this can indicate that there is no bacterial growth in the genus Clavibacter.

2.2.5. Test media NBY (colony color in NBY). Testing with NBY media can be an indication for Clavibacter with the presence of white changes. Because for some genera the color shown is yellow.

2.2.6. Pathogenicity Test. Pathogenicity testing using eggplant plants. This is done by providing 24 eggplant plants. Then a single bacterial colony obtained from the results of bacterial isolation on culture media was made a concentrated suspension by adding MgSO4·H2O, then injected into the eggplant plant, on the stem. Then the observation was made.

2.3. Molecular Identification by PCR Method

2.3.1. DNA Extraction and Purification. Samples were taken from plant tissue 0.2 gr and put into 2.0 ml eppendorf tubes then suspended and homogenized with 700 ul ml of extraction buffer water (CTAB 1%), Tris HCL 50 Mm Ph 8.0, NaCl 0.7 M and Na2ED 10 Mm and added with 1% mercaptoethanol and vortexed until the suspension is evenly distributed. The DNA was extracted by adding 1x the volume of Phenol: Chloroform: Isoamilalkohol (25: 24: 1) with a pH of 7.6. The mixture was reversed thoroughly by turning the tubes carefully, then centrifuging at 12,000 rpm for 12 minutes. The upper layer (supernatant) phase is taken into the new eppendorf tube. DNA samples in the supernatant were extracted again by adding a solution of chloroform: isoamilalkohol (24: 1) as much as 0.5x the volume of solution. The tubes are turned back and centrifuged at a speed of 12,000 rpm for 5-10 minutes. The upper liquid phase (supernatant) is transferred to the new eppendorf tube and 0.6x is added to the volume of cold isopropanol to precipitate the DNA, then incubated in the freezer (-20°C) for 1 hour or more. DNA deposits are separated from the solution by centrifuging them at 12,000 rpm for 12 minutes. The
The supernatant is removed using a pipette. Next the pellets are washed with 70% cold ethanol and vacuum dried.

The pellet is suspended by adding 1x TE (10 mM Tris - 1 mM EDTA pH 8.0) as much as 100 ul. At the stage of DNA purification from possible contamination by RNA, RNA was removed by adding 0.1x RNAase (1 μg / μl) and incubated for 1-2 hours at 37°C. The suspension was added with 0.5x the volume of 7.5 M ammonium acetate, followed by adding 2x the volume of 95% cold ethanol and being centrifuged at a speed of 10,000 rpm for 12 minutes. After centrifugation, the obtained pellets were washed with 70% cold ethanol, dried at 40°C and dissolved with 100 ul 1x TE.

2.3.2. Polymerase Chain Reaction (PCR). This procedure is done on samples from DNA extraction. Previously a PCR (PCR mix) reaction mixture was made for the volume of 25 μl in each tube plus; 2.5 μl of PCR buffer 10 x 0.5 μl dNTPs 2 mM; 1 μl each primer (10 pmol/μl) consisting of Cms 50F Primer and Cms 50R Primer, 2 μl DNA template; and 12.5 μl. Top tags, 7 μl of sterile bides. PCR Grade Amplification was carried out in 40 cycles using a thermalcycler PCR machine. The amplification condition is:

1. Denaturation 94°C 45 seconds
2. Annealing 55°C 60 seconds
3. Extension 72°C 10 seconds

4. Denaturation 95°C 45 seconds
5. Annealing 55°C 40 seconds
6. Extension 72°C 15 seconds
7. Final Extension 72°C 5 seconds

2.3.3. Total DNA Electrophoresis and PCR Products. Electrophoresis of PCR Products is by using 1.5% Agarose, 90 volts for 20 minutes, while total DNA electrophoresis is 0.8% Agarose, 90 volts for 30 minutes. The visualization of DNA profiles was carried out by staining etidium bromide (1 μg/ml) for 15 minutes, followed by washing with sterile bides for 5 minutes, then detected using UV light on a UV-cabinet. Gel documentation was then carried out using Geldoc (UVP UPLAND CA UK).

2.3.4. Observation. Detect the presence of Clavibacter michiganensis subsp. sepedonicus bacteria in each column of treatment carried out by observing the presence of DNA band 224 bp. If there is a DNA band, the seeds are positive for Clavibacter michiganensis subsp. sepedonicus bacteria but if it does not mean the potato seeds do not contain Clavibacter michiganensis subsp. sepedonicus.
3. Results

3.1. Symptoms of ring rot disease
Ring rot that attacks potato plants shows the initial symptoms of wilting at the edges of the leaves, especially on the lower leaves and the color of the leaves becoming pale green and the symptoms on Cms infected tubers are clearly visible when the tuber is cleaved, as shown in the image below.

![Symptoms of ring rot disease](image)

**Figure 1.** Symptoms in potato plants infected with Cms on cropping fields (a) and ring rot symptoms in infected potato tubers (b).

3.2. The intensity of disease attacks in the warehouse
The highest intensity of Cms attacks in storage is seen in Gowa district, which is 33.60%, and followed by Enrekang district at 20.80%, Bantaeng at 21% and lowest disease intensity seen in Jeneponto district at 16.60%.

![Intensity of disease attacks in storage](image)

**Figure 2.** Average intensity of ring rot disease in potatoes *Clavibacter michiganensis* subsp. *sepedonicus*) in storage.
3.3. Attack intensity on cropping land

The intensity of Cms attacks on potato plantations was the highest seen in Gowa district at 37.66%, and followed by Enrekang district at 26%, Bantaeng at 24.33% and lowest disease intensity seen in Jeneponto district at 19%.

![Intensity of disease attack (%)](image)

**Figure 3.** Average intensity of ring rot disease in potatoes *Clavibacter michiganensis* subsp. *sepedonicus* in the planting area.

3.4. Bacterial isolates in potatoes

From the results of isolation of bacteria taken in the plantations, several bacterial isolates were obtained. The color of the colony is white and looks like creamy white milk and the shape of the colonies is irregular, not slimy.

| No. | Isolates | Gram Test | Aerobic bacteria | Anaerobic bacteria | YDC | NA |
|-----|----------|-----------|------------------|--------------------|------|----|
| 1   | KMN12    | +         | Yellow           | Blue               | White| White |
| 2   | KBTG2    | +         | Yellow           | Blue               | Yellow| White |
| 3   | KJPT4    | +         | Yellow           | Blue               | Milky white| White |
| 4   | KJPT2    | +         | Yellow           | Blue               | Milky white| White |
| 5   | KBTG 12  | +         | Yellow           | Blue               | Milky white| White |
| 6   | KJPT20   | +         | Yellow           | Blue               | Milky white| White |
| 7   | KER 2 (NA) | +         | Yellow           | Blue               | Milky white| White |
| 8   | KER2(NBY) | +         | Yellow           | Blue               | Milky white| White |
| 10  | KMN16    | +         | Yellow           | Blue               | Milky white| Cream |
| 11  | KMN17    | +         | Yellow           | Blue               | Milky white| White |
| 12  | KJPT 21  | +         | Yellow           | Blue               | Milky white| White |
| 13  | KBTG 3   | +         | Yellow           | Blue               | Milky white| White |
| 14  | KBTG 8   | +         | Yellow           | Blue               | Milky white| White |
3.5. PCR testing
From the test results using the PCR method (polymerase chain reaction), positive (+) results were obtained for Clavibacter michiganensis subsp. sepedonicus (Cms) in several districts in South Sulawesi.

![Figure 4. Results of DNA amplification of eight samples from several districts in South Sulawesi. Note: (1) = 100 bp Marker, (2) = KMN1; Tuber samples from Malino, (3) = KER1; Tuber samples from Enrekang, (4) = KBTG; Tuber samples from Bantaeng (5) = KJPT1; Tuber samples from Jeneponto, (6) = KMN; Tuber samples from Malino, (7) = KER2; Tuber samples from Enrekang (8), KBTG2 = Bulb sample from Bantaeng, (9) = KJPT2; Bulb sample from Jeneponto, (10): Negative control (-).](image)

4. Discussion
The attack of ring rot on the plant will show the initial symptoms of wilting on the edges of the leaves, especially on the lower leaves and the color of the leaves turn pale green when compared to the color of leaves in plants that are not attacked, and the base of the stem if cut and squeezed. Symptoms of tubers infected with Cms are clearly seen when the tubers are divided into ring-shaped vascular parts with pale yellow to light brown. This is consistent with the statement [7] which states that the initial symptoms shown by Cms are symptoms of wilting on leaves that begin at the edges of the leaves, mainly the lower leaves and are shown in pale green, and finally leaf tissue and stems turn yellow. Symptoms in the tuber are seen when cleaved. It appears on the vascular tissue resembling a ring, and the tuber will emit creamy milk exudates.

Based on the results obtained, it is known that the intensity of the disease attack which is suspected to be detected by ring rot Clavibacter michiganensis subsp. sepedonicus on potato planting land in several districts, namely Gowa district ranges from 37.66%, Enrekang district ranges from 26%, Bantaeng district ranges from 24.33 %, and Jeneponto district ranges from 19%. The results table shows that the highest incidence of disease is in Gowa district at 37.66%. This can be caused by pathogens caused by the bacteria Clavibacter michiganensis subsp. sepedonicus is a seed-borne disease, so that the spread of this disease can spread so quickly.

This is in accordance with the opinion [8], which states that one of the most important spreads of ring rot is through seed. In addition, the rapid spread of this disease is supported by the trade in inter-island potato seeds that occurs at the farmer level, because due to the large need for potato seeds while sometimes the availability of potato seeds is limited in an area so that buying potato seeds in other regions, this sometimes makes farmers not paying attention to the seeds used, seeds that are less healthy and not certified, so it is quite risky to get infected with the disease. The same thing was stated by [5] which states that the spread of pathogens of the bacteria Clavibacter michiganensis subsp. sepedonicus can be transmitted through seed trade and Indonesia is a country that often imports potato seeds from abroad and trades inter-island potato seeds.
Compared with the intensity of attacks in the other three regions, namely enrekang, bantaeng and jeneponto. The intensity of attacks is lower compared to the Gowa area, this is because these three regions often use local potato seeds or seedlings from their own nurseries, and rarely buy from other regions, so that the wider spread of the disease can be minimized. In addition, these three regions tend to grow other vegetables, such as cabbage, leeks, carrots, corn compared to potatoes. Meanwhile, Malino is the most potato-producing center. The spread of disease by a pathogen can also occur through land, water, and agricultural equipment and transportation, therefore cleanliness and sanitation of a cropping area need to be considered so as not to trigger infection and the spread of a pathogen. Because the remains of infected plants or tubers on the farmer's land can cause these bacteria to survive in the soil, therefore when the land is harvested it must be completely clean and no tuber is followed when stored in the storage warehouse. This is in line with the opinion [8] which states that in addition to spreading through seeds, ring rot can also spread through water, soil and agricultural equipment. Apart from being planted, the intensity of the attack of ring rot can be seen in the postharvest storage warehouse. Based on the results obtained it can be seen that the incidence of attacks in storage warehouses for the Gowa area ranges from 33.60%, Enrekang 20.80%, Bantaeng 21% and Jenepongo 16.60%. From this value, the highest intensity of attacks is found in the Gowa area. The high level of attack in the warehouse, is greatly influenced by attacks on the field before the harvest. This is because the lowest disease of the seed is systemic and latent, which makes the potato plant which is attacked during planting the symptoms are in all parts of the plant, namely wilting, leaf color changes. The infection is latent, which means that the symptoms caused have a long incubation period, so that the pathogen can survive in the plant until after harvest. This is in accordance with [9] which states that Cms pathogens cause wilting in potato plants and degraded vascular tissue so that they cause rotting potato tubers, but can last latently in the tubers for a long time without causing symptoms.

Storage of crops is very necessary to pay attention to the temperature and cleanliness of the storage warehouse. If the potato bulbs are stored by not sorting with tubers which are indicated to be attacked by pathogens, when friction or contact with healthy tubers occurs, the pathogens can easily spread. This is because the exudate in the sick tuber will easily spread to healthy tubers because of the temperature and humidity that supports pathogens to develop and survive and spread rapidly. *Clavibacter michiganensis* subsp. *sepedonicus* bacteria carries seeds into the stem and leaves through vascular tissue so that the pathogens will begin to show symptoms rather slowly that is mid-season before infecting new tubers. Internal symptoms appear in the tuber at harvest, and are observed after some time in storage. The disease develops rapidly at 18 - 22°C. CMS bacteria can survive and remain contagious for several years on the storage of potatoes due to exudates on the surface wall of potatoes that are removed by infected potatoes so that they contaminate and transmit to other tubers [8, 10]. From the isolation of potatoes taken from plantations in several districts, namely Gowa, Enrekang, Bantaeng, and Jenepongo, 14 isolates belonging to the positive gram bacteria with white cream colonies were said to be said because they resembled creamy milk with irregularly shaped colonies, sometimes oval round sometimes shaped rod. According to [10] which stated that the Cms colonies on NCP-88 media or even NBY after 5 days were round and irregular in creamy white color and after incubation 10-12 days, the colony became pale yellow.

Bacterial isolates Cms grown on NA or NBY media, can grow at temperatures below 30°C. Bacterial culture of Cms can be grown on NBY media for 3-5 days at temperatures around 22°C. Cms bacteria, classified as bacteria that require media specifically to grow properly because these bacteria need more nutrition or nutrition. Bacteria Cms is an aerobic bacterium and requires more nutrients to grow on the media, therefore it requires special media NCP 88 or NBY media and Cms colonies to be seen within 5 days at 25°C [10, 11]. Pathogenicity testing uses eggplant plants because eggplant is still a family with potato plants, and in preparation for eggplant plant nursery for applications it does not take long. After the application of bacterial Cms, the egglplant plants will show symptoms of wilting, which starts at the edges of the leaves, and over time will cause necrosis, the younger vulnerable eggplant plants are far more sensitive than the old ones. The infected plants do not immediately die, the longer the period the symptoms arise the greater the chance of survival. Tests on eggplant plants were used to detect the
presence of Cms in the supernatant from infected potato tissue. Eggplant plants will show wilting green tissue of wilting leaves, and for a long time will become necrotic. Young eggplants are susceptible to being more sensitive to old eggplants, and there are latent infections so the plants do not die immediately [10].

The spread of Cms disease, apart from being the main factor which is the lowest seed, there are several other factors that can trigger rapid spread, among others; the use or selection of seeds used by farmers, the need for farmers to seed which is sometimes quite high causes farmers to buy seeds from outside their territory. Some farmers in several districts of South Sulawesi often buy seeds from West Java, while the spread of Cms is detected for the first time in West Java. Ring rot by Cms pathogens in West Java is positive for bacterial Cms based on the results of serological testing using the DAS-ELISA method [5]. There are also several other factors that trigger the spread of this disease, namely in terms of cultivating potatoes, among others, paying little attention to maintaining the main land when the harvest period, often the remnants of infected tubers, are left in the field for a long time. So that the soil can be contaminated, and when transporting the crop sometimes there are infected tubers followed, so this will cause the spread of bacteria Cms on the crop of potato tubers in the warehouse to occur [12]. The presence of ring rot by Cms pathogens can be detected using several methods. One of them is by using the serological method namely Elisa or PCR. However, the PCR method will be better used in detecting ring rot disease than using Elisa, because testing with PCR has a higher sensitivity level in detecting \textit{Clavibacter michiganensis} subsp. \textit{sepedonicus} in potato tubers or potato plant tissue. [13] who stated that the comparison of testing using the PCR and ELISA methods to detect Cms in potatoes, the sensitivity would be higher in the PCR testing method, even if the test using commercial ELISA procedures was used.

The method using PCR is very well used for serological testing in addition to its high sensitivity, the testing time is fast and more accurate. However, the costs used are more expensive. Whereas for ELISA testing is a test that is generally the most widely used, this is because the method is simpler and costs less. The disadvantage of ELISA testing is that the results used can be inaccurate due to the occurrence of cross reactions. According to opinion [13] which states that ELISA is the most commonly used method to identify serologically. But the weakness of this test is that there is a cross reaction that can occur due to cross-reaction of antibodies with remnants of plants or non-pathogenic bacteria. The PCR method was carried out by taking a sample of potato tubers which showed symptoms of Cms infection and DNA extraction was performed. In the process of extracting DNA using RNAse A is added to eliminate RNA contaminants. The suspension is added with buffer and ethanol before being centrifuged again. Buffer for cleaning and precipitating DNA. Ethanol functions to concentrate, separate DNA from solution and precipitate DNA. After being centrifuged the DNA will settle, while contaminants such as proteins will efficiently be eliminated through the washing process and dried. DNA deposits are dissolved by adding TE buffer. The extraction process is very important to be used to secure the quality of DNA in plant tissue if it is to be stored for a long time. Based on the results of electrophoresis, the results of DNA extraction showed that there were bands of eight samples, sometimes at several times the testing of the quality of DNA was smeared or dirty because DNA was not purified so that the DNA had not been separated from RNA and the rest of the debris.

5. Conclusion
The intensity of bacterial ring rot on potato plantations was highest in Gowa district, which was 37.66%, and followed by Enrekang district at 26%, Bantaeng at 24.33% and the lowest disease intensity seen in Jeneponto Regency at 19%. The highest Cms attack intensity in the storage warehouse was seen in Gowa district, which was 33.60%, and followed by Enrekang district at 20.80%, Bantaeng at 21% and the lowest disease intensity seen in Jeneponto district at 16.60%. The use of PCR with specific primers that can detect the presence of \textit{Clavibacter michiganensis} subsp. Epiconicus is indicated by the appearance of bands at 224 bp.
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