First report of *Enterobacter cloacae* in shallot (*Allium cepa* L. *Aggregatum* group) in Indonesia

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Abstract. Shallot (*Allium cepa* L. *Aggregatum* group) is one of the important vegetable crops in Indonesia. Shallots are very susceptible to diseases caused by pathogenic bacteria. Bacterial pathogens that infect onions generally can invade and infect species of other the genus *Allium* members. This study aims to identify the bacterial pathogens associated with shallot bulbs disease with phenotype and genotype approach (molecular). Sampling shallot bulbs disease conducted at two locations shallot production centers in Indonesia, namely the Regency of Bantul and Nganjuk in August-September 2012. Isolation initial bacterial pathogens of shallot bulbs disease using culture methods or pure culture. For phenotyping approach is done through observation of the colony and cell morphology, biochemistry, and physiology test bacteria. Whereas the genotyping approach, the identification is made based on the analysis of 16S rRNA sequences of technique Polymerase Chain Reaction (PCR). The results show that the identification of phenotypically UBNG21 isolates has characteristics in similarity with the characteristics of *Enterobacter cloacae*, causing bulb decay. Amplification of the 16S rRNA gene using PCR UBNG21 isolates produce 1500 bp fragment size. The results obtained by sequencing isolates UBNG21, which has a value of 97% homology with *E. cloacae*. The results of this study are the first report of the presence of *E. cloacae* associated with disease shallot bulbs in Indonesia.

1. Introduction
Shallot (*Allium cepa* L. *aggregatum* group) is a main commodity in the priority development of lowland vegetables in Indonesia. This commodity has a high economic value and large market opportunity as a food flavoring ingredients (seasoning) for household consumption, industrial materials processing food and medicine, as well as meet the needs of export [1]. In shallot cultivation often face a number of obstacles, especially the presence of plant diseases caused by pathogenic bacteria in the field, transport, and storage. According to Bodnar (1998), the shallot is very susceptible to bacterial pathogens. Bacteria *Pectobacterium carotovorum* subsp *carotovorum* recorded as a pathogen causing soft rot disease in shallot bulbs [2].
In Indonesia, shallot diseases caused by pathogenic bacteria classified as new diseases and scientific information about attacks against bacterial pathogens of shallot bulbs are still very limited, but some bacteria such as *Pseudomonas viridiflava*, *Dickeya chrysanthemi*, *P. carotovorum* subsp. *carotovorum*, *Pantoea ananatis*, *Enterobacter cloacae*, *Burkholderia gladioli* pv. *aliiicola*, and *Burkholderia cepacia* reportedly has been associated with diseased onion bulbs [3]. The attack bacterial pathogens estimated can cause yield loss of up to 40%, especially in favorable climatic conditions [4]. According to [5], a bacterial pathogen that infects onion bulbs can invade other *Allium* species. Shallots most infected by the same disease with a disease that infects onion [6]. Accordingly, this study then specifically is conducted to isolate and identify bacterial pathogens that cause disease in shallot bulb in Indonesia through phenotypic and genotypic approaches.

2. Methods

2.1. Isolation of pathogenic bacteria

Shallot bulbs were showing symptoms of decay bulb obtained from locations the shallots crop in Nganjuk and Bantul Regency, Indonesia. The diseased bulb is cut into small pieces (using a scalpel), then cleaned and washed thoroughly using running water [7]. Subsequently, the pieces included in 0.1 ml of sterile PBS solution (0.05 M KH2PO4; 0.85% NaCl at pH 7.4) and the suspension in gojog, then streaking using a needle loop on the surface of the medium peptone yeast glucose (YPG) agar in a petridish [8]. YPGA medium composition consisted of 7 g of yeast extract, 7 g of peptone, 7 g of glucose and 15 g of agar / l; 1000 ml of sterile distilled water; pH 7.2, was then added cycloheximide 20 ug/ml (antibiotics) to prevent mold growth. Petridishes were incubated for 48 hours at a temperature of 28°C (Roumagnac et al., 2004). Colony grows taken with a sterile toothpick and then transferred to a new medium YPGA.

2.2. Hypersensitivity test

Pure cultures of bacteria were grown and propagated for 24 hours on the medium YPGA, then was made the suspension with a concentration 10^6 cfu/ml. Suspension of 0.5 ml/leaf is injected into the intercellular space (empty space), the young leaves of the host plant age one month [9], or tobacco (*Nicotiana tabacum*) using a hypodermic syringe. Plants that have been injected bacterial suspension was incubated at a temperature of 27 - 32°C. The onset of symptoms in the form of small or necrotic spots on the leaf area at the injection occurs within 24-48 hours indicates the occurrence of hypersensitivity reactions. The results were positive in the hypersensitive reaction test showed that the bacteria are bacteria and plants reasonably suspected pathogens, including bacteria [10].

2.3. Pathogenicity test

Culture isolates of bacteria, grown and propagated in sub medium YPGA. Bacterial cultures were incubated at room temperature for 72 hours. Then suspended in sterile water as inoculum of bacteria with a density of 108 cfu/ml. Inoculation of bulb performed using healthy onion bulbs and surface sterilized with 70% alcohol [11]. Bulb inoculated artificially was made an open wound with a diameter of about 0.5 cm using a sterile toothpick. Subsequently, the suspension was inoculated with a syringe at the wound site, and the bulbs are placed on top of petridish that has been coated with sterile cotton wet. As a control, the other onion bulbs inoculated with 0.5 ml of distilled water. Bulb tissue showed a necrotic fowl beyond the point of inoculation is evidence of pathogenic bacterial infection has occurred [12]. For visualization of the pathogenicity of pathogens, bulbs incubated at room temperature for two weeks. Observation of the results of inoculation is done every day since the plants were inoculated until symptoms (incubation period). Symptoms were observed for the first time the formation of bulb rot, is the beginning of infection. In the pathogenicity tests, isolates pathogenic bacteria tested were from the same location with the location of origin of shallots cultivars were inoculated, namely Nganjuk and Bantul. Furthermore, pathogen isolates used in the test cell and colony morphology,
biochemistry and physiology, and molecular showed is isolates one of the fastest incubation periods on pathogenicity testing.

2.4. Morphology observation of bacteria

a. Colony morphology. To determine the bacterial colony morphology, done by strike bacterial culture in medium YPGA. Bacterial colonies that grow on the medium in bacterial culture was observed after 48 hours.

b. Cell morphology. The shape and size of bacterial cells observed using Transmission Electron Microscopy (TEM) [13].

c. Characteristics physiology and biochemistry. Pathogenic isolates were grown and propagated on the surface of the medium with the past YPGA incubated for 28 hours at room temperature. Furthermore, pathogen isolates tested biochemical and physiological characteristics that include gram test, oxidation fermentation glucose metabolism, presence of oxidase, urease activity, levan production, the presence of fluorescent pigments, arginine hydrolysis, indole production, gelatin hydrolysis, starch hydrolysis, catalase production, nitrate reduction, tween 80, acid production from carbohydrates, tolerance to temperature, pH and NaCl [10].

d. Extraction of DNA. Bacterial isolates derived from a single colony grown on a slant YPGA medium in a test tube and incubated in an incubator at 30°C for 48 hours. Bacterial isolates were grown and moved back into 5 ml of liquid YPGA medium, shaken on a shaker with a speed of 125 rpm for 16-24 hours at room temperature. The extraction of bacterial DNA is done by using the Wizard Genomic DNA Purification Kit from Promega (Madison, USA) following the protocol suggested by the manufacturer.

e. Amplification of the 16S rRNA gene. Extraction of genomic DNA used for amplification of the 16S-rRNA gene using PCR system 2400 machine GenAmp (PERKIN ELMER). DNA primers used are kit template was amplified with Ready To Go PCR Beads (Pharmacia-Biotech) using universal prokaryotic specific primers (Marchesi et al., 1998), namely 8-27f (5'-AGAGTTTGATCCTGCTCAG-3') and 1492 -1509r (5'-GTTACCTTGGTACGAGTTTTCA-3') (Delgado et al., 2006). PCR reaction mixture (50 µl) containing 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM MgCl₂, 2 mg of bovine serum albumin per ml, 16 mM (NH₄)₂SO₄, 100 µM (each) primer and 0.1 U Taq polymerase super (HT biotechnology, Cambridge, England). PCR was performed with the following conditions: PCR reaction mixture was incubated with 0.5 µg DNase I, which is active on dsDNA (dsDNA, Boehringer, Mannheim, Germany), for 15 min at room temperature, and then subjected by inactivating DNase for 10 minutes at a temperature of 95°C. Then by 5 µl cell lysate containing the target DNA was added to the PCR mixture and PCR on the run for 30 cycles per minute at 94°C and for 10 seconds at a temperature of 72°C and 1 min at 55°C in a thermocycler. DNA amplification products were analyzed using agarose gel electrophoresis. After amplification, a total of 5 µl of accelerated amplification products on a 1% agarose gel in 0.5 × TBE. DNA bands detected by staining with ethidium bromide and visualized with UV light photography [14].

f. Sequencing of the 16S rRNA gene. Results of DNA amplification by PCR, sequencing later in the sequencing service company PT Indonesia Genetics Science (1st BASE Pte Ltd Singapore) to determine the base composition of genes encoding 16S rRNA bacterial isolates. The results of the data in the form of tracing DNA sequence (amino acids) examined the quality use DNA Baser program, then analyzed the data sequence identity to the most similar (highly similar sequences) or kinship genetic similarity with the target bacteria in Genbank using NCBI BLAST-N program (Basic Local Alignment Search Tool) on-site http://www.ncbi.nlm.nih.gov/BLAST/. The next highest level of similarity to the sequences of data recorded.

g. Analysis of phylogenetic. Phylogenetic tree analysis performed using the MEGA 5.05 program, Neighbor-Joining method (NJ) with a bootstrap of 1,000 x. or protein and DNA
alignment programs in the Genes Work package (Intelli Genetic Inc.). A phylogenetic tree is useful to show the kinship of each species seen by molecular characteristics between species or between strains of the same species [15].

3. Results and discussion

3.1. Hypersensitivity test
Hypersensitive test results that the five isolates showed a positive reaction to the pathogen of tobacco leaf symptoms shown by the formation of water soaking, chlorosis (yellow), and necrosis (brown) on the surface of tobacco leaf tissue after 48 hours (figure 1).

3.2. Pathogenicity test
Pathogenicity test results showed that the five isolates of pathogens of shallot decay bulb disease symptoms in the form of infected bulb tissue appear damaged on the inside (scales) but remained intact without change as pulp, soft, gently, not runny, not slimy, brown, or blackish and slightly foul odor issue. There are clear boundaries between the disease and healthy when cut (arrows) (figure 1 CD). The symptoms appear between 8-14 days after inoculation. While the control (sterile water inoculated bulbs), does not cause any symptoms at all in the bulb (figure 1).

![Figure 1](image)

**Figure 1.** Hypersensitivity reaction of test bacteria on the leaves of tobacco (A); control: sterile water was inoculated on healthy bulb do not cause symptoms of the disease (B); pathogenicity test: infected bulb tissue is damaged but still intact, not changes such as slurry, soft, gently, not runny, not slimy, odorless, brown or black (CD)

The average incubation period is necessary to express the symptoms of pathogen isolates rot of onion bulbs ranged from 8.00 to 11.33 days after inoculation (table 1). Isolates UBNG21 has an average incubation period is faster than the isolates UBBT17, UBBT19, UBBT27, and UBBT29 Bantul origin. To ensure the tested isolates are pathogenic bacteria in shallot bulbs, then selected one of the isolates tested for biochemical and molecular 16S rRNA genes in order to determine the species based on the fastest incubation period was chosen to obtain pathogen isolates, namely UBNG21.

| Number | Location research (village) | Bacterial isolates | Incubation period |
|--------|-----------------------------|--------------------|-------------------|
| 1      | Tirtomulyo (Kretek Subdistrict, Bantul Regency) | 1. UBBT17 | 11.33 |
|        |                             | 2. UBBT19 | 10.00 |
|        |                             | 3. UBBT27 | 10.33 |
|        |                             | 4. UBBT29 | 10.66 |
| Average|                             |          | 10.06 |

**Table 1.** The average incubation period of shallot bulbs
3.3. The morphology of bacterial colonies
The result of observation on pathogenic bacteria colony morphology the origin of shallot bulbs that are grown on the surface of the medium YPGA showed that colonies are round, the surface (elevation) convex, flat edge, hard and not slimy (nonmucoid), lustrous (shiny), fine texture, white color, not fluorescent, opaque and does not fluorescent (figure 2).

![Figure 2](image1.png)

**Figure 2.** *Enterobacter cloacae* colonies on the medium, according to Takahashi *et al.* PSA., (1997) (A); colony UBNG21 isolated on YPGA medium (B).

3.4. The morphology of bacterial cells
The result of observation on cell morphology of pathogenic bacteria using Transmission Electron Microscopy (TEM) shows that the rod-shaped cells elongate, straight, with the size of the cell size between 0.6 to 1.1 x 1.1 to 2.6 µm. End of round cells have flagella peritrichous (arrows) located around the bacterial cells and serves as a means of motion by pushing the bacteria in the fluid, such as water. Bacteria, including gram-negative, facultatively anaerobic, not encapsulated, and do not form spores (figure 3).

![Figure 3](image2.png)

**Figure 3.** *Enterobacter cloacae* cells by Takahashi *et al.*, (1997) (A); UBNG21 cells isolation results (B).

3.5. Characteristics physiology and biochemistry
Isolates of pathogens showed a gram-negative reaction. Furthermore, these isolates have a negative reaction to the test oxidase, urease, levan, formation fluorescent pigment, dehidrolisis arginine, indole
production, and hydrolysis of starch. Pathogen isolates reacted positively to the catalase test, nitrate reduction, hydrolysis of gelatin and tween 80, and able to grow under aerobic and anaerobic conditions. Carbohydrate components that can be used as a carbon source are arabinose, cellobiose, fructose, glucose, sucrose, lactose, trehalose, sorbitol, glycerol, and dulcitol. The best growth of all isolates on media containing NaCl between 0 - 6%. Although the isolates were able to grow at temperatures between 4 - 41°C but optimum growth temperature is between 25 – 37°C. The degree of acidity (pH) in the range of 4-10 with an optimum pH of 5.0 to 8.5.

3.6. Molecular analysis of the 16S rRNA gene

3.6.1 Amplification of 16S rRNA genes by polymerase chain reaction (PCR). DNA amplification to isolate UBNG21 using universal primers for 16S rRNA with the sequence of bases 8-27F (5′AGAGTTTGACTCAG-3′) and 1492-1509R (5′GGTTACCTTGTTACGACTT-3′) (Delgado et al., 2006) resulted in fragments of the gene 16S rRNA single (single band) with a size of 1500 bp (figure 4).

![Figure 4. Results of amplification DNA-PCR isolates UBNG21](image)

The presence of DNA fragments is indicated that the isolates pathogenic bacterial targets (Walcott, 2003) because of the amount of the fragment size was in the range of the size of prokaryotic genes encoding 16S rRNA, i.e., between 1300 to 1600 bp. This suggests that the amplification of DNA fragments according to the size of the expected DNA band bacteria, which measures 1500 bp DNA sequences.

3.6.2. DNA sequence analysis of bacterial isolates. The results of the analysis of the sequence alignment using the NCBI BLAST program show that UBNG21 isolates have the highest similarity with the bacterium E. cloacae is the percentage of similarity reaches 97% (table 2).

| Isolates              | Kinship nearby bacteria              | Homology (%) | Access Code   |
|-----------------------|--------------------------------------|--------------|---------------|
| UBNG21                | Enterobacter cloacae strain M         | 97%          | EF633997      |
|                       | Enterobacter cloacae strain LSRC11    | 96%          | JF772071      |
This value the percentage of indicates that isolates UBNG21 is the same species or closely related to *E. cloacae* based on the criteria used for comparison of 16S rRNA sequences is when two bacteria have a 16S rRNA sequence similarity values of more than 97% then it will be grouped in a single species, or represent the same species. Whereas, if the two bacteria have a sequence similarity value between 93-97% is considered to represent the identity at the level of the same genus but different at the species level.

Early identification of pathogens to the existence of new pathogenic bacteria can help prevent attacks of pathogens in the shallot production process, and anticipating the next infection in plants around it. This is because the disease-causing bacteria may be able to invade and spread rapidly to endanger other shallots crop. The identification of pathogenic bacteria can be carried out with a non-molecular approach (phenotype) and molecular (genotype). Phenotypic testing is usually done by observing the symptoms and signs of disease, followed by microbiological testing, isolating, and identifying pathogens using culture methods [16]. Other methods are still frequently used are a hypersensitive test, pathogenicity test, and determination of physiological and biochemical tests. According to [17], physiological and biochemical testing is considered to have enough to be used in identifying the bacteria because the test can already know the physiological and biochemical characteristics of pathogenic bacteria; or are able to distinguish the important characteristics of a pathogenic bacterial species with other species. This test is expected to provide an overview of the colony and cell morphology, physiology, and biochemistry, as well as the characteristics of pathogenic bacteria [18]. Furthermore, the genotyping test (molecular) can be done through the analysis of 16S rRNA gene sequences for the target bacteria by engineering Polymerase Chain Reactions (PCR) and sequencing [19]. This molecular approach has been widely used to reinforce or support the result of pathogenic identification bacteria phenotypically involving 16S rRNA gene sequences [20].

Hypersensitivity tests on tobacco leaves showed that all isolates tested caused a positive reaction. A positive reaction is shown by the formation of necrotic symptoms, or chlorosis (Lelliot & Stead, 1987) indicates that these isolates are pathogenic bacteria. According to Kerr & Gibb, if the bacteria were tested in the form of bacterial pathogens, there will be symptoms of necrosis or chlorosis on tobacco leaves as a result of the incompatible relationship between pathogen and host. Hypersensitivity reactions are rapid plant defense responses in the face of attack by pathogens, such as the death of the cells rapidly on the network in the area of the injection of bacterial suspension [21]. Cell death in these tissues to limit the spread of pathogens in a place invasion by inhibiting multiplication [22].

Symptoms of the disease appear in the pathogenicity test showed that the five isolates estimated as shallot bulb decay disease pathogen. Symptoms of the disease include discoloration of the inner bulbs to brown, or blackish, there is damage to the infected tissue but remained intact but does not turn into pulp, soft, gently, not runny, not slimy, and secrete less foul odor. There is a clear difference between aching parts and healthy when the bulbs split. These symptoms have similarities with symptoms of disease onion bulb decay reported by [23–25]. Attacks of pathogenic bacteria causing decreased production, in the size and quality of the bulbs in the field, then continues with the rot of bulbs in storage. According to [24], it is very difficult to detect the presence of *E. cloacae* bacteria cause bulbs decay in the field except by dissecting the bulb. This is because *E. cloacae* rarely cause symptoms on
the leaves, but more often attack and infect bulbs directly causing bulb decay disease (Zaid et al., 2012). Affected leaves usually show premature die back. On the field, the bacteria that infect onion will persist as a latent infection at the time of harvest and move from the field to storage [25], but the symptoms of the disease usually occur only on a bulb in storage [6].

Pathogenicity test results showed that isolates UBNG21 tend to be more virulent than pathogenic isolates of Bantul origin based in the fastest incubation period of disease symptoms, i.e., eight days after inoculation. According to [26], a more virulent strain of the pathogen has an incubation period of most rapid. Isolates UBNG21 is selected pathogens and the next used in biochemistry and molecular testing. The result of observation colony morphology showed that isolate UBNG21 has similarities with bacterial colonies of *E. cloaca*, which are round, convex, smooth texture, does not fluorescent [27], no slimy, shiny, flat edge [11,28] and white [29]. Similarly, cell morphology UBNG21 isolate resemblance to bacterial cells of *E. cloaca*, which is a straight rod-shaped cell with rounded edges, measuring between 0.3 - 1.0 x 1.0 - 3.0 µm, flagellum peritrichous and do not form spores [27,29,30].

Identification through observation of cell morphology and bacterial colonies certainly not enough to give satisfactory results, thus required further identification. Based on physiological and biochemical characteristics, it can be strongly suspected that the UBNG21 isolates of *E. cloaca* are a pathogenic bacteria that invade and infect shallot bulbs because it has characteristics in similarity with the bacteria *E. cloaca* described by [24,31,32].

![Diagram](image-url)

**Figure 5.** Phylogenetic tree UBNG21 isolates were identified as *E. cloaca*

The results of reconstruction phylogenetic trees showed that UBNG21 isolates have a closer kinship with *E. cloaca* when compared with *Pseudomonas viridiflava*, *Dickeya chrysanthemi*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pantoea ananatis*, *Burkholderia gladioli* pv. *aliiicola* and *Burkholderia cepacia* bacteria (figure 5). The whole bacterial pathogens are known to be associated with onion bulbs disease [3]. Thus, it can be said that the UBNG21 isolates form *E. cloaca* a species based on 16S rRNA gene analysis. Based on the colony and cell morphology, physiology and biochemical characteristics, as well as the molecular characterization, can be concluded that bacterial pathogens (UBNG21 isolates) were found associated with disease shallot bulbs is *E. cloaca*. In Indonesia, shallot plant diseases caused by pathogenic bacteria is a new disease. The presence of *E. cloaca* bacteria becomes important and very dangerous when it has been in parts of Indonesia, especially in the areas of production centers because of the possibility it may cause serious damage to the crop both in the field, on transportation and storage. For the identification of the...
activities carried out in order to obtain information about the presence of pathogenic bacteria in order to support control efforts that will be conducted.

*E. cloacae* were described as the bacteria that cause an onion decay bulb in storage. In general, bulb decay is a disease of a minor, but when environmental conditions favor, it can cause significant yield loss. For the seed industry, the loss could reach more than 100% because if there is only one infected seed can cause all the seeds denied, and there is a risk of additional costs for extermination infected seed  [28,29]. *E. cloacae* bacteria reported spread in several countries with attacks on different hosts, such as the U.S., Japan, Australia, Malaysia [28], and China [27]. For example, on internal bulb decay on onions (*Allium cepa* L.) in the USA, papaya fruits (*Carica papaya* L.) in Hawaii, and leaf rot disease on odontioda orchids (*Odontioda* sp.) in Japan [28]. Besides attacking onion bulb, *E. cloacae* species are also found in some plants such as elm trees, coconut, orchid, corn, apple, papaya, mung beans sprouts, [33], cassava, ginger, macadamia, dragon fruit, and mulberry (Santana et al., 2012). *E. cloacae* is a soil-borne pathogenic bacteria [34], but the spread of pathogens can be through insect vectors such as fruit flies [11] and farm equipment [28]. In nature, *E. cloacae* bacteria are often found in water, plants, insects, waste, consumables harvested vegetables [34], and seed plants [5]. On the field, the spread of bacteria through the rain splash on contaminated soil pathogens [35].

4. Conclusion

The results of biochemical and molecular tests based on the analysis of 16S rRNA sequences of technique *Polymerase Chain Reactions* (PCR) showed that the UBNG21 isolates were an *E. cloacae* bacteria pathogen. The results of this study are the first report of the presence of *E. cloacae* associated with disease shallot bulbs in Indonesia.

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