Detection of *Pseudomonas aeruginosa oprL* Gene Based on Polymerase Chain Reaction

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Abstract. Infectious diseases are among the main cause of mortality in the world. The most common of pyogenic germs causing infection, yet scattered in the human bodies are *Pseudomonas aeruginosa*. *P.aeruginosa* is a pathogenic, Gram-negative bacterium, which often causing difficult-to-trat infection because it is resistant to most antibiotics. Early detection by Polymerase Chain Reaction (PCR) is among the most important step to eradicate *P.aeruginosa*. Among important target gene in *P.aeruginosa* detection by PCR is *oprl gene.* This study aimed to conduct *oprl* detection of *P.aeruginosa* BP-1 (boy's pus-1) originated from pus wound by PCR-based method. The BP-1 isolate has not been molecularly identified before. After subculture, the isolated genome of *P.aeruginosa* BP-1 was used as template in PCR using a pair of *oprl* primer: F= 5'-CTTCTTCAGCTCGACGCGACG-3' and R= 5'-ATGGAAATGCTGAAATTCGGC-3'.The obtained PCR product was then Sanger-sequenced, and the obtained sequence was checked using BLAST (Basic Local Alignment Search Tool) program. As result, the oprL gene was detected as amplicon on electrophoresis gel as a single band sized ~500 bp. After sequencing, the gene fragment has a size of 504 bp with the highest degree of similarity of 99.21% of with the *oprL* gene sequence of the bacterium *Pseudomonas aeruginosa* strain NCTC12903.

Keywords: *Pseudomonas aeruginosa*, *oprL* gene, Polymerase Chain Reaction

1. Introduction

Infectious diseases are among the main cause of death in the world. About 53 million deaths occurred worldwide in 2002, some due to infectious diseases. In Indonesia, infectious diseases are one of the important health problems (RI, 2011). Every year it is reported that nine million people die due to infectious diseases, including children under the age of 5 who experience infections that cause lifelong disability (WHO, 2018). One of the key processes in development of infectious diseases is pus occurring in the wound. Pus formation is a form of acute reaction to tissue damage caused by bacterial infection (Muthukrishnan et al., 2019)

There are many species of pyogenic germs spreading in the human body. The most common of them are *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Singh et al., 2013). *P.aeruginosa* is a

Gram-negative bacterium that is pathogenic for humans. It can cause difficult-to-treat infections because the bacterium is resistant to most antibiotics (Gellatly & Hancock et al., 2013). *P.aeruginosa* is an opportunistic pathogenic bacterium in humans, which can enter areas of the defense system that are not normal, which can cause mucous membranes and skin to open due to injuries (Mayasari, 2005).

Pyogenic infections are common in developing countries and their treatment is a great challenge, despite advances in microbiological examination techniques. To ensure that therapy is efficient, it is necessary to identify and treat inflammation that is focused on. (Chong and Soon, 2009). Identification of the *oprL* gene using *P.aeruginosa* isolate in wound pus stored in Microbiology Laboratory, Universitas Muhammadiyah Semarang (UNIMUS), Indonesia. Identification of the *oprL* gene in the isolates has not been reported. In this study, we wanted to find out whether *P.aeruginosa* stored in the Microbiology Laboratory could produce protein encoded by the *oprL* gene which will later help with treatment.

Research Identification of skin infection wound pus found *P.aeruginosa* with a percentage of 90.7% and S aureus with a similar percentage of 91.5% in pus (pus) samples (Ekawati et al., 2018). Research on clinical isolates of *P.aeruginosa* using the *oprL* gene was marked by the readability of the band at 504 bp in the sample, which is the band size of the bacterium Pseudomonas aeruginosa (Abdulhaq, 2020). Clinical isolates of the lasl and lasR genes at Denpasar General Hospital in 2013-2016 used 140 isolates which were detected using PCR. In this study, the results showed that the prevalence of clinical isolates of *P.aeruginosa* with the lasl and lasR genes was 93.57% and it could be concluded that the prevalence of clinical isolates with these genes was very high (Sanjaya et al., 2019).

Polymerase chain reaction (PCR) is a method used to multiply the DNA of an organism. This PCR method is often used in the identification of organisms, both through DNA fingerprinting and DNA barcording (Shaheen et al., 2020). The PCR method is generally used for detection of hereditary diseases, contamination detection, and paternity testing (Sasmito., 2014). PCR techniques are used to detect pathogens quickly, for example, by detecting *P.aeruginosa* contamination in wound pus samples. Research conducted by Milanda (2014), explains that in Indonesia the detection of *P.aeruginosa* contamination by the PCR method is still very limited in hospital equipment and to test for bacterial resistance.

In detecting *P.aeruginosa* bacteria, this study used the *oprL* gene primer. The *oprL* gene is one of the main protective antigens for *P.aeruginosa* (Gong et al., 2022). The *oprL* gene has a high sensitivity compared to other genes such as the exoA (exotoxin A), *oprL* (small lipoprotein) and *algD* (alginate) genes. The gene can be used as a tool to identify *P. aeruginosa* (Huynh et al., 2022). The *oprL* gene also has a more appropriate specificity than culture to show bacterial colonization. One of the studies used the PCR gene *oprL*, namely research (Hamasalih et al., 2021) to detect *P.aeruginosa* contamination in contact lense samples.

The *oprl* gene is commonly used to detect bacterial contamination up to the strain species P.aeruginosa (Gholami et al., 2016). In this study, the identification of *oprL* gene from *P.aeruginosa* isolate from wound pus stored at the UNIMUS Microbiology Laboratory was conducted. Identification of the *oprL* gene in these isolates has not been reported. This also aimed to determine the virulence potential of the bacteria so that it can support the diagnosis of *P.aeruginosa* infection in wound.

2. Materials and Method

2.1. Material

Sample used in this study was the *P.aeruginosa* BP-1 (boy's pus-1) bacterium originating from a boy's wound pus which had not been identified molecularly. Primers *oprL*-F-5-ATGGAAATGCTGAAATTCGGC-3' and *oprL*-R-5-CTTCTTCAGCTCGACGCGACG-3' were used to amplify the *oprL* gene (Ahmed et al, 2022)

2.2. Method

2.2.1 Subculture of bacterial isolates

The bacterial isolates used in this study were obtained by subculture of isolates that had been stored at the Microbiology Laboratory, UNIMUS. The isolate originated from the wound of a small buy which had secreted pus. The pus sample was then taken and put into the brain heart infusion broth (BHIB) media.

2.2.2 Preparation of Brain Heart Infusion Broth (BHIB) media

As much as 4.44 g of BHIB powder was put into a 250 mL Erlenmeyer and redissolved with 120 mL of distilled water, made as needed. The pH of solution was measured by pH indicator of 7.4 ± 0.2. stirring the mixture was done by heating until well dissolved, after that, sterilize by autoclaving at 121°C for 15 mins (Darmayani et al., 2017).

2.2.3 Preparation of Mac Conkey Agar (MCA) Media

A total of 5.20 g of Mac Conkey powder was weighed and put into a 100 mL Erlenmeyer. As much as 100 mL of distilled water was pipetted into the Erlenmeyer. Then the solution was heated while stirring until it is homogeneous. After that, it was sterilized using an autoclave for 15 mins at 121°C with a pressure of 2 atm (Ngajow et al., 2013)

2.2.4 Bacterial innoculation

Bacterial isolation was carried out on the sample in the culture tube, pipette 10 mL of Brain Heart Infusion Broth (BHIB) media and put it in a test tube. 2 cc of blood was taken in a culture tube and put into 10 mL of BHIB media then homogenized and incubated for 24 h at 37°C (Rafika et al., 2020).

2.2.5 Purification of Bacterial Cultures

Pure culture of *P. aeruginosa* was purified by single cell culture technique by back-scratching on NA medium. Commencement of purification by selecting colonies around which there is a clear zone. The loop needle was sterilized, then touched to the purification of the bacterial colony after which it was inoculated on the surface of NA and Mac Conkey medium using the Goren method to obtain separate colonies. Then incubated at 37°C for 2 x 24 h. This culture purification stage can be carried out 2 to 3 times, to make sure that the colonies formed are truly pure (Yuliana et al., 2015).

2.2.6 Differential Staining of Bacteria

Bacterial staining begins with 1-2 drops of sterile distilled water placed on a glass object, an ose of colonies taken from the media and placed on top of sterile distilled water and leveled, leave to

dry. After the spread is completely dry, drop crystal violet on the preparate glass that has been treated with bacteria until all the bacteria in the preparation are stagnant and left for 1 min. After that, the preparation is rinsed using distilled water, then drops of iodine solution and left for 2 mins, then washed with distilled water and dried. After drying, drip with 95% ethanol solution for 30 s, then rinse with distilled water and dry. Then drip the covering agent or safranin solution and leave it for 30 s, then wash or rinse with distilled water and dry. Then observed using a microscope. The indication of the staining is that Gram-positive will have a violet color (Waluyo, 2010).

2.2.7 Preparation of Biochemical Test Media

2.2.7.1 TSIA media (Triple sugar iron agar)

Preparation of TSIA medium with as much as 6.5 g of TSIA medium dissolved in 100 mL of distilled water, pH 7.4 was made, then heated while stirring until homogeneous. After that, the solution was divided into 4 Erlenmeyer pieces of 25 mL each. Then the mouth of each Erlenmeyer was closed using aluminum foil and then sterilized in an autoclave at 121 °C and 1 atm pressure for 15 mins (Lamid et al., 2011).

2.2.7.2 Media SIM (Sulfid Indole Motitily)

Weigh as much as 3 g of SIM medium dissolved in 100 mL of distilled water to make a pH of 7.3. Then heated while stirring until homogeneous. Then divided into 4 Erlenmeyer pieces of 25 mL each then the Erlenmeyer mouth was closed using aluminum foil and then sterilized in an autoclave with a temperature of 121 °C for 15 mins (Sulviana et al., 2018)

2.2.7.3 Media MR-VP (Methyl Red-Voges Proskauker)

MR-VP media was prepared by weighing 1.7 g of MR-VP medium dissolved in 100 mL of distilled water, then made at 6.9 pH. Heat the chili stir until homogeneous. Then the solution was divided into 4 Erlenmeyer pieces, each filled with 25 mL. Then the Erlenmeyer's mouth was closed using aluminum foil and then sterilized in the autoclave at 121 °C for 15 mins (Lamid et al., 2011).

2.2.7.4 Medium Urea

Urea media was prepared by weighing 2.1 g of urea base medium dissolved in 100 mL distilled water, which was then sterilized at 40 °C, for 15 mins then added 2 g of urea PA and then sterilized at 121°C (Lamid et al., 2011).

2.2.7.5 Citrate Media

As much as 22.5 g of citrate medium was dissolved in 100 mL of distilled water. Then heated and stirred until homogeneous. Then the solution was divided into 4 Erlenmeyer pieces of 25 mL each. then the Erlenmeyer mouth was closed using aluminum foil and then sterilized by autoclaving at 121 °C and 2 atm pressure for 15 mins (Lamid et al., 2011).

2.2.8 Biochemical Test

2.2.8.1 TSIA (Triple Sugar Iron Agar) Test

The TSIA test was made with 1 bacterial isolate taken from culture stock using straight loops and then inoculated on TSIA medium, after which the center of the TSIA was pierced. Incubation for 1x24 h at 37 °C (Anastiawan, 2014).

2.2.8.2 MR (Methyl Red) Test

total of 1 Round Ose of bacterial isolates from culture stock was taken and inoculated on liquid MR-VP medium in a test tube. Then incubated for 5x24 h at 37 °C. Then 5 methyl-red was added on top of the bacterial isolates. A positive result will be formed if a pink to red complex is formed indicating that the microbe produces acid (Anastiawan, 2014).

VP Test (Vogen Proskauer)

Bacterial isolate BP-1 taken from culture stock was inoculated on liquid MR-VP medium in a test tube. After that it was incubated for 3x24 h at 37 °C. Then 0.2 mL of 40% KOH medium and 0.6 mL of alpha-naphtol were added and shaken for 30 s. A positive result is seen if the medium turns purple (Anastiawan, 2014).

2.2.8.3 Motility Test

A total of 1 straight ose of culture stock isolates was then inoculated by means of vertical stabs on SIM medium, then incubated at 37 °C for 2x 24 h. Motile positive results can be seen if there are propagations around the ose needle puncture site in the medium (Anastiawan, 2014).

Citrate Test

A total of 1 ose of culture stock was then inoculated with soursop and then incubated for 24 h at 37 °C (Anastiawan, 2014).

Urea test

A total of 1 straight loop of isolates was taken from stock culture and then inoculated with soursop then incubated for 24 h at 37 °C (Anastiawan, 2014).

Catalase Test

A loopfull of bacteria was taken from each culture stock and then it was dipped into the H_2O_2 reagent filled in the test tube. A positive result is seen if gas bubbles form in the loop and a negative result if no gas bubbles form (Isnaeni & Rahmawati, 2016).

2.2.9 Isolation of Bacterial DNA

Molecularly identified Mac Conkey Agar from *P.aeruginosa* DNA extraction protocol was used for the isolation of genomic DNA contained in the Geneid PrestoTM Mini gDNA Bacterial Kit. The supernatant contained in the tube was DNA extract (Susilaningsih et al., 2019)

2.2.10 Quantification and Purity Test of DNA Extract

The extracted DNA stock was pipetted 1 µl and then analyzed quantitatively using a NanoDrop spectrophotometer at λ 260 and λ 280. The purity of DNA was determined by calculating

the ratio of absorbance at A260 to A280 (Ratio A260:A280). A DNA molecule is said to be pure if its absorbance ratio is in the range of 1.8 – 2.0. A purity level that exceeds 2.0 indicates that the DNA sample is impure, which can result from the presence of ethanol residue or the presence of residual battery secondary metabolite content extracted (Harun et al., 2018).

2.2.11 Purification of isolated DNA results

Purification of the results of DNA isolation is carried out if the DNA ratio results are less than 1.8. Purification was carried out by adding extracted DNA isolates into a spin column that had been given 25 mg of silica gel and centrifuged at 12000 rpm for 1 min. This aims to separate contaminants in the form of protein from DNA extracts. The purification results obtained can be used for the next process, namely DNA amplification using the PCR method (Nugroho et al., 2015).

2.2.12 PCR amplification of the *oprL* gene

Sample amplification was performed using DNA extracted from each sample. The total reaction volume used was 25 µL, which contained 12.5 µL of Taq mix (GoTaq, Green Master Mix), 5 µL DNA template which had been extracted from the sample, nuclease free water and forward and reverse primer solutions with various concentrations. namely 0.2 µM (Billard-Pomares et al., 2011), 0.4 µM (Zeitoun et al., 2015), 0.8 µM (Williams et al., 2010). Negative control was carried out by replacing 5 µL of DNA extracted from the bacterial culture of *P. aeruginosa*. All of these PCR components (see Table 3) were carried out by DNA amplification using a Thermalcycler. The PCR profiles used were as follows: initial denaturation temperature 95 °C for 5 mins, followed by denaturation 95 °C for 30 s, annealing 55, 59, and 64b°C for 30 s and primary extension 72 °C for 1 min with a final extension of 72 °C for 10 mins with each totaling 30 cycles (Zeitoun et al., 2015) and 40 cycles (Williams et al., 2010).

Table 3. Composition of polymerase chain reaction (PCR) reagents for *Pseudomonas aeruginosa* BP-1 *oprL* amplification in this study

PCR component	Stock concentration	Final concentration	Volume (µL)
DNA template	100 ng	8 ng	2
Primer Forward	10 µM	0,8 µM	2
Primer Reverse	10 µM	0,8 µM	2
Mastermix	-	-	12,5
ddh_2O	-	-	6,5

2.2.13 Electrophoresis

Electrophoresis via agrose gel is a simple, fast and precise separation technique for separating DNA molecules (Ethica et al., 2013). To carry out the electrophoresis process, an agarose gel is prepared first. The steps for making agarose gel with a concentration of 2%, namely weighing 2 grams of agarose powder, then putting it into an Erlenmayer flask. The agarose powder that has been weighed is added 1x TAE solution and added 10,000x flourovue then put in the microwave for ± 2 mins. Furthermore, the solution is poured into the electrophoresis tray, then a comb is installed to print "wells". The solution is allowed to stand until it cools and hardens. After the agarose gel had hardened, the comb mold was removed.

The next process is filling the tank by inserting 1x TAE buffer into the electrophoresis tank. Pipette 10 µl of marker and then put it in the "well". 8 µl of agarose gel was put into the DNA and then 2 µl of Loading dye (Thermo-Scientific) was added. After all the "wells" were filled, running

electrophoresis was carried out. Then it was transferred and the agarose gel was soaked using a UV Transluminator (Fakhardo, 2021).

2.2.14 OprL gene sequencing

The nucleotide sequence of the purified PCR product will be determined using a sequencing method. At the sequencing stage the PCR products were sent to PT. Genetica Science Tangerang and will be used in sequencing analysis. The primers in the PCR stage are also used in sequencing, it's just that each primer is used separately in one sequencing cycle (forward only or reverse only). DNA sequences are formed from the alignment of reverse and forward primer readings which are generally called consensus sequences. These consensus sequences were then compared in the database using certain software (Akihary and Kolondam, 2020). The analysis of the sequence results used the Boedit DNA Base Assembeler software, which then matched the data with data in the *Basic Local Alignment Search Tool* (BLAST) gene bank at the *National Center for Biotechnology Information* (NCBI), National Institute for Health (Ethica et al., 2018).

BLAST is alignment of a sequence with other sequences that have been registered with Genbank so that it can be seen which sequence has the highest percentage of similarity to the sequence being analyzed. It is performed on the resulting alignment and consensus sequences by (Ethica, 2019):

1) Both sequences are highlighted with the mouse.
2) BLAST selected sequence menu is selected.
3) After the NCBI BLAST website opens, press the blast button by leaving all requirements by default.
4) Analysis of the percentage similarity of the 16S rDNA gene sequence is performed to determine the genus or species.
5) Phylogenetic tree information can be obtained automatically from the NCBI BLAST output.

3. Results and Discussion

3.1 Results

Research on the detection of the *oprL* gene based on Polymerase Chain Reaction (PCR) in *P. aeruginosa* BP-1isolate pus was carried out from May to June 2022, the results obtained were that the sample used was isolate pus on the legs of a 5-year-old boy who is the result of sub-culture that has been stored in the Microbiology Laboratory of UNIMUS Semarang.

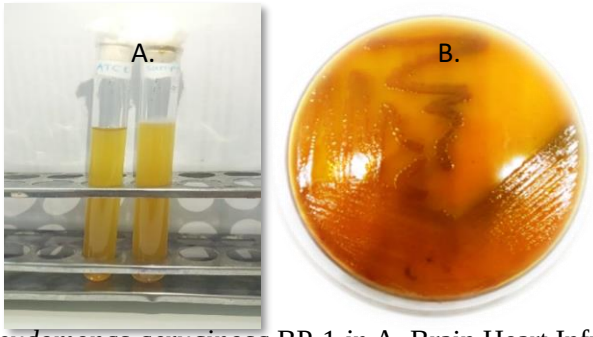

Figure 1. Culture of *Pseudomonas aeruginosa* BP-1 *in* A. Brain Heart Infusion Broth (BHIB) medium B. Mac Conkey (MCA) solid media

3.1.2 Isolation and identification of Bacterial Colonies

The sub-culture or rejuvenation results from bacterium *P. aeruginosa* wound pus isolate were obtained in BHIB and Mac Conkey Agar (MCA) media (**Figure 1**). Subculture functions to maintain microbial cultures by transferring cultures that vary according to the type of microorganism, media and storage conditions (Wahyuningsih and Zulaika, 2019). Rejuvenation of bacteria starts metabolism again after preparation. Bacterial rejuvenation was carried out streaking a bacterial single colony on the agar culture with a slanted surface, then incubating at 37 °C for 24 h (Wijayati et al., 2014).

The results of the BHIB media wound pus isolates were Gram stained to see the characteristics and types of bacteria. The results of Gram-staining of bacterial colonies observed under an optical microscope can be seen in the Table 1.

Table 1. Morphological Characteristics of *Pseudomonas aeruginosa* BP-1

Isolate Code	**Colony shape**	**Color**	**Size**	**Edge**	**Elevation**	**Consistency**	**Cell shape**	**Arrangement**	**Gram-staining**
BP-1	*Circullar*	Green	Medium	*Undulate*	Convex	Dry	Rod	Soliter	Negative

3.1.3 Gram stain

From the isolates that have been found, gram staining is carried out to see the characteristics and types of bacteria. The results of Gram-staining of bacterial colonies were observed under an optical microscope with 100x magnification. Gram stain is used to identify bacteria including Gram-positive or Gram-negative.

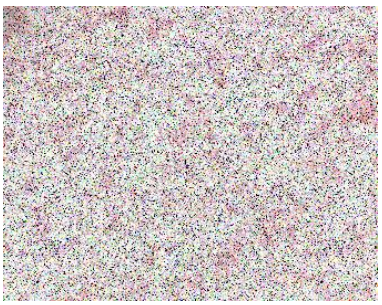

Figure 2. Gram-staining results of bacterial cells of *Pseudomonas aeruginosa* BP-1

Gram stain is a very useful and widely used differential stain in microbiology laboratories (Patel, 2019). Based on table 6 it is known that bacterial isolates subjected to gram staining have Gram-negative characteristics in the form of bacilli with a solitary arrangement.

3.1.4 Biochemical Test

Bacterial colonies were then subjected to biochemical tests consisting of MR, VP, Citrate, Indole, Urea, TSIA, Moltility, Lactose, Glucose and Sucrose Tests. The results were presented in **Figure 3** and **Table 2**.

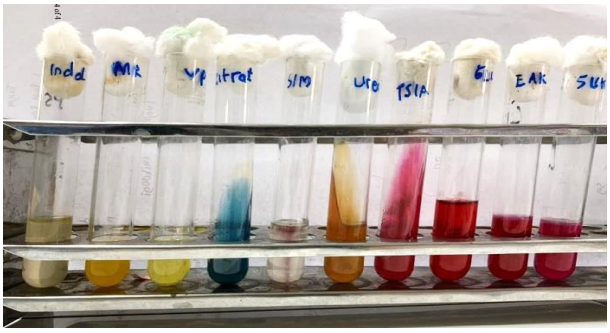

Figure 3. Biochemical identification test results of of *Pseudomonas aeruginosa* BP-1

Table 2. Biochemical identification test results of of Pseudomonas aeruginosa BP-1

Biochemical parameter	Results
MR	Negative
VP	Negative
Indol	Negative
Citrate	Positive
Motility	Positive
Urea	Negative
TSIA :	K/K
H_2S	Negative
Gas	Negative
Lactose	Negative
Glucose	Negative
Sukrose	Negative

As seen in **Table 2**, the results of the bacterial biochemistry test showed negative results in the indole, MR, VP, urea, glucose, lactose, and sucrose tests. Positive results were found in the motility test and citrate test and TSIA test results on the slopes and bottom red 9K/K without the formation of gas and sulfur.

3.1.5 Bacterial DNA Isolation

Isolation of Bacterial DNA from Mac Conkey Agar culture results from *P.aeruginosa* bacteria identified molecularly. The DNA extraction protocol was used for isolation of genomic DNA contained in the Geneid PrestoTM Mini gDNA Bacterial Kit (Susilaningsih et al., 2019). The extraction results (**Figure 4**) were then measured for the purity of the bacterial DNA extract using a Nanodrop spectrophotometer.

Figure 4. Extracted total DNA of *Pseudomonas aeruginosa* BP-1

3.1.5 Quantification of Absorbance Value

Isolation results for measuring the purity of bacterial DNA extracts using a Nanodrop 2000 spectrophotometer (Tthermo Scientific). The purity of the results of DNA isolation can be seen in table 8:

Table 3. Concentration and purity level of isolated DNA of *Pseudomonas aeruginosa* BP-1

Isolate code	Concentration (ng/Ml)	A260	A280	Absorbance Ratio (purity level)
BP-1	145,85	2,917	1,525	1,931
ATCC	160,68	3,735	1,931	1,955

Based on measurements of concentration and purity of DNA from DNA extraction using a NanoDrop spectrophotometer, pure results were obtained by absorbing DNA purity from ATCC and BP-1. It can be seen that the ƛ = 280 nm. The maximum value of DNA that can be absorbed with a ƛ = 260 nm while the maximum value of protein residues that can be absorbed with a ƛ = 280 nm. DNA is said to be pure if it has a 260/280 ratio between 1.8 – 2.0 so that the visualization of the DNA bands on the electrophoretic gel can be read (Harin et al., 2018).

3.1.6 PCR amplification of the oprL gene

Bacterial DNA that has been isolated will be used as a template for *oprL* gene amplification. The method used is the Polymerase Chain Reaction (PCR). PCR is used to amplify single genes, gene segments, or non-coding DNA sequences and is an *in vitro* DNA synthesis and amplification technique. The primers used were universal primers for the Forward and Reverse *oprL* genes. The PCR product was inserted into the well contained in the agarose gel which is at the negative pole, and run with a voltage of 100 V. The results were run on 2% agarose gel electrophoresis and then visualized using a UV transilluminator. The results of the amplification of the *oprL* gene in a sample of wound pus isolates having a size of ~500 bp can be seen in **Figure 5**.

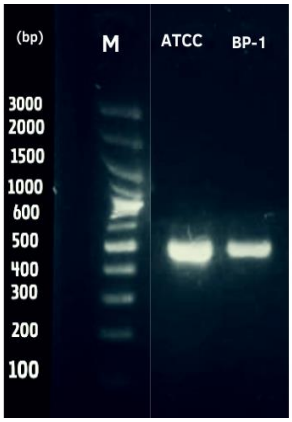

Figure 5. Electrophoresis visualization of amplified *oprL* gene of *Pseudomonas aeruginosa* BP-1 and ATCC (as control) on agarose gel of 2% conducted at PT Genetika Science.

3.1.6 Sequencing Results

The sequencing process was carried out at PT Genetika Science in Tangerang, Indonesia for the analysis of nucleotide sequences. The sequencing results obtained were then bioinformatically analyzed based on forward and reverse alignment to form DNA which is called the consensus sequence using the DNA Baser assembler software. The consensus sequence of the *oprL* gene for strain BP-1 was then analyzed using BLAST on the NCBI website (Ethica, 2019). The obtained consensus is displayed in **Figure 6**.

>*Pseudomonas aeruginosa* gen *oprL* strain BP-1
TTATGGAAATGCTGAAATTCGGCAAATTTGCTGCGCTGGCTCTGGGCCATGGGCTGTGGGCTGTGGGTT
GCTCCTCCAAGGGCGGCGATGCTTCCGGTGAAGGTGCCAATGGCGGCGTCGATCCGAACGCAGGCTATG
GCGCCAACAGCGGTGCCGTTGACGGCAGCCTGAGCGACGAAGCCGCTCTGCGTGCGATCACCACCTTCT
ACTTCGAGTACGACAGCTCCGACCTGAAGCCGGAAGCCATGCGCGCTCTGGACGTACACGCGAAAGACC
TGAAAGGCAGCGGTCAGCGCGTAGTGCTGGAAGGCCACACCGACGAACGCGGCACCCGCGAGTACAACA
TGGCTCTGGGCGAGCGTCGTGCCAAGGCCGTTCAGCGCTACCTGGTGCTGCAGGGTGTTTCGCCGGCCC
AGCTGGAACTGGTTTCCTATGGTAAAGAGCGTCCCGGTCGCTACCGGCCACGACGAGCAGTCCTGGGCT
CAGAACCGTCGCGTCGAGCTGAAGAAG

Figure 6. Consensus of sequenced *oprL* gene of *Pseudomonas aeruginosa* BP-1 amplified by Polymerase Chain Reaction (PCR) obtained using DNA Baser software (Heracle BioSoft SRL Romania).

The results of DNA sequencing are formed by the alignment of reverse and forward primers and are generally referred to as consensus sequences. Then this consensus sequence will be compared with sequence data available in the database using certain software (Rinanda, 2011).

Table 4. Results of NCBL BLAST Gen *oprL* strain BP-1

Isolate code	***Acession Number***	**Size**	**Top hits**	***Query Cover***	Homology level
BP-1	LR134309.1	504 bp	*Pseudomonas Aeruginosa*	99%	99,21%

From the **Table 4** it is known that the bacterial isolate BP-1 has a 99% similarity with the bacterium *P.aeruginosa* NCTC12903 (GenkBank access code: LR134309.1). The results of the consensus sequence of the *oprL* gene using DNA baser assembler software obtained a DNA sequence with a size of 504 bp.

4. Discussion

This study used a sub-culture of wound pus isolates from the UNIMUS Microbiology Laboratory with the code BP-1 and control ATCC. Then planting is carried out on BHIB media, the use of BHIB media functions as a fertilizer medium for bacterial growth and the presence of bacterial growth on the media is marked by the media becoming cloudy (Rahman et al., 2022). Bacterial growth occurs with the formation of turbidity on the BHIB media as shown in **Figure 1**.

After the rejuvenation of the bacteria, a DNA extraction procedure followed by purification process was carried out, namely the purity of the results of bacterial inoculation to obtain pure bacterial colonies. The goal is to extract bacterial DNA, so that DNA can be separated from cell components such as proteins, cell membranes and cell components (Wasdili & Gartinah (2018).

Isolation and purification of bacteria using MCA media to obtain pure and single ones by streaking on MCA media and incubating for 24 h at 37 °C. BP-1 isolate in **Figure 1** and **Table 1** shows colonies with a greenish color that are circular in shape with medium size, convex in shape and have uneven edges. These results match to characteristics of *Pseudomonas aeruginosa*.

Observation of the characteristics of bacterial colonies needs to be done so that bacteria do not accumulate and can facilitate observation of colony morphology. After observing the morphology of the colonies, proceed with gram staining. Bacteria can be seen the type and shape microscopically through gram staining. On Gram-staining it is known that bacteria are classified as Gram-negative or negative. The BP-1 bacterial isolates were read under a microscope with a magnification of 100x with light and using oil immersion to see the types of Gram-negative and Gram-positive bacteria. In this study, BP-1 isolates obtained Gram-negative bacteria.

Based on Gram staining, bacteria are grouped into 2, namely Gram-positive and Gram-negative. Positive bacteria are bacteria that retain gram A dye containing crystal violet, when gram staining is carried out. This type of bacteria will appear purple under a microscope, while gram-negative bacteria will appear red or pink. The difference in classification between these bacteria is mainly based on differences in the structure of the cell wall (Rachmawaty et al., 2009).

Biochemical tests were carried out on samples of bacterial isolates BP-1. Biochemical tests were carried out, namely indole, SIM, MR-VP, urea, citrate, TSIA, and sugar media, including lactose, glucose and sucrose. Biochemical tests were carried out to obtain bacterial species through their physiological characteristics (Inayatul et al., 2018). The results of the sulfide (-), indole (-) and motility (+) tests showed that the bacterium *P.aeruginosa* did not produce sulfide because the bacteria were unable to reduce thiosulfate so that they did not produce hydrogen sulfide which caused the media to not produce a black color. Indole test results showed negative or did not form a red ring after Erlich A and B (1:1). This is because the bacteria do not form indole and tryptophan as a carbon source. Positive motility is indicated by the growth and spread of bacterial turbidity throughout the media (Sulviana et al., 2018). The results of the citrate test indicated by the presence of bacterial growth and the occurrence of a color change in the media from green to blue caused by an increase in the pH of the media above 7.6 due to the presence of ammonia produced which comes from monoammonium phosphate contained in the media (Sardiani et al., 2015) . Based on the results of the biochemical test showed that the BP-1 isolate sample was *P. aeruginosa* according to its biochemical properties.

Isolation of bacterial DNA is the first step in the molecular identification test. Isolation of bacterial DNA was carried out using the Kit method under the brand name Geneaid PrestoTM Mini gDNA Bacteria Kit including sample preparation, lisilan, DNA binding, washing, and DNA elution. The kit method has many advantages, it is fast and easy to process and the quality of the resulting DNA is relatively pure and is widely used by researchers in examining genetic material. The lacquering step is used to remove DNA. According to Wasdili and Gartinah (2018), proteinase K functions to lyse cells or tissues. This chelation process is also supported by increasing the temperature of 70 °C through incubation to support protein degradation in cells. The addition of ethanol aims to degrade DNA so that DNA will precipitate and will bind to silica in the column under the appropriate conditions (Saiz et al., 2021) The last stage of DNA isolation is DNA elution, DNA elution is the process of extracting DNA from the solid phase of the spin column by adding Elution Buffer (Wasdili and Gartinah., 2018)

The results of bacterial DNA extraction were carried out by quantification tests to determine the concentration and purity of DNA using the NanoDrop spectrophotometer at 260 nm and 280 nm. DNA purity is determined by calculating the ratio of absorbance at A260 to A280 (Ratio A260 : A280). A DNA molecule is said to be pure if its absorbance ratio is around 1.8 – 2.0 (Harun et al., 2018). The results of the DNA quantification test of the bacterial isolate BP-1 obtained the purity value of the absorbance DNA ƛ260/280 which was 1.931 and that of the ATCC isolate the absorbance of ƛ260/280 was 1.955. Based on the quantification results, the value of the DNA samples in this study still met the requirements for the DNA amplification test using the PCR technique

The results of DNA isolation from BP-1 and ATCC isolates were then carried out by amplification of the *oprL* gene DNA fragment using a conventional PCR machine (thermalcyler) using *oprL* forward primer and *oprL* forward primer with a band size of 500 bp. The PCR results were then electrophoresed via 2% agarose and then visualized using a UV transluminator. Based on the results of Figure 14, the *oprL* gene amplification results obtained an *oprL* gene band with a size of 500 bp. After DNA amplification of the gene fragment using PCR was carried out, it was followed by the sequencing stage. The results of sequencing the *oprL* gene amplification using Forwar and Reverse primers were obtained in Figure 14. Based on the results of the sequencing in Figure 14, the *oprL* gene band was obtained at 500 bp.

The results of DNA sequencing using the Sanger method were used to determine the nucleotide sequence amplified by the *oprL* gene by PT. Genetika Science. Sequencing analysis is considered to be able to answer various problems related to identification based on conventional microbiology (Rinanda, 2011). Molecular-based identification methods with nucleic acid amplification and sequencing show advantages in terms of better time, sensitivity and accuracy (Nolte, 2008). The results of DNA sequence alignment using the BLAST program showed that the BP-1 strain had similar homogeneity to the Pseudomonas aeruginosa sequence with a query cover value of 99%. The similarity percentage is the ratio of all parallel columns with identical nucleotides in the two sequences (Girgis, et al., 2021). Query cover is the percentage of nucleotide lengths aligned with the database on BLAST (Ethica, 2019).

5. Conclusion

As conclusion, bacterial isolate BP-1 (Boy's Pus-1) could be biochemically identified as Pseudomonas aerugiosa and it was confirmed by its oprL sequenced gene with the highest similarity level (99.21%) to the that of Pseudomonas aeruginosa strain NCTC12903 (accession code LR134309.1)

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