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ORIGINAL ARTICLE

Molecular characterization of proteolytic bacteria associated with Malaria vectors: *Anopheles sundaicus* and *Anopheles vagus*

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ABSTRACT

BACKGROUND

Anopheles (An.) sp. transmits *Plasmodium* parasites that cause malaria. In its life cycle in the mosquito's body, *Plasmodium* passes through 2 mosquito organs, namely the salivary glands and midgut. The bacterial community (symbiont bacteria) in these organs has been known to influence and/or inhibit the development of the *Plasmodium* life cycle by producing specific proteases. This research aims to isolate and characterize symbiotic bacteria with proteolytic activity from 2 important malaria vectors in Indonesia: *An. sundaicus* and *An. vagus*.

METHODS

A total of 183 bacterial originating from the salivary glands and midgut were successfully isolated. Initial screening was carried out based on morphological differences, followed by purification of the selected isolates to obtain single colonies. The selected isolates were then subjected to an initial proteolytic ability test using skim milk agar media. Only isolates with proteolytic activity were further characterized with the 16SrDNA molecular marker. The isolates were pabs5 from the salivary glands and pabs3 from the midgut of *An. vagus*, while pdbs3 and ecbs4 were isolates from the salivary glands and midgut of *An. sundaicus*.

RESULTS

Morphological and molecular characterization showed that both pabs5 and pabs3 isolates were *Pseudomonas (Ps.) aeruginosa*, while ecbs4 was *Enterobacter cloacae* and pdbs3 was Pantoea dispersa. These species were first discovered in *Anopheles vagus* and *Anopheles sundaicus*.

CONCLUSION

The ability of *Ps. aeruginosa* and *Pantoea dispersa* to produce proteases indicated their potential role in the exploration of new strategies to control mosquito vectors that transmit pathogens.

Keywords: *Anopheles*, molecular identification, malaria vectors, bacteria, midgut

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INTRODUCTION

Malaria is a vector-borne disease (VBD) which is transmitted through the hematophagous activity of mosquitoes as the vector. Malaria is caused by *Plasmodium sp.* and transmitted by *Anopheles* mosquitoes. Transmission of malarial parasites depends on the vector's ability to support the development of the parasite in the midgut and the *Plasmodium* sporozoite phase in the salivary glands.[1] Various studies show that these two organs are not only a place for parasites to develop but also contain various types of microbiota. Some of the microbiota associated with mosquitoes that have been studied include bacteria, fungi, protists, viruses, and nematodes.[2] More specifically, the presence of bacteria associated with mosquito vectors (symbiont bacteria) is known to be one of the mosquito defense systems when infected with *Plasmodium* parasites or other pathogens.[3,4] Several bacterial genera that have been successfully characterized from *Anopheles* salivary glands include *Klebsiella*, *Serratia*, *Lactobacillus*, *Pseudomonas*, *Streptococcus*, and *Bacillus*. Meanwhile, the genera *Pseudomonas*, *Serratia*, and *Actinobacter* have been isolated from the midgut.[5]

When the *Anopheles* vectors obtain *Plasmodium* through the blood feeding mechanism, the symbiont bacteria are able to help the vector become resistant to *Plasmodium* thereby blocking its transmission.[6] In the process of parasite transmission, symbiotic bacteria inside the mosquito have anti-plasmodial properties that can stimulate the mosquito's immune response, thereby suppressing parasite development and reducing the mosquito's ability to transmit parasites to new hosts.[5] This has been conducted by activating several genes that play a role in the mosquito immune system, such as the immune deficiency pathway (IMD), Janus kinase/signal transducers and activators of transcription (JAK-STAT), Toll, and RNA interference (RNAi) pathways. This mechanism can cause an increase in reactive oxygen species (ROS) and the formation of antimicrobial peptides (AMPs) or antiviral peptides.[7] An increase in ROS in cells will cause oxidative stress which can cause damage to several molecules that are susceptible to damage such as fats, proteins, and nucleic acids, thereby disrupting parasite development. Meanwhile, the formation of AMPs and/or antiviral peptides can induce the expression of several genes that play a role in the mosquito's natural immune system, such as cecropin, gambicin, and attacin. Antimicrobial peptides and/or antiviral peptides are hypothesized to be proteases and have been known to be produced by several symbiotic bacteria including *Escherichia coli, Bacillus subtilis, Micrococcus luteus, Proteus sp.*, and *Paenibacillus sp.*[4,8]

Although research has been conducted to identify the bacterial microbiota of malaria vector mosquitoes, several important *Anopheles* species remain unstudied or with very limited information being available. This is also because the *Anopheles* vector diversity is quite high. Among the 24 *Anopheles* species confirmed as malaria vectors in Indonesia, exploration regarding the *Anopheles* symbiont bacteria has not been carried out. The few published and unpublished data from GenBank showed a high degree of polymorphism in internal transcribed spacer 2 (ITS2) sequences of *Anopheles* in the Indonesian mosquito population, to the extent that every individual was different. One species of *Anopheles* that is specific to Indonesia is *An. sundaicus* which has been found in the eastern part of East Java [9] and also identified from several parts of Sumatra, and the provinces of West Java, as well as West and East Nusa Tenggara.[10] Several other *Anopheles* species that have vector potential include *An. vagus, An subpictus, An. aconitus* and *An. barbirostris.* [11] *An. vagus* has been discovered in several parts of Sumatra, and the central and eastern parts of East Java.[11,12] This species has been confirmed to have vectorial capacity for *Plasmodium falciparum* in Indonesia.[13] However, there is no data available on its associated bacteria which will be important in relation to its transmitted malarial pathogen. This study aimed to screen and characterize proteolytic bacteria from these 2 important malaria vectors in Indonesia: *An. sundaicus* and *An. vagus*.

METHODS

In silico analysis of bacterial symbionts from *Anopheles*

In silico analysis was carried out to give an early overview of the diversity of bacteria in mosquito organs based on the previous research database by using clustering, metagenomic, or DNA metabarcoding approaches.[14,15] This is necessary to determine the possible diversity of *Anopheles* symbiont bacteria as initial data that can be used as a reference for the results from laboratory analysis. This *in silico* analysis was carried out using a clustering approach to the 16S rDNA sequence of *Anopheles* symbiont bacteria

from the NCBI GenBank database. 16S rDNA data was collected into fasta format. Bacterial diversity abundance analysis was carried out via the Galaxy webtools (https://usegalaxy.org/) with the Kraken2 algorithm. The abundance of bacterial diversity was visualized in the Krona data format with a clustering system from taxa from kingdom to genus.

Landing collection and mosquito identification

Anopheles mosquitoes were obtained from landing collections from Bangsring - Watudodol, Banyuwangi, with an aspirator for catching adult mosquitoes from December 2022 to May 2023. Larvae were also collected which were then reared on a laboratory scale. Mosquito rearing activities were carried out in the insectarium at a temperature of ± 28˚C (room temperature). The mosquitoes obtained were then identified morphologically using a mosquito identification key.[16] To confirm the identity of the vector, identification was continued with DNA barcoding using ITS2 (Internal Transcribed Spacer-2). Identification based on DNA molecular markers is very important because the potential for identification errors is high if we consider only the morphological characters. [11,17] The correctness of the *Anopheles* vector identification process will determine whether the bacterial isolate obtained is truly a symbiont bacterial isolate associated with the mosquito vector, and not contamination from the environment.

Isolation of bacteria from midgut and salivary glands

Ten of the mosquitoes that had been identified were then killed by placing them in the freezer for ± 1 minute, then soaking them in sterile 75% ethanol for 1-5 minutes. Mosquito dissection was carried out using a sterile stereo microscope (which had been sprayed with 70% alcohol), and the organ samples obtained were then put into a microtube containing 100 µl of sterile PBS [18] and crushed with a micro-pistil. The homogeneous solution was then diluted 5-fold in 900 µl of PBS and 100 µl of this suspension was taken to be grown in nutrient agar medium (NA, containing meat extract, peptone, and agar) in petri dishes and incubated at 37°C for 48 hours.[18] The bacteria that grew were then purified based on their colonies on a new NA medium in petri dishes using the streak quadrant technique and incubated at 37°C for 48 hours until pure bacterial colonies were obtained. The bacterial stock that was obtained was then subjected to morphological identification based on Bergey's Manual of Determinative Bacteriology and to molecular identification based on the 16S rDNA sequence. Gram staining was also performed according to Leboffe and Pierce [19] with minor modifications. The Gram staining bacteria were observed by using light microscopy and were compared with *Escherichia coli* as the Gram negative control while the Gram positive control was represented by *Bacillus subtilis*.

Proteolytic assay of selected bacterial isolates

The initial proteolytic test carried out was a general protease test using skim milk agar (SMA) media. The presence of proteolytic enzymes produced by the bacteria can be seen from the presence of a clear zone around the selected bacterial isolate colony as a result of the degradation of proteins in the media.[20] The selected bacterial isolate will then be cultured to obtain putative extracellular proteases which are then isolated from the media. One bacterial isolate each from the salivary gland and the midgut of *An. vagus* and *An. sundaicus* that had the potential to have protease activity (proteolytic bacteria) was then further characterized morphologically, biochemically, and molecularly based on 16S rDNA. The proteolytic ability shows the bacterial isolate's potential of intervening in the life cycle of the *Plasmodium* parasite in the body of the *Anopheles* vector.

Identification of bacteria based on 16S rDNA

Molecular identification based on DNA barcoding was carried out using 16S rDNA sequences. Therefore, the genomes of selected bacterial isolates were extracted using the freeze and thaw isolation technique.[21] The working principle of the freeze and thaw method is to damage the membrane structure and bacterial cell walls with heat shock treatment so that the total cell genome is obtained. The genome obtained was then used as a template to amplify the 16S rDNA sequence. Amplification of DNA (16S rDNA) encoding 16S rRNA was carried out *in vitro* using a PCR machine with a forward primer 27F (5' AGA GTT TGA TCM TGG CTC AG 3'), and a reverse primer 1492R (5' GGT TAC CTT GTT ACG ACT T 3'). The PCR conditions used were initial denaturation at 98 °C for 5 minutes followed by 35 cycles of the following stage: denaturation at 95 °C for 35 seconds, annealing at 55 °C for 35 seconds and extension at 72 °C for 90 seconds. The PCR results were then purified using the Wizard SV Gel and PCR Clean-Up System

(Promega, USA). This DNA barcoding-based identification can be done after the 16S rDNA PCR result sequence has been determined with the help of 1st BASE (Singapore). This sequence data was then analyzed using Bioedit bioinformatics software and the resulting DNA sequences were then compared with the 16S rDNA sequence database in GenBank using the Basic Local Alignment Search Tool (BLAST) online software (www.ncbi.nlm.nih.gov). The resulting sequence was reconstructed as a phylogenetic tree using Mega 6 software and the method used was Neighbor Joining Tree.

RESULTS

In silico analysis using a clustering approach to the 16S rDNA sequences of *Anopheles* symbiont bacteria from the NCBI GenBank database was carried out to map the diversity of bacteria in mosquito organs based on a database of previous research results.[14] *An. gambie* and *An. stephensi* are two *Anopheles* vectors that have been widely studied by researchers regarding the interaction of vector, pathogen, and human host. Results of the in silico analysis at the genus level are shown in Figures 1 and 2. *Bacillus, Asaia, Pseudomonas, Micrococcus,* and *Acinetobacter* are the main bacterial genera associated with mosquito vector *An. gambie* and *An. stephensi.* This data will be a reference on the probability of the diversity of symbiont bacteria that will be isolated from *Anopheles* samples of this study. From several sampling times, a total of 760 mosquitoes were collected. The morphology-based characterization results are relevant to the ITS2 DNA barcoding results which showed that *An. vagus* and *An. sundaicus* were the dominant mosquitoes from the sampling area (Table 1).

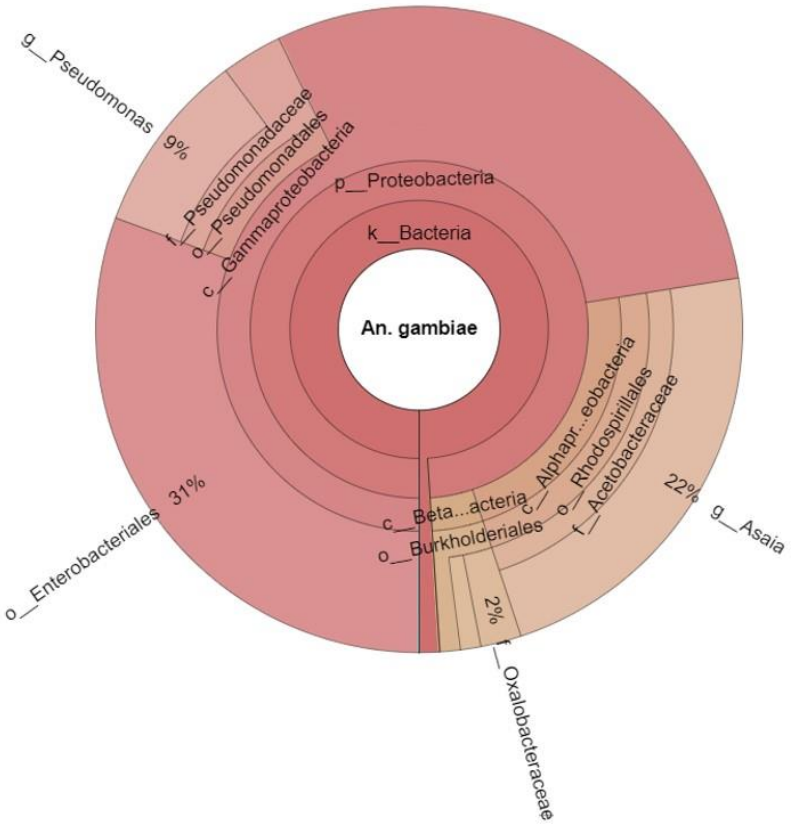

Figure 1. *In silico* analysis results of 16S rDNA based symbiotic bacterial diversity at genus level from mosquito vector *An. Gambiae*

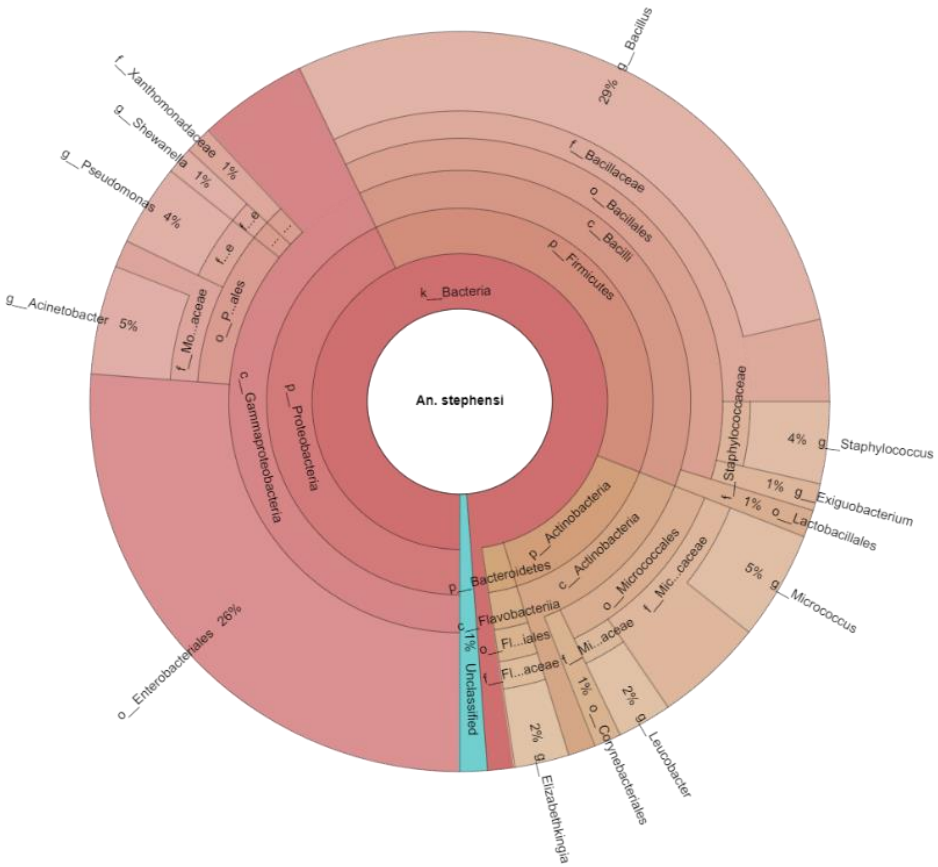

Figure 2. *In silico* analysis results of 16S rDNA based symbiotic bacterial diversity at genus level from mosquito vector *An. Stephensi*

Table 1. Internal transcribed spacer-2 molecular based identification of *An. vagus* and *An. sundaicus*

Description	Max score	Query cover	E-value	Percent identity	Accession number
Internal transcribed spacer-2 (ITS2)					
Anopheles vagus					
Anopheles vagus 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	1164	100%	0.0	99.69%	FJ654649.1
Anopheles vagus voucher 1841-AN4 internal transcribed spacer 2, partial sequence.	1160	100%	0.0	99.69%	MN203100.1
Anopheles sundaicus					
Anopheles sundaicus isolate CT 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1003	100%	0.0	99.46%	GQ284825.1
Anopheles sundaicus internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	1003	100%	0.0	99.46%	AY768541.1

Salivary gland and midgut organs were successfully dissected from identified mosquitoes which were then extracted to obtain potential bacterial isolates that be used as model species. Plating of the extracts from salivary glands and midgut of both *Anopheles* resulted in a total number of 245 colonies of bacterial isolates (Figure 3). Several colonies with morphological differences were selected for initial proteolytic screening as in the methodology. The four selected isolates with proteolytic activity were given the notation: pdbs3, ecbs4, pabs3, and pabs5 (Figure 4). Molecular characterization results showed that the isolates were identified as *Pantoea dispersa* (pdbs3); *Enterobacter cloacae* (ecbs4), and *Pseudomonas aeruginosa* (pabs3 and pabs5). The results of the 16S rDNA phylogenetic construction of these isolates can be seen in Figures 5, 6, and 7.

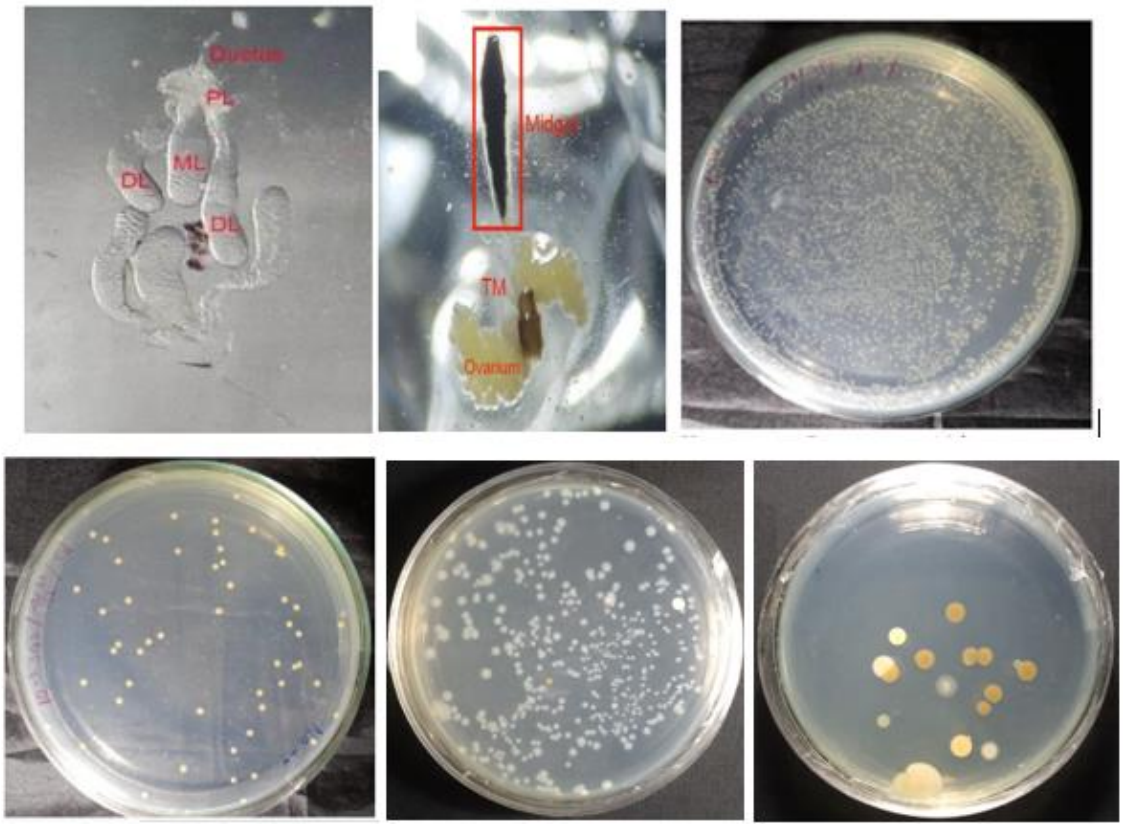

Figure 3. Clockwise: salivary gland and midgut of *Anopheles* sample with stereo microscopy at 50× magnification, some bacterial plating from these organs

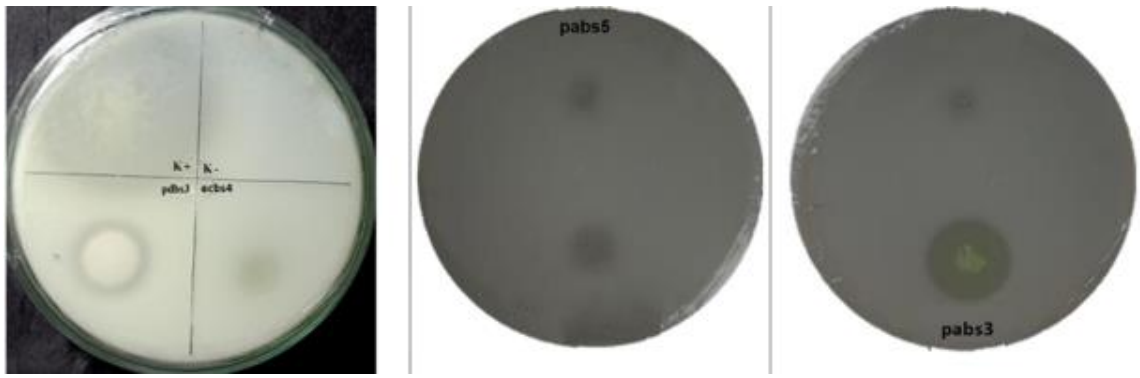

Figure 4. Preliminary proteolytic test using skim milk agar revealed that some bacterial isolates may produce extracellular proteases

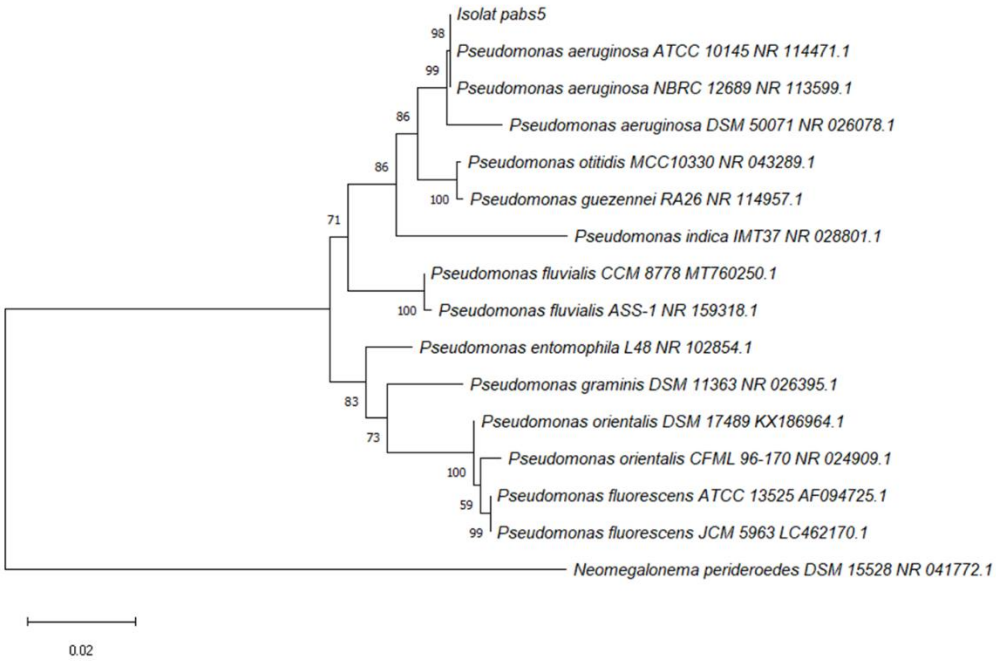

Figure 5. 16S rDNA based phylogenetic tree construction of *Pseudomonas aeruginosa* isolated from salivary gland and midgut of *An. vagus*
Note: isolat pabs5 = pabs5 isolate

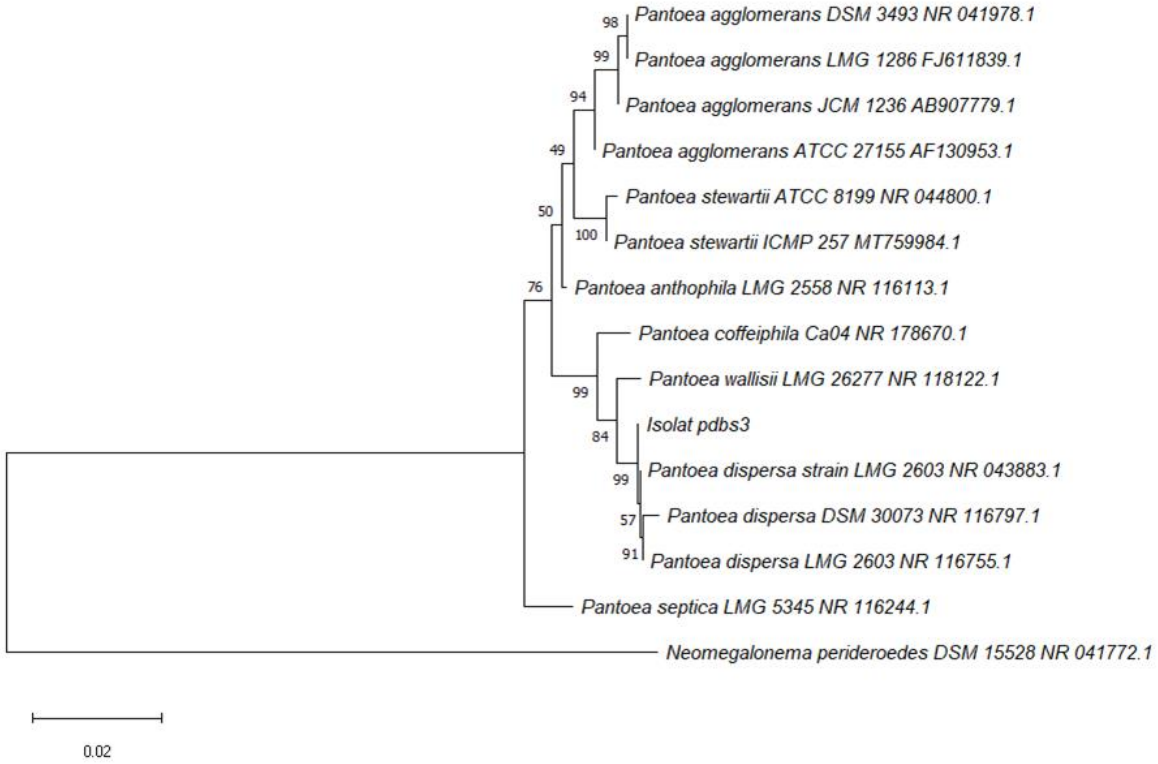

Figure 6. 16S rDNA based phylogenetic tree construction of *Pantoea dispersa* isolated from midgut of *An. sundaicus*

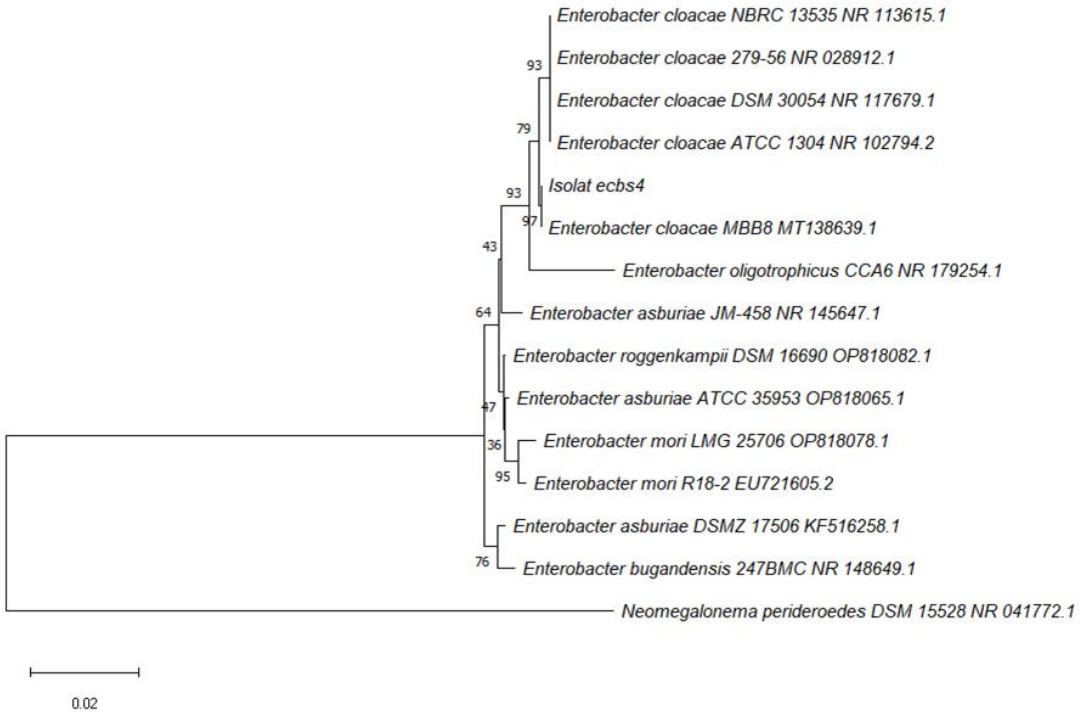

Figure 7. 16S rDNA based phylogenetic tree construction of *Enterobacter cloacae* isolated from the salivary gland of *An. sundaicus*

DISCUSSION

Gram staining results showed that all selected bacterial isolates from the salivary glands and midgut belonged to the Gram negative group. This is in accordance with the study by Berhanu et al.[5] who described that mosquito symbiont bacteria are dominated by Gram negative bacteria from the mosquito salivary glands, midgut, and reproductive organs. Further analysis with molecular markers of the 16S rRNA coding gene showed that the two isolates (pabs3 and pabs5) from the salivary glands and midgut of *An. vagus* were identical, namely *Pseudomonas aeruginosa* with a homology percentage reaching 99.9% and query cover of 100% (**Figure 5**). This study reported for the first time the existence of *P. aeruginosa* in the salivary glands and midgut of *An. vagus*. Previously, *P. aeruginosa* had been found in other Anopheles mosquitoes such as *An. stephensi*,[22] *An. albimanus*,[23] *An. culifacies*,[24] and *An. arabiensis*.[5] The microbiota found in mosquito bodies, both in the salivary glands and midgut, are known to have the ability to inhibit the development of parasites and play a role in generating mosquito defense responses.[22] This result is relevant to the results of the in silico analysis above which shows that the dominant bacterial genus found in *Anopheles* is *Pseudomonas*.

P. aeruginosa is predicted to act by initiating the mosquito's immune response to more effectively produce several inhibitory compounds. The initiation of the immune response carried out by *P. aeruginosa* bacteria is generally the same as in other symbiont bacteria. After blood feeding, bacterial growth triggers an immune response through the immune-deficiency (IMD) pathway, which causes the synthesis of antimicrobial peptides and immune effectors possessing antiparasitic effects. Prodigiosin pigment derivative has been reported to be found in *P. aeruginosa* with the capability of inhibiting *P. falciparum*.[25] Apart from this pathway, *P. aeruginosa* also has a periplasmic protein called azurin which has the function of inhibiting the development of the plasmodium.[26] Like other bacteria, *P. aeruginosa* is also known to produce proteolytic enzymes. Cytotoxic metalloproteases are an example reported by Azambuja et al.[23] to be found in *P. aeruginosa*. According to Elkington et al.[27] matrix metalloproteases (MMPs) are a group of proteolytic enzymes that play many roles in the normal immune response to infections including *Plasmodium* parasite infections.

The pdbs3 isolate from the midgut of *An. sundaicus* was identified as *Pantoea dispersa* with a homology percentage of 98.9% and query cover of 99% (Figure 6). To the best of our knowledge, this is new information on the discovery of this

bacterium from *An. sundaicus*. The *Pantoea* genus is a group of Gram-negative bacteria, does not have a capsule, and has a rod shape. *Pantoea* forms associations with a variety of hosts, including insects.[28] *P. dispersa* is a member of the *Pantoea* genus which has the following characteristics on culture plates: facultative anaerobic bacteria with yellow pigment and colonies of round shape and smooth surface that is raised above the medium.[29] The *Anopheles* midgut has been reported to be dominated by the genus *Pantoea*, therefore this genus has been proposed for a paratransgenesis implementation.[30] Paratransgenesis is a strategy to control vector-borne diseases by using microbiota (bacteria and fungi) derived from insect vectors that are genetically manipulated to inhibit or kill disease-causing pathogens. Bacteria are isolated and genetically modified in vitro to produce anti-pathogenic factors that function to disrupt the life cycle of disease-causing pathogens.[31] The genus *Pantoea* was previously identified from the mosquitoes *An. stephensi*, *An. gambiae*, *An. funestus*, *An. coluzzii* and *An. darlingi*. *Pantoea dispersa* has also been found specifically in *An. darlingi* which originates from the Brazilian Amazon.[32]

The results of sequence analysis and phylogenetic tree construction for the ecbs4 isolate from the salivary gland of *An. sundaicus*, showed that this isolate was *Enterobacter cloacae (E. cloacae)* with a homology percentage of 99.4% and query cover reaching 100% (**Figure 7**). *E. cloacae* has also been found in the salivary glands of *An. arabiensis* maintained in the laboratory. [33] *E. cloacae* is dominant in female *Anopheles* especially in *An. funestus* and plays an immunological role as a bacterial symbiont residing in the midgut.[5] The bacterium *E. cloacae* has been proven to influence the immune system of mosquito vectors by limiting the development of *P. berghei* and *P. falciparum* in *An. stephensi* and *P. vivax* in *An. albimanus* [34,35] *E. cloacae* can also interfere with transverse ookinete invasion of the midgut. *E. cloacae* bacteria can also induce *An. stephensi* serine protease inhibitor (AsSRPN6) in mediating the anti-plasmodium response in the midgut of *An. stephensi.*[34] This bacterium has been tested for paratransgenesis with genetic modification to express antiplasmodium effector molecules in *An. stephensi*. The bacterium *E. cloacae* is a potential candidate to be used to strategically block the development of *Plasmodium* in the body of the mosquito vector.[36] However, for vector *An. sundaicus*, this new characterized isolate has to be further elaborated to understand its role in transmission and/or inhibition of malaria pathogens.

To our knowledge, this is the first report on culturable proteolytic bacteria associated with *Anopheles* mosquitoes in Indonesia. Understanding the bacterial composition and structure in *Anopheles* mosquitoes may be important in future investigations regarding the influence of the interactions between the microbiota – Anopheles vector – *Plasmodium* on mosquito development and control. Identification of symbiotic bacteria that can be used against *Plasmodium* development inside the mosquito and that play a major role in the anopheline lifespans will give the potential public health benefit of new tools to reduce or even eradicate malaria. Furthermore, although it is still in the conceptual stage, another potential implementation of these associated bacteria consists of an effective and scalable paratransgenesis-based malaria control strategy. Still, issues related to the efficacy and biosafety need to be considered and a regulatory framework for this specific application in the future needs to be established.

CONCLUSION

This research succeeded in isolating and identifying *Pseudomonas aeruginosa* (GenBank Accession Nr. OR794204), *Pantoea dispersa* (GenBank Accession Nr. OR801039), and *Enterobacter cloacae* (GenBank Accession Nr. OR801006) from the 2 main vectors of malaria in Indonesia, namely *An. vagus* and *An. sundaicus*. The proteolytic potential of both *Pseudomonas aeruginosa* and *Pantoea dispersa* isolates is an important indicator for further exploration regarding their role in influencing the life cycle of pathogens in the mosquito vector's body.

Conflict of Interest

All authors declare that they have no conflicts of interest.

Acknowledgement

None.

Author Contributions

K.S. contributed to the conception, design of the study, data analysis, manuscript preparation and the final version of the manuscript. TYMR, SW, RO & AL involved in design of the study, data analysis, and the final version of the manuscript. NPCA, DPA, DA & DAU performed

data acquisition and experimental laboratory works. All authors have read and approved the final manuscript. All authors will take public responsibility for the content of the manuscript submitted to Universa Medicina.

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Data Availability Statement

All data generated or analysed during this study are included in this published article. DNA sequences are available at the National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov.

Ethics Approval and Consent to Participate

Not applicable.

Declaration of Use of AI in Scientific Writing

The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript, nor for the creation of images, graphics, tables, or their corresponding captions.

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