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Molecular detection of pathogenic bacteria in *Rhipicephalus sanguineus* (sensu lato) ticks from Bitung, North Sulawesi, Indonesia

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Abstract. *Tahulending J, Tulung M, Pelealu J, Doda DV. 2022. Molecular detection of pathogenic bacteria in* Rhipicephalus sanguineus *(sensu lato) ticks from Bitung, North Sulawesi, Indonesia. Biodiversitas 23: 6164-6170.* Domesticated dog populations have grown throughout Indonesia, notably in Bitung City. Dogs are frequently kept as home guardians and reside with their owners. Dogs are typically attacked by ectoparasites, parasites that adhere to the skin's surface and potentially spread the disease microorganisms to humans, animals, and the environment. As a specific host, ticks are frequently found in dogs. This research aims to identify and analyze the molecular character bacterial of *Rhipicephalus sanguineus* ticks in Bitung City. A hypervariable region of 16S rRNA called V3-V4 was selected for the DNA barcode area. Dogs' ticks have been collected from several areas in Bitung. The microbes inside the digestive tract were analyzed using genomic DNA with a gSYNC DNA extraction kit, and PCR amplification using KOD FX Neo and MyTaq HS red mix. At First Base Singapore, gel electrophoresis and sequencing were carried out. The collected sequencing data were analyzed using MEGA X and the BLAST, connected online with the NCBI's GenBank. The nine distinct bacteria were identified inside the digestive tract of ticks. *Pseudomonas stutzeri* (35%), *Brevundimonas* sp. (21%), and *Aquamicrobium lusatiense* (17%) were the pathogens that were most frequently found. These results enhance knowledge of the pathogen microbes that may impact either humans or animals.

Keywords: *Aquamicrobium lusatiense, Brevundimonas*, genomic DNA, *Pseudomonas stutzeri*, *Rhipicephalus sanguineus*

INTRODUCTION

Rhipicephalus sanguineus was reported in Indonesia as a common parasite of dogs (Hadi et al. 2016). Dogs have been in close contact with humans for a long time; thus, humans are at risk, particularly for zoonotic tick-borne pathogens (TBPs) (Corales et al. 2014). The tropical climate of Southeast Asian countries, which include Bitung, North Sulawesi, Indonesia, the presence of stray or neglected companion animals, and the high popularity of dog ownership all contribute to favorable conditions for tick survival and reproduction. Ticks are known to be capable of transmitting more pathogens to humans and animals than any other arthropod (Baneth 2014). The incidence of tick-borne diseases (TBDs) has been reported to have increased worldwide in recent years. Ticks are the most important vectors of vector-borne diseases in terms of human and animal health, responsible for the transmission of many infectious agents such as bacteria (*Borrelia*, *Anaplasma*), viruses (tick-borne encephalitis), and even parasites (*Babesia*, *Theileria*) (Dantas-Torres et al. 2012). Ticks are vectors of various pathogenic bacteria (Sperling et al. 2017).

In the last decade, studies on the molecular detection of TBPs in dogs through PCR have been increasing. Dogs have been in close contact with humans for a long time; thus, humans are at risk, particularly for zoonotic TBPs (Galay et al. 2018). Microbes transmitted from animals to humans and cause many new diseases have been reported recently (Estrada-Peña 2015). However, some zoonotic diseases must be wary of public health. Most information available in the scientific literature on bacterial transmission between pets and humans relates to human pathogen bacteria. Dogs and cats are potential sources of various zoonotic bacteria that can be transmitted *via* vectors (i.e. ticks). Pet-associated zoonoses are usually sporadic and their frequencies are not easily determined because of the difficulty in recognizing and validating disease transmission from pets (Tan 1997). However, epidemiological data in Bitung are needed because surveys were limited to small areas of the city. Furthermore, previous studies mostly included pet dogs that showed clinical signs and presented in a few veterinary clinics.

Rhipicephalus sanguineus, the brown dog tick is the most common ectoparasite of dogs in the world (Dantas-Torres 2010) and also in Indonesia (Hadi and Rusli 2006). *R. sanguineus* tick is a vector of many disease agents, such as *Coxiella burnetii* (Khalili et al. 2018), *Ehrlichia canis* (Rochelle et al. 2018), *Rickettsia conorii* and *Rickettsia rickettsia* (Solomon et al. 2022). The tick can carry and spread a range of blood-borne diseases that can affect both animals and humans. These include Q fever as one of the tick-borne zoonotic diseases (Norris et al. 2013). Moreover, in the era of globalization and climate change, the brown dog tick has become increasingly relevant from a public health perspective. This tick has also been implicated in the transmission of pathogens of zoonotic concern. *R. sanguineus* is the most widely distributed tick, prevalent

throughout the year in tropical and subtropical areas. It is a three-host tick, dropping from its host after each blood meal and molting in the environment to the next stage. Thus, this tick can utilize a different host for every blood meal, and therefore has a higher chance of spreading pathogens it might carry to other hosts (Dantas-Torres and Otranto 2015). Tick-borne diseases are among the most prevalent problems diagnosed in both medical and veterinary medicine and their spectrum has recently increased (Dantas-Torres et al. 2012). Ticks are obligate hematophagous arthropods that can be transported over large distances by animals and serve as vectors of pathogens, they have short feeding structures (Bonnet et al. 2016).

The identification of bacteria using a metagenomic approach with 99% of accuracy in samples (Trinh et al. 2018) and the 16S RNA gene empathetic to identify bacteria (Koo et al. 2018). However, data are needed for investigation of the pathogenic bacteria as well as for preventing the risk of transmission to humans. This research used a metagenomic approach to identify the bacteria in the digestive tract of *R. sanguineus* ticks, it has not previously been reported. Thus, this study was conducted to further establish the topic. These results extend the knowledge of the potentially pathogenic bacteria affecting human and animal health in the future.

MATERIALS AND METHODS

Study sites

This study was conducted in April 2022, in three areas in the City of Bitung, North Sulawesi, Indonesia, namely Matuari, Girian, and Aertembaga (Figure 1). Located between: Matuari (1º26'15.5" N and 125º06'25.7" E), Girian (1º26'32.2" N and 125º07'58.1" E), and Aertembaga (1º28'13.7" N and 125º12 '02.7" E). The samples of dogs were collected from three areas in each plain. Areas were selected based on dog population and the number of infected dogs.

Sample collection

Samples of dogs from several areas in Bitung City were conducted using a purposive sampling method, 15 dogs with criteria (a) having owners, (b) being kept freely, without being tied up or caged, and (c) dogs with different owners. Ticks were collected from areas of five body parts, namely the head and neck, back, abdomen, legs, and tail. The procedure for taking ticks: (a) using gloves, 50 adult ticks were taken from each area, (b) then put into a bottle that has been labeled with the location, filled with 70% alcohol, and brought to the laboratory, (c) then the body structure of the tick was observed and measured using a Hirox KH8700 3D microscope equipped with measurement software. Then each of the six ticks was used for genomic DNA extraction. The specimens were separately transferred into labeled tubes containing 70% absolute ethanol, the tick samples were stored at -20°C until DNA extraction. In the laboratory, ticks were examined under a Hirox KH8700 3D microscope at 250x magnification. All morphological identifications were confirmed by amplification and sequencing of the 700 bp fragment of the mitochondrial 16S rRNA gene. Samples were then transported to the Microbiology and Chemistry of Pharmaceutical Analysis Laboratory at Sam Ratulangi University, North Sulawesi, Indonesia. Samples were stored in a refrigerator at -20°C before being used for total DNA genomics extraction.

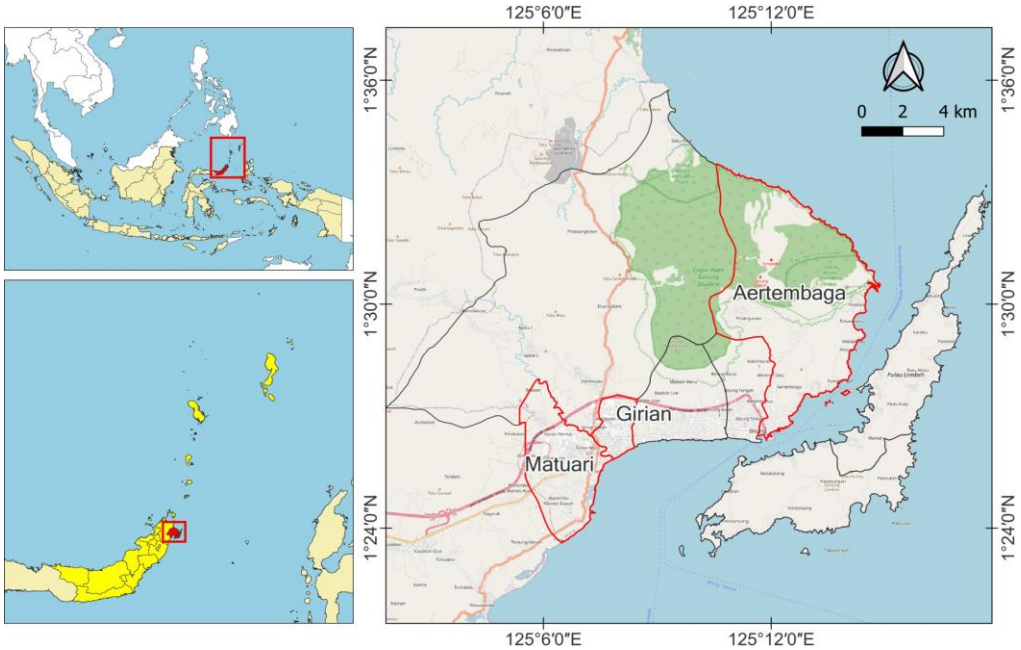

Figure 1. The locations used for sampling *Rhipicephalus sanguineus* (sensu lato) in Matuari, Girian, and Aertembaga of Bitung City, North Sulawesi, Indonesia

Molecular characterization

The total genomic DNA was extracted from the digestive tract of ticks. DNA extraction steps are lysis, binding, washing, and elution, then coding for the tick DNA. Ticks were extracted using the CTAB/SDS method. The extraction results become a template for the CO1 gene amplification stage. DNA isolation was carried out using the fungal/bacterial quick-DNA and Quick-DNA plant/seed mini kit (Zymo Research, D6005) and Quick-DNA Plant/Seed Miniprep Kit (Zymo Research, D6020). The DNA isolation steps follow its manufacturer protocol. DNA Amplification was performed with PCR Thermocycler using two CO1 primers. The amplified and sequenced of mitochondrial DNA specifically the CO1 gene using the following primers: 27F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' GGH TAC CTT GTT ACG ACT T 3'. The V3-V4 hypervariable regions were selected from the 16S rRNA gene for this metabarcoding process. The area was amplified using MyTaq ™ HS Red Mix (HS Red Mix, 2X (Bioline, BIO-25048) & KOD FX Neo (Toyobo, KFX-201). The amplifications were carried out under the following condition: 1 cycle of initial denaturation at 95°C for 1 min, then followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, and a final extension to complete the process at 72°C for 10 sec (Table 2). The electrophoresis of PCR products was run on a 0.8% and 1% agarose gel centrifugation with Red Mix (Bioline) and buffered with BashingBead™ (Table 3). All amplification samples were sent to First Base Singapore through PT. Genetika Science Jakarta (Indonesia) for purification and sequencing.

Based on the results of genomic DNA extraction of ticks using nanodrop conducted a concentration of 72.4 ng/µL, 63.8 ng/µL, and 127.9 ng/µL. Furthermore, DNA purity detected was 1.97, 1.99 and 1.97 (A260/A280) while with Nanodrop 2.23, 2.29 and 0.45 (A260/ A230). The results obtained can be continued for amplification of the 16S rRNA gene was carried out in regions V3 and V4 (Table 3).

Data analysis

Raw nucleotide sequences and chromatograms were viewed using BioEdit. Additional sequences from GenBank were added as a dataset. The genetic distance was calculated by the Kimura 2-parameter method. The neighbor-joining (NJ) method was used to generate the phylogenetic tree using MEGA software (Kumar et al. 2018). BLAST searches were used to compare the 16S rRNA gene sequences in this study with the GenBank database to determine the closest matches.

RESULTS AND DISCUSSION

Results

In this study, a total of 150 ticks were randomly collected from 15 dogs. All ticks were morphologically identified to be *R. sanguineus* (sensu lato) regarding the standard characteristics including red-brown color, elongated body shape, and hexagonal basis capituli (Figure 2). Ticks samples were prepared for DNA extraction after identification and sorting. The presence of symbiont bacteria communities in the digestive tract of ticks was analyzed using the metabarcoding approach. The DNA barcode region used in this approach was V3-V4 which is a hypervariable region of 16S rRNA. The result of this metabarcoding shows a total of 3 phyla that can be identified from the digestive (Table 4). The most abundant phyla originated from *Pseudomonas stutzeri* (35%), followed by *Brevundimonas* sp. (21%) and *Aquamicrobium lusatiense* (17%) (Figure 4). After obtaining the sequence readings, sequences were compared to reported isolates using the Basic Local Alignment Search Tool (BLAST) of the U.S. National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The control genes actin and mt-rrs were successfully detected in all tick DNA samples, respectively. Following nested PCR using screening primers, the DNA of bacterial pathogens of *P. stutzeri*, *Brevundimonas* sp., and *A. lusatiense*. All obtained sequences for each pathogen were 100% homologous and were found to share 99.86-99.93% identity to *P. stutzeri* (Accession number: KM278988.1, CP025149.2, CP027664.1, CP011854.1, CP062162.1, CP091174. 1, OK618380.1, OK618369.1, MZ596191.1, MZ508319.1). All positive amplicons share 99.92-100% sequence identity to *Brevundimonas* sp. (Accession number: KY486816.1, CP039382.1, LC324685.1, LC3246841), and 97-98% identity to *A. lusatiense* (Accession number: KU525645.1, KU525640.1, AM884147.1, KM210272.1, MK396598.1).

Table 2. PCR conditions

Phase	Temperature (°C)	Duration (seconds)	Cycle
Initiation	95	60	35 x
Denaturation	95	15	
Annealing	55	15	
Final Extension	72	10	

Table 3. Genomic DNA extraction of *Rhipicephalus sanguineus* (s.l.)

Nucleic Acid	Nanodrop (ng/µL)	A260/A280	A260/A230	Volume (µL)
Isolate A	72.4	1.97	2.23	35
Isolate M	63.8	1.99	2.29	35
Isolate G	127.9	1.97	0.45	35

Table 1. 16S rRNA gene-targeted PCR primers used in this study

Primer name	Target group	Sequence (5′ to 3′)	*E. coli* position	References
27F	Bacteria	AGA GTT TGA TCC TGG CTC AG	8-27	Weisburg et al. (1991)
1492R	Archaea and bacteria	GGH TAC CTT GTT ACG ACT T	1492-1510	Weisburg et al. (1991)

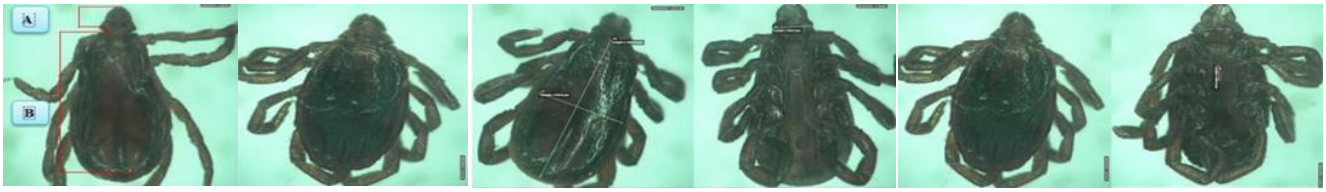

Figure 2. *Rhipicephalus sanguineus* (sensu lato) from Bitung, North Sulawesi, Indonesia (A. Matuari, B. Girian, C. Aertembaga)

Table 4. The bacteria species in *Rhipicephalus sanguineus* (sensu lato) ticks

Location	Family	Genus	Species	Group
Matuari	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas stutzeri*	Gram- negative bacteria
Girian	Phyllobacteriaceae	*Aquamicrobium*	*Aquamicrobium lusatiense*	Gram- negative bacteria
Aertembaga	Caulobacteraceae	*Brevundimonas*	*Brevundimonas* sp.	Gram- negative bacteria

Discussion

The different microbiomes composition found comparatively in the samples can be explained by the tick's intrinsic mechanisms and environmental factors, like life stage and the collection site (Kueneman et al. 2021). However, this difference was not greatly expressed comparing the number of species in the samples. Three phyla found in our samples (*Pseudomonadaceae, Phyllobacteriaceae, Caulobacteraceae*) were composed of adult ticks. Members of this phylum are gram-negatives, which compose animal and the human microbiome from urinary tract infections. Ticks acquire different bacteria in the microbiome depending on the environment, in the nest, or affected by blood diet, the bacterial presence in the DNA could be originated from the tick's contact with the animal-host skin (Jose et al. 2021).

Compared with the results of other studies, that *Coxiella, Rickettsia,* and *Bacillus* are the most pathogens in ticks from France, Senegal, and Arizona (René Martelle et al. 2017). Similar results were also reported by Khalili et al. (2018), *Coxiella burnetii* is the most pathogen in ticks collected from infested dogs in Iran. Geurden et al. (2018), the *Rickettsia* is the most pathogen in ticks from dogs and cats in different European countries. The most common bacteria found in general in the microbiome of adult ticks are *Coxiella, Francisella, Anaplasma, Borrelia, Ehrlichia,* and *Rickettsia* (Greay et al. 2018b). Meanwhile, the test results of molecular detection of tick-borne pathogens in stray dogs and *R. sanguineus* (sensu lato) ticks from Bangkok found that *Babesia vogeli* is the most pathogen (Thom et al. 2021). Tick microbiome can be different depending on the tick genotype, biogeographical area, and male or female (Luzzi et al. 2021). The environmental microorganisms can be found in the tick microbiome and may be difficult to separate from where they belong (O'Neal et al. 2021) because these bacteria might have originated from dogs' skin, blood, or even saliva (Luzzi et al. 2021).

Based on this table, the most common bacterial detected belongs to the group of Gram-negative bacteria. Other researchers have also agreed that the most of symbiotic bacteria included of Gram-negative group (Boulanger et al. 2019). This obligate intracellular Gram-negative bacterium protects itself in hostile environments by forming spores that can survive for long periods, for example, 586 days in tick feces at room temperature (Philip 1948). The pathogen can be transmitted vertically between invertebrates through life stages or be transmitted horizontally from invertebrates to vertebrates during feeding on its host (Weinert et al. 2009).

Based on this chart, it is confirmed that there are several species. The *P. stutzeri* is responsible for 35% of the total bacterial population, followed by *Brevundimonas* sp. (21%), and *A. lusatiense* (17%). Previous surveys have shown that *R. sanguineus* (sensu lato) ticks were mainly infected by the pathogenic bacterial genera *Coxiella* and *Rickettsia* and *Bacillus* (René-Martelle et al. 2017), *Francisella, Anaplasma, Borrelia* and *Ehrlichia* (Greay et al. 2018b). The abundance of pathogen bacterial in the digestive tract of ticks is presumably related to the differences in the structure of the microbiota, based on the tick's genotype, geographical origin, and life cycle of ticks (nymphs, adult males and females) (René-Martellet 2017).

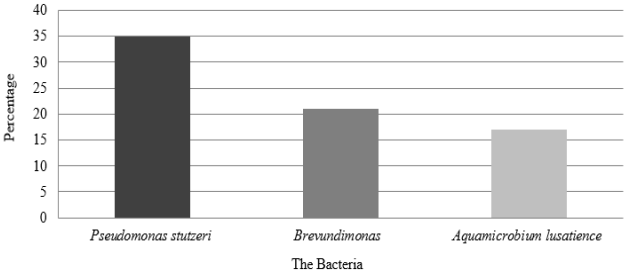

Figure 4. The percentage of bacterial in the digestive tract of *Rhipicephalus sanguineus* ticks

Table 5. The specimens sequenced for this study with collection locality and NCBI GenBank accession numbers

Species name	Locality	Homology	No. accession
Pseudomonas stutzeri strain SGAir0442	Singapore	99.93%	CP025149.2
Pseudomonas stutzeri strain 1W1-1A	China	99.93%	CP027664.1
Pseudomonas stutzeri strain SLG510A3-8	China	99.93%	CP011854.1
Pseudomonas stutzeri strain 2020WEIHUA_G	China	99.93%	CP062162.1
Pseudomonas stutzeri strain XX1.	China	99.93%	CP091174.1
Pseudomonas stutzeri strain B-38-7.	India	99.93%	OK618380.1
Pseudomonas stutzeri strain B-34-6.	India	99.93%	OK618369.1
Pseudomonas stutzeri strain GR1.4.	Indonesia	99.93%	MZ596191.1
Pseudomonas stutzeri strain GR 114.	Indonesia	99.93%	MZ508319.1
Pseudomonas stutzeri strain ZH-1.	China	99.86%	KM278988.1

Table 6. The specimens sequenced for this study with collection locality and NCBI GenBank accession numbers

Species name	Locality	Homology	No. accession
Aquamicrobium lusatiensese strain XJ-9.	China	98%	KU525645.1
Aquamicrobium lusatiensese strain XJ-3.	China	97%	KU525640.1
Aquamicrobium lusatiensese strain 854/1.	Germany	98%	AM884147.1
Aquamicrobium lusatiensese strain ADC-22.	Greece	98%	KM210272.1
Aquamicrobium lusatiensese strain P4N-04.	Korea	98%	MK396598.1

Table 7. The specimens sequenced for this study with collection locality and NCBI GenBank accession numbers

Species name	Locality	Homology	No. accession
Brevundimonas sp. strain EN14ES7.	Poland	99%	KY486816.1
Brevundimonas sp. 266XY5.	China	100%	KF818659.1
Brevundimonas sp.	China	99%	CP039382.1
*Brevundimonas*sp. FrW-Asx16.	Egypt	99%	LC324685.1
Brevundimonas sp. FrW-Asx5.	Egypt	99%	LC324684.1
Brevundimonas sp. GW460-12-10-14- LB2.	Poland	99%	KY486816.1

Pseudomonas stutzeri is a specific strain of the genus *Pseudomonas*, rod-shaped cells, 1-3 µm long and 0.5 µm wide with a single flagellum. The widespread geographic distribution in nature, moist areas found in water and soil, occupies diverse ecological niches and has been isolated as an opportunistic pathogen of humans (Lalucat et al. 2006). Osteomyelitis, arthritis, bacteremia, endocarditis, endophthalmitis, pneumonia, empyema, urinary tract infections, and meningitis are human infections caused by *Pseudomonas* species (Bennett 2020; Goldman 2020). Humans can be exposed in various ways because these bacteria also grow on fruits and vegetables, or in humid public areas such as public baths, bathrooms, kitchens, and sinks (Bhargav 2016).

The genus *Brevundimonas* has first proposed by Segers et al. (1999), *Brevundimonas* species are aerobic Gram-negative, oxidase and catalase positive, non-fermenting rods 1 to 4 mm in length and 0.5 mm in width, they are aerobic with optimal growth temperatures of between 30-37°C, belonging to the *Alphaproteobacteria* class and *Caulobacteraceae* family. The group can survive in a wide variety of environments including different water sources (aircraft water, bottled water, hospital water, purified water) (Handschuh et al. 2017), and usually resistant to a wide array of antimicrobials (Flores-Trevino et al. 2014). The bacteria can infect patients or individuals with underlying medical conditions and diseases (co-morbidity), *Brevundimonas*-associated bacteriemia conditions (Swain and Rout 2017), urinary tract infections, empyema, and foot ulcers (Chandra et al. 2017; Sánchez-Montes et al. 2021). The scientific literature showed multiple types of infections resulting from *Brevundimonas* spp., it indicates that the genus may be more widespread pathogen, invasive and severe (Ryan and Pembroke 2018).

The genus *Aquamicrobium* was first proposed by Bambauer et al. (1998), and its still relatively new bacterial species. Currently, 7 species in the genus have been isolated and taxonomically identified, which were *Aquamicrobium defluvii*, *Aquamicrobium aerolatum*, *Aquamicrobium ahrensii*, *Aquamicrobium segne*, *Aquamicrobium aestuarii*, *Aquamicrobium soli*, *Aquamicrobium terrae*, and *A. lusatiense*. Interestingly, all members have been isolated from pollutant-loaded environments such as wastewater treatment plants, activated sewage sludge, and biofilters. It is a gram-negative bacteria, aerobic bacteria, this genus is found in the activated sludge, air, and chemical industry center, and can degrade thiophene-2-carboxylate, petroleum, and polychlorinated biphenyls (PCB). The degradation abilities

of some strains have been investigated for specific pollutants (Wu et al. 2021).

The habitat of *R. sanguineus* tick is endophilic (living indoors), but the tick also survives in the environment (Dantas-Torres 2010). The tick's habitat is composed of a variety of living and non-living things in the space in which it lives. Ticks are adapted to two contrasting components of their habitat, the physical environment and their host (Barker and Walker 2014).

BLAST analysis indicated that isolates samples from Matuari belong to *Pseudomonas* genus. The 16S rRNA sequence of isolate Matuari is 99.86-99.93% identical to that of *P. stutzeri* from Singapore, China, India, and Indonesia. Based on data in Table 6, thus it can be concluded that the bacterial isolates of Girian samples showed 97-98% homologous with *Aquamicrobium* sp. from China, Germany, Greece, and Korea. Based on the BLAST analysis, it was concluded that the bacterial isolate from Aertembaga showed 99-100% homologous with *Brevundimonas* sp. from Poland, China, and Egypt. The species found have never been reported. *P. stutzeri*, *Brevundimonas* sp., and *A. lusatiense* are pathogens potentially infected humans.

In conclusion, the study shows the bacterial in the digestive tract of *R. sanguineus* (sensu lato) ticks using the V3-V4 hypervariable region of the 16S rRNA gene marker. *P. stutzeri*, *Brevundimonas* sp., and *A. lusatiense* dominated most of the species. This study can provide data on the pathogenic bacteria in *R. sanguineus* (sensu lato) ticks. As the result, the study provides scientific information of bacteria in the ticks through a metagenomic approach. However, further research will be required to determine the specific relationship between ticks, host, and ecological.

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