The diversity and composition of new pathogenic bacteria in cat fleas

by Mokosuli Yeremia

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Research Article

"The diversity and composition of new pathogenic bacteria in cat fleas."

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ABSTRACT
Ctenocephalides felis is specific ectoparasites in cats. As a pet, C. felis has the potential not only to transmit pathogenic bacteria in cats but also in humans. Metagenomic research on the digestive tract and body surface of C. felis has been conducted. DNA genomics was extracted from the body surface and digestive tract of C. felis. Metagenomic analysis has using the 16S rRNA gene (V3-V4 region). Sequencing was carried out using New Generation Sequencing at the First BASE Laboratory, Singapore. Metagenomic analysis has found 15 families and 19 bacterial genera. The results of this study become an important reference pathogenic bacteria that have the potential to be transmitted to humans and animals through C. felis. In the future, it is necessary to study the resistance of bacteria isolated from C. felis to antibiotics.

Keywords: metagenomic, 16S rRNA, Ctenocephalides felis, bacteria diversity

INTRODUCTION

Microbes that are transmitted from animals to humans and cause many new diseases have been reported recently. Cats are pets that are widely spread in the world. However, many cats live wild around human settlements. Ctenocephalides felis are ectoparasites in cats. C. felis is the most widely spread ectoparasites on earth. This is caused by human migration which also brings domestic cats to various regions of the world. Previous studies of C. felis were associated with various types of bacteria. Some bacterial species have been isolated from C. felis from manado on agar nutrient media, showing resistance to some antibiotics (Rombot et al., 2019). Previous studies have identified bacterial isolates from C. felis in Manado to be resistant to 14 types of antibiotics. However the position of species of bacteria remain unclear.

Insects have carried out symbiotic coevolution with various types of bacteria (Kanan et al., 2020; Rotty et al., 2018) Metagenomic analysis has made it possible to examine the diversity of symbiotic and non-symbiotic bacteria in insects, especially species of bacteria that cannot be cultured in a laboratory (Oliveira et al., 2017) (Simandjuntak & Samuel, 2018). However, the genome and behavior of these bacteria is the object of very deep biological studies and holds a mystery. Metagenomic exploration in insects has been carried out lately. Several species of insects have been performed metagenomic analysis including Apids (De Clerck et al., 2015) (de clerck et.al., 2015), Megaphragma amalphitanum (Nedoluzhko et al., 2017) (Nedoluzhko et al., 2017), blowflies and house flies (Junqueira et al., 2017), (Singh et al., 2012) (Junqueira et.al. 2017; Singh et al. 2015), Dendroctonus valens and D. Mexicanus (Hernández-García et al., 2018) (Hernández-García et.al. 2018). All metagenomic research results on these insects are reported to be genera and bacterial species that have never been known to be associated with insects before. 16s RNA proved to be very good in identifying bacteria, especially eubacteria (Suddin et al., 2019).

Research has been conducted to treat and obtain the bacterial composition of cat fleas with a metagenomic approach. Metagenomic identification of bacteria is expected to break the bacterial species found on the surface of the body

and digestive tract of C. felis. Knowing the diversity of bacterial species in C. felis is very important for preventing the transmission of pathogenic bacteria and knowing the species of bacteria that have the opportunity to infect humans in the future.

MATERIALS AND METHODS

Samples

C. felis cat fleas were obtained from several areas in the city of Manado. Morphological identification was carried out in the biology laboratory of Manado State University. Samples were prepared with 70% alcohol before being used for total DNA genomics extraction.

Fig.1:Ctneocephalides felis samples from Manado North Sulawesi, Indonesia

Extraction of DNA genomic

Total DNA genome from the body and digestive tract C. felis was extracted using the CTAB / SDS method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng / µL using sterile water.

Amplicon Generation

16S rRNA genes of distinct regions 16S V3-V4 were amplified used specific primer with the barcode. The primer was use follow :

Hypervariabel region	Primer	References
V3	28F: 5'-GAGTTTGATCNTGGCTCAG-3' ; 519R: 5'-GTNTTACNGCGGCKGCTG-3'	Suchodolski et al. (2012) (Suchodolski et al., 2012) Turner et al. (1999) (Turner et al., 1999)
V4	515F : 5'-GTGCCAGCMGCCGCGG-3' 907R : 5'-CCGTCAATTCMTTTRAGTTT-3'	Lane et al. (1991) (Weisburg et al., 1991)

All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

PCR Products quantification and qualification

Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400-450bp were chosen for further experiments. The quality and quantity of the V3V4 amplicons were measured using Tapestation 4200, picogreen and nanodrop. All the samples passed the QC measurement and proceed straight for 2nd PCR-step of the library preparation.

PCR Products Mixing and Purification

PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries generated with TruSeq® DNA PCR-Free Sample Preparation Kit and quantified via Qubit and Q-PCR, would be analysed by HiSeq2500 PE250.

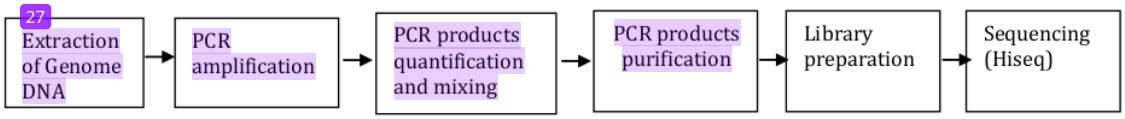

Fig.2: Metagenomic research workflow

The quality of the libraries was measured using TapeStation4200, Picogreen and qPCR. All libraries passed the QC measurement. The library was then pooled according to the protocol recommended by the Illumina and proceed straight to sequencing using MiSeq platform at 2x301PE format. The libraries were prepared using Illumina 16s metagenomics library prep kit and their quality and quantity were determine using Agilent Tapestation 4200 and Picogreen (Appendix 1).

New Generation Sequencing of DNA

Amplicon was sequencing on Illumina HiSeq paired-end platform to generate 250 bp paired-end raw reads (Raw PE), and then assembled and pretreated to obtain Clean Tags.

Data analysis procedures

For the veracity of sequencing data analysis, raw data would be merged and filtered to get clean data. The effective data is used to do OTU cluster and species annotation for the respective sequence of each OTU. Thus the relative species, evenness and abundance distribution can be analyzed with Alpha diversity, Beta diversity according to the results of Singapore's First BASE metagenomic sequencing analysis.

RESULTS AND DISCUSSION

Results

a. Extraction and Purification of DNA genomics

The results of genomic DNA extraction of C. felis obtained a concentration of 75 ng / ul were detected with Picogreen while detection using Nanodrop obtained a concentration of 550.18 ng / ul. Furthermore, DNA purity detected by Picogreen was 1.75 (A260 / A280) while with Nanodrop 0.79 (A260 / 230) (Table 1). The results obtained can be continued for amplification of the 16S rRNA gene. Application of the 16S rRNA gene was carried out in regions V3 and V4 (Table 1).

Table 1. Genomic DNA extraction of Ctenocephalides felis.

No	Sample code	Picogreen (ng/ul)	Nanodrop (ng/ul)	A260/A280	A260/A230	Description
1	RN1	75	550,18	1,75	0,79	Continuing metagenomic sequencing.

b. Amplification of the 16S rRNA gene by the DGEE PCR method.

The results of the measurement of the quality and quantity of the V3 and V4 amplicons with Tapestation 4200, picogreen and nanodrop obtained by the concentration of the amplicon was 44.2 ng / uL with a library size of 573 bp (Table 2).

Table 2. Library concentration and size of the amplified 16S rRNA gene of Ctenocephalides felis.

No	Sample	Concentration (ng/uL)	Molarity (nM) qPCR method	Library Size Tape Station 4200	Description
1	RN1	44,2	2,97	573	Continuing metagenomic sequencing.

Electrogram visualization of the 16S rRNA gene amplicon (regions V3 and V4) of the cat flea templete DNA obtained by the detected amplicon at 600 bp to 1000 bp (Figure 3). The size of the library obtained is 573 bp.

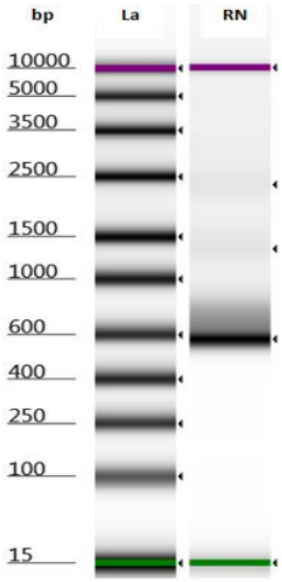

Fig.3: Electrogram PCR 16S rRNA gene (Description: bp = length (base pair); La: ladder, RN = C. felis sample from Manado City)

The quality of the 16S rRNA gene library was measured using Tape Station 4200, Picogreen and PCR. All libraries pass quality control measurements. The library is then collected according to the protocol recommended by Illumina and proceed directly to sequencing using the MiSeq platform in the 2x301PE format. The measurement results obtained by the 16S rRNA gene amplification products region V3 and V4 showed a concentration of 75 ng / ul on picogreen while 550.18 on nanodrop (Table 4). These results are based on the NGS First Base metagenomic sequencing requirements that meet the metagenomic sequencing standards.

Table 4. The concentration of 16S rRNA amplicon of V3 dan V4 region

Sampel	Concentration (ng/ul)		Pass/fail
	Picogreen	Nanodrop	
RN	75	550,18	Pass

c. Library Construction

The results of library construction obtained 573 library size with a concentration of 44.2 ng / ul (Table 5). Based on the NGS First Base standard, it is stated that it can be continued for metagenomic sequencing.

Table 5. The Library size 16S rRNA region V3 and V4

Sample	Library Size	Picogreen	Molarity (nM) qPCR method	Pass/fail
RN	573	44,2	2,97	Pass

The cluster generation describes the stages of metagenomic sequencing that have been carried out. Metagenomic sequencing was carried out with Singapore's First Base NGS (Table 6).

Table 6. The summary of cluster generation sequencing gen 16S rRNA region V3 dan V4

Level	Yield Total (G)	Projected Total Yield (G)	Aligned (%)	Error Rate (%)	Intensity Cycle 1	% > 0,30
Read 1	5,19	5,19	5,39	1,98	65	75.09
53 read 2 (1)	0,12	0,12	0	0	825	90,72
Read 3 (1)	0,12	0,12	0	0	501	93,76

Read 4	5,19	5,19	5,31	3,33	50	66,13
Non-Indexed Total	10,38	10,38	5,35	2,66	58	70,51
Total	10,62	10,62	5,35	2,66	360	71,1

d. Classification of bacteria as a result of metagenomic sequencing.

Based on metagenomic analysis obtained 15 bacterial families found on the surface of the body and digestive tract of C. felis from the city of Manado, North Sulawesi, Indonesia. The scale of the number of families based on the color on dendrogram. The family with the most species members is Rickettiaceae. Whereas the family with the smallest number of species members has Burkhoideriaceae (Figure 4 and Figure 5). The results of metagenomic analysis at the genus level obtained 19 genera of bacteria. As in the family level dendogram at the genus level the number of families is also based on color. The genus with the highest number of species is Wolbachia (Figure 4). The genus of bacteria with the smallest species members is Burkholderia.

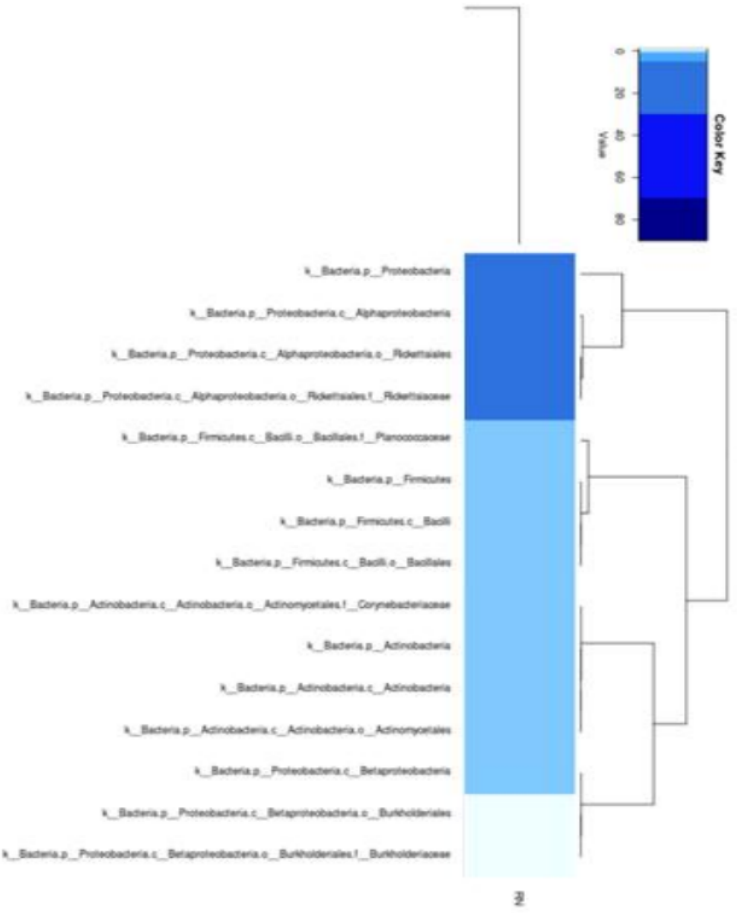

Fig.4:The familiy level dendogram results from metagenomic analysis.

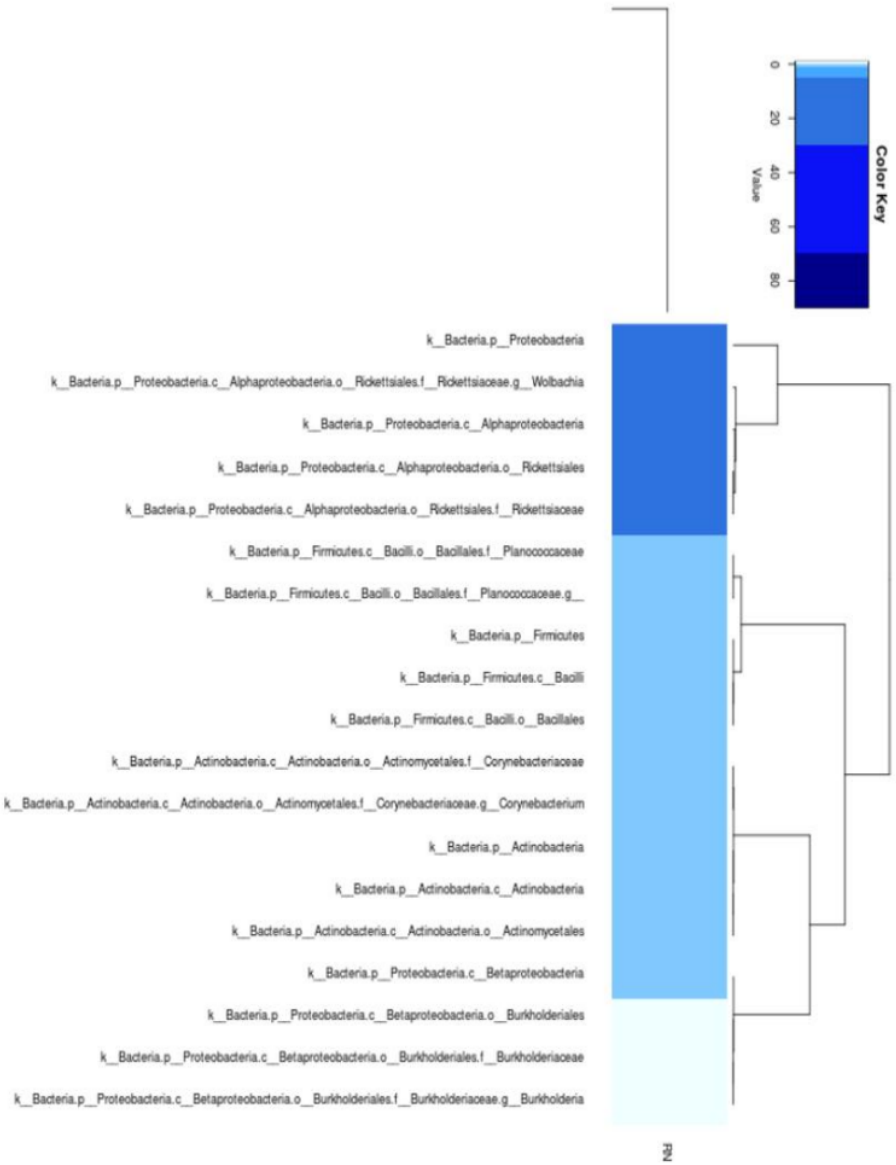

Fig.5:Genus level dendogram results from metagenomic analysis.

Description: The dark blue color indicates the number of families between 25 to 80, the light blue color indicates the number of families 5 to 30, while the blue color is almost white the number of families between 0 to 5.

The results of chrona analysis showed that the composition of bacteria in C. felis, 90% included in the genus Wolbachia, 88% of the Rickettsiaceae family and 0.1% could not be ascertained its taxonomic position (Figure 6).

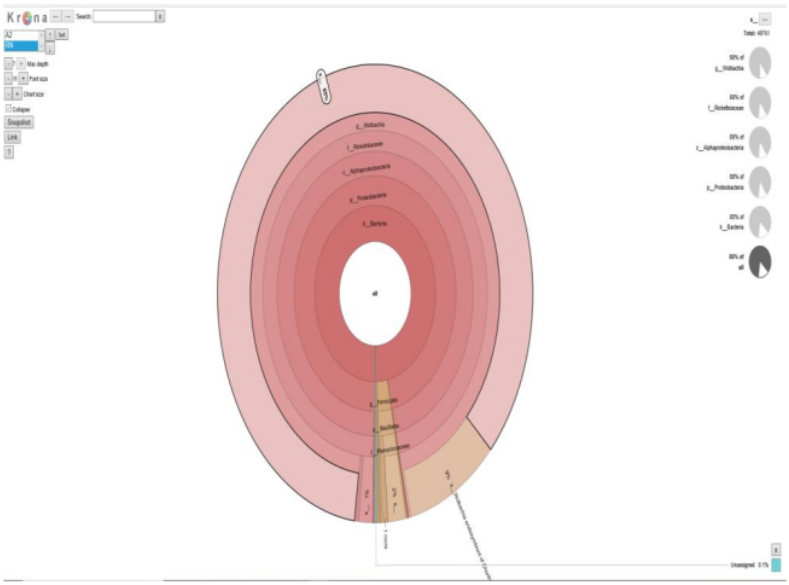

Fig.6:The Crona analysis of Metagenomic bacteria from C. felis

Phylogeny

The phylogeny tree from the C. felis metagenomic analysis was shown at the genus and family level. At the level of the family C. felis is given the symbol x (RN) while the comparison / control is symbolized Δ (A2). Rickettsiacea is the most abundant family in the body and digestive tract of C. felis from Manado. Besides Rickettsiaceae, Burkhoidericeae, Pseudomonadaceae, Planoccocaceae, Corrynebacteriaceae, Caulobacteriaceae, Isosphaeraceae, and Staphylococcaceae (Figure 7) were found.

Family level phylogeny tree construction produces four monophyletic branches. The four monophyletic branches consist of three branches namely Rickettsiaceae and one branch consists of several families namely Burkhoidericeae, Pseudomonadaceae, Planoccocaceae, Corrynebacteriaceae, Caulobacteriaceae, Isosphaeraceae, and Staphylococcaceae (Figure 7). Rickettsiaceae dominates bacteria found on the surface of the body and in the digestive tract of C. felis fleas in the city of Manado.

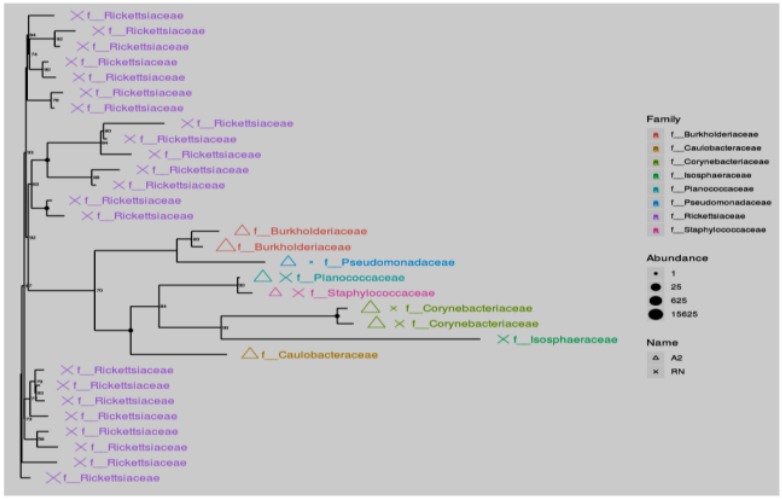

Fig.6:The phylogeny tree at family level

At the genus level, four monophyletic groups are formed. As at the family level, three monophyletic groups belong to the Rickettsiaceae family. The first monophyletic group is the genus Wolbachia, the second monophyletic group has Rickettsia, Wolbachia and one genus that is unknown or has not been recorded in the NCBI gene bank. The third monophyletic group has the genus Burkhoideria, Pseudomonas, Staphylococcus, Corynebacterium, and 3 genera that have not been recorded in the NCBI gene bank. The fourth monophyletic group consists of only one genus, Wolbachia (Figure 8). The results of this study found that there was a genus of four bacteria that existed on the surface of the body and / or the digestive tract of C. felis from Manado whose genus name was unknown and had not even been reported.

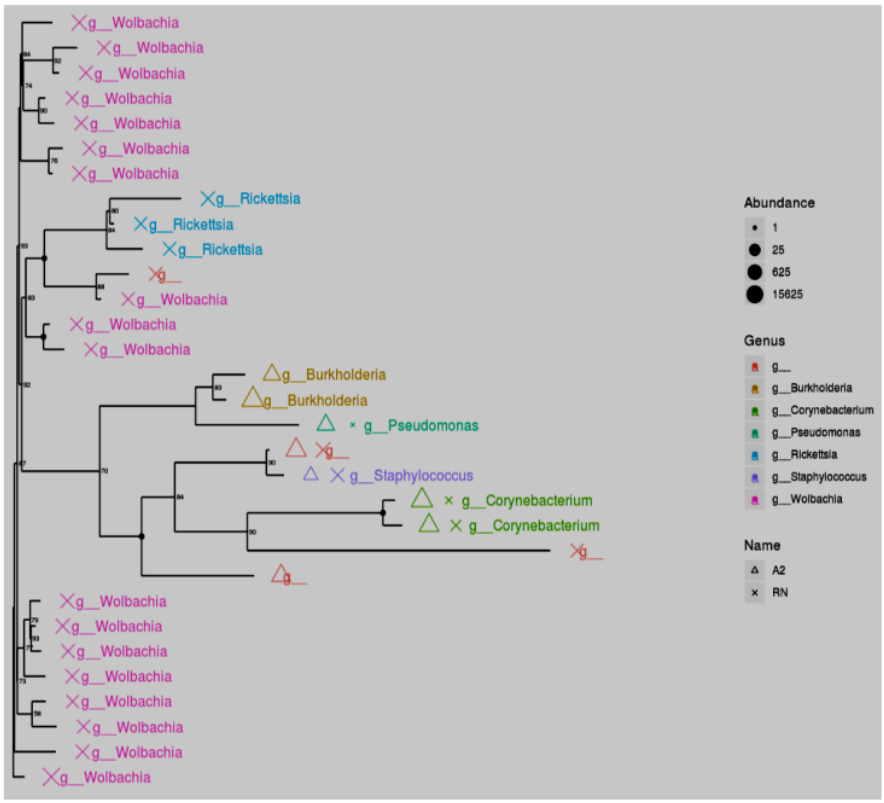

Fig.8:The phylogeny tree at genus level

DISCUSSION

Rickettsia spends part of its life cycle on Arthropods, then transmitted to humans or other mammals through arthropod bites including ticks and mites (Gupta & Mok, 2007), (Stenos & Walker, 2000) (Gupta and Mok, 2007; Stenos and Walker, 2000). Metagenomic analysis of C. felis in this present study found Rickettsiaceae as the most dominant bacteria. Rickettsia is a gram-negative bacterium that is very sensitive to environmental exposure, so it lives obligate through intracellular infection. Rickettsia bacteria are in the digestive tract or internal organs. Rickettsia rickettsii is an example of a prototype species that infects many organisms. R. felis was identified as being a common cause of fever in Africa. Knowledge of the characteristics of R. Felis is still very limited (Brown & Macaluso, 2016) (Brown et.al. 2016).

Research conducted by (Horta et al., 2007) Horta, et al., (2007) in Brazil C. felis felis using molecular markers of the gltA, htrA, ompA and ompB genes, found Rickettsia in all phases of the C. felis life cycle starting from eggs, larvae , cocoon, adult male and adult female. Each phase of the C. felis life cycle potentially transmits bacteria from the genus Rickettsia. Some family and genus of bacteria found in this study have never been reported in cat fleas. The family that has never been reported in C. felis is

Burkholderiacea. The Burkholderiacea family has several pathogenic species namely Burkholderia mallei which causes glanders and Burkholderia pseudomallei which causes meliodosis (Sawana et al., 2014) (Sawana, et al., 2014). Furthermore, cat fleas are also the main vector of Bartonella. About 17 new Bartonella species have been reported (Gutiérrez et al., 2015) (Gutiérrez et.al. 2015).

The results of this study indicate that a new family and genus of bacteria were first reported in cat fleas. The genus has many species of species that are pathogenic in humans, namely the genus Staphylacoccus, Corynobacterium, and the family Plannococcaceae. It is necessary to study more deeply the potential of C. felis to transmit other pathogenic bacteria to humans.

CONCLUSION

From the results of this study it can be concluded that the dominant bacterial genus in cat fleas is Wolbachia. There is an identified genus of pathogenic bacteria associated with C. felis that has never been reported. These genera have many species of pathogens in humans.

Conflict interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

We would like to acknowledge the chair and staff of the bioactivity and molecular biology laboratory, State University of Manado, for assisting in the identification of molecular bacteria.

Authors' Contributions

All authors designed the experimentsAll authors are involved starting from the preparation of research designs, research, data analysis, discussion, writing journal articles. All authors read and approved the manuscript.

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Data Availability

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

Ethics Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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The diversity and composition of new pathogenic bacteria in cat fleas

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