Molecular Identification of Pathogenic Bacteria in *Sarcophaga peregrina* Larvae as a Cause of Ophthalmomyiasis

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Abstract
Ophthalmomyiasis, a rare but severe parasitic infestation of the eye, is commonly associated with fly larvae, particularly *Sarcophaga peregrina*. This study aims to identify pathogenic bacteria present in *S. peregrina* larvae, which may exacerbate infections in ophthalmomyiasis cases. Samples were collected from high-risk areas in Bitung and Manado, Indonesia, where fly populations thrive in human and livestock environments. Molecular identification of the larvae was conducted using COI gene sequencing, while bacterial identification employed 16S rRNA analysis. The results confirmed *S. peregrina* as the primary fly species responsible for ophthalmomyiasis. Bacterial analysis revealed the presence of *Stenotrophomonas maltophilia*, *Bacillus subtilis*, *Proteus mirabilis*, and *Enterobacter hormaechei*, all of which are known to contribute to secondary infections and antimicrobial resistance. The study highlights the role of *S. peregrina* as both a direct cause of myiasis and a vector for pathogenic bacteria, emphasizing the need for early detection and preventive measures. The findings have significant implications for public health, particularly in tropical and subtropical regions where poor sanitation and high fly populations increase the risk of infection. Improved hygiene, rapid bacterial identification, and targeted antimicrobial treatments are essential to reducing the morbidity associated with ophthalmomyiasis. Future research should explore the full spectrum of microbial interactions within *S. peregrina* to enhance control strategies and therapeutic approaches.
Keywords: Ophthalmomyiasis, *Sarcophaga peregrina*, Pathogenic Bacteria, Molecular Identification

INTRODUCTION

Myiasis is the infestation of fly larvae in vertebrate tissues, including humans, and is classified based on the affected body region. One of the more severe but rare forms is ophthalmomyiasis, where fly larvae infest the eye, either on the surface or within the intraocular space (Ayalon et al., 2020). Cases have been reported in several countries, often linked to the presence of fly larvae in local environments.

Human infestations are more common in rural areas, particularly among communities living near livestock, leading to zoonotic transmission. Myiasis is more prevalent in tropical and subtropical regions, such as Indonesia, due to poor hygiene, ecological factors, and the abundance of susceptible fly and animal populations (Ślapeta, 2022; Pather et al., 2013). Environmental conditions, such as high humidity and warm temperatures during the rainy season (September–November), favor fly reproduction and increase infestation risks (Partoutomo, 2000).

The incidence of myiasis is also influenced by individual risk factors, including malnutrition, chronic illnesses, mental disorders, and immunosuppression (Hugo et al., 2003). Elderly individuals (>60

years) are more susceptible, and cases occur in both urban and rural areas, with variations based on local hygiene and living conditions (Jokar, 2022). Between 1997 and 2017, 464 cases of human myiasis were reported globally, involving 41 fly species, though most cases (99.4%) were caused by a single species (Benhardt et al., 2019). Ophthalmomyiasis accounts for less than 5% of total human myiasis cases, with 312 cases recorded worldwide as of December 2022 (Bada et al., 2023; Hugo et al., 2023).

In Indonesia, human ophthalmomyiasis cases remain underreported, but livestock infestations are frequently documented in North Sulawesi, South Sulawesi, East Nusa Tenggara (NTT), and West Nusa Tenggara (NTB), increasing the risk of transmission to humans (Partoutomo, 2000). Identifying the species responsible is crucial for understanding infestation mechanisms, determining treatment strategies, and developing preventive measures. Ophthalmomyiasis is classified into external, internal, and orbital forms, with orbital ophthalmomyiasis being the most severe, potentially leading to blindness or fatal complications if the larvae invade the central nervous system (Pauline et al., 2022; Sigauke et al., 2003).

Several fly species are implicated in ophthalmomyiasis, including Oestrus ovis, Dermatobia hominis, Musca domestica, Hypoderma tarandi, Sarcophaga spp., and Chrysomya bezziana (Shankar et al., 2012; Wakamatsu et al., 2006; Tommy et al., 2013; Lagace et al., 2008; Mitsuo et al., 2005; Khurana et al., 2010). Among these, Sarcophaga peregrina has a high level of human interaction, thrives in waste-rich environments, and is widely distributed in Southeast Asia and China (Lipin et al., 2022).

In addition to larval infestations, bacterial infections often exacerbate the condition. The ocular surface contains normal microbiota, but trauma or infection can disrupt the balance, leading to pathogenic activity. Common bacteria found in ophthalmomyiasis wounds include Staphylococcus aureus, Proteus mirabilis, Pseudomonas, and Moraxella spp. (Islam et al., 2015; Jayaprakash & Kartikheyan, 2019). Genetic identification through PCR can help determine bacterial virulence and guide appropriate treatment (Travis et al., 2018). As a tropical country with high humidity, Indonesia is highly susceptible to fly infestations. Early detection and preventive measures, including sanitation improvements and bacterial identification, are essential in reducing ophthalmomyiasis cases and improving public health outcomes.

MATERIAL AND METHODS

Study Sites

This study was conducted in August 2022 at three locations in North Sulawesi, Indonesia, specifically in the cities of Bitung and Manado. The selected study sites in Bitung included Winenet Market (1º26'45.3" N, 125º10'32.1" E) and a residential house with a chicken coop (1º27'12.5" N, 125º11'05.8" E). In Manado, the study was conducted in the Wenwin residential area (1º29'05.2" N, 124º59'47.6" E) (Figure 1). These locations were chosen based on their high fly population, close proximity to livestock, and environmental conditions that facilitate the development of fly larvae. The experimental procedures, including surgical incisions and the initial sample collection, were carried out at the Laboratory of Prof. R.D. Kandou Hospital. Following these procedures, the test subjects were transferred to their respective observation sites for further study and monitoring.

Figure 1. The locations used for sampling ophthalmomyiasis in Winenet Market, Bitung City and Wenwin residential area, Manado City, North Sulawesi

Sample Collection

This study used Rattus norvegicus (Wistar rats) with an average body length of approximately 25 cm and a weight of 200–300 grams. Wistar rats were chosen due to their genetic, anatomical, and physiological similarities to humans, as well as their high pain sensitivity, making them suitable models for ophthalmomyiasis research.

To induce ophthalmomyiasis and collect samples, sterile incisions measuring 1–2 cm in length and 0.5 mm in depth were made on the eyelid and cornea using a surgical scalpel. After the procedure, the rats were placed in sterile cages for post-operative care. The larvae were collected from the wounds using sterile forceps and stored in sterile containers. Live larvae were sent to the FMIPA Laboratory for bacterial analysis, while preserved larvae were sent to First Base Singapore for molecular identification using the COI gene.

For genetic analysis of the larvae, the DNA barcoding method (COI gene) was employed. DNA extraction was performed using the Genomic DNA Mini Kit (Geneaid) and GS300 (Genomic DNA extraction with gSYNC DNA Extraction Kit). PCR amplification was conducted using KOD FX Neo (Toyobo, KFX-201) with LCO1490 and HC02198 primers. The amplified DNA was analyzed through agarose gel electrophoresis, and sequencing was performed at First Base Singapore. Phylogenetic analysis was conducted using the Neighbor-Joining method, and sequencing data were processed using BLAST and MEGA X, with phylogenetic tree construction done in Geneious v5.6.

Bacterial analysis of the larvae and infected wounds was conducted through bacterial isolation using the spread plate method from Lindquist on Nutrient Agar. Bacterial characterization was performed using Bergey's Manual of Determinative Bacteriology (Brenner et al., 2005), including Gram staining, catalase testing, carbohydrate fermentation tests, motility tests, and oxidase tests. For molecular identification of bacteria using 16S rRNA, DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). PCR amplification was carried out using MyTaq HS Red Mix 2X (Bioline, BIO-25048) and KOD FX Neo (Toyobo, KFX-201). The sequencing was performed at First Base

Singapore through PT. Genetika Science Indonesia. The sequencing data were analyzed using BLAST, and a phylogenetic tree was reconstructed using the Neighbor-Joining method.

RESULTS AND DISCUSSION

Result

The study focused on fly larvae infesting wounds in the eye region, including the palpebra and cornea, using Rattus norvegicus placed in high-human-mobility areas such as markets, residential areas, and homes with animal enclosures. These locations were chosen due to their abundant food sources, attracting flies, and the common history of livestock ownership or animal contact among reported ophthalmomyiasis cases.

The larvae had an oval, elongated shape with pointed ends, measured 1–1.5 cm, and were whitish to yellowish with a segmented body. Morphological identification was challenging due to similarities among species in the Sarcophaga genus, which includes over 3,092 species. Consequently, identification was mainly possible at the family level.

Given the limitations of morphological identification, the study employed DNA-based methods to accurately determine the species. Molecular identification provided greater accuracy, efficiency, and ease of sample preservation and transfer between laboratories.

A. Identification of COI Gene Sequence Characteristics In Bitung Area

Species identification based on morphology is challenging due to structural similarities and limited taxonomic literature. Therefore, a molecular approach using Cytochrome c Oxidase Subunit I (COI) gene was employed for species identification. The COI gene, found in the mitochondrial DNA of flies, has been proven effective in identifying species within the Sarcophagidae family. In this study, COI gene amplification from Bitung larval samples produced a 550 bp fragment, which aligned with previous studies, such as 465 bp (Sharma, 2015) and 555 bp (Guo, 2012). BLAST analysis with the GenBank NCBI database revealed a 99% similarity to S. peregrina (accession number AF259509.1), a species previously reported in China and associated with human myiasis cases. The identified larvae showed close evolutionary relationships with species found in the Indomalayan region, including Thailand, Korea, and Malaysia. A phylogenetic tree constructed using the Neighbor-Joining method indicated that the larvae formed a distinct monophyletic clade, while maintaining evolutionary similarities with larvae from China. These findings confirm that Sarcophaga species thrive in tropical environments near human habitation, making them potential disease vectors (myiasis) and significant in forensic entomology.

Table 1. BLAST Results of Larval Samples from Bitung

BLAST Identification	Similarity Level (%)	Accession Number
S. peregrina COI gene partial	99.71	AF259509.1
S. peregrina isolate CSU150701CS5	99.56	KY001833.1
S. peregrina COI gene	99.56	JN604569.1
S. nathani	99.42	MN255738.1
S. highlandica	99.42	JF500463.1
S. peregrina mithondrion complete genome	99.42	KF921296.1
S. forinosensis mithocondrion complete genome	99.27	MF688548.1
S. peregrina isolate Fg4	99.27	JX861412.1

S. peregrina voucher CSU 130512CS18	99.27	KF037991.1
S. nathani voucher BN26	99.12	KT694985.1

In Manado Area

Larvae samples collected from Manado underwent total genomic DNA extraction using primers LCO1490/HCO2198, resulting in an amplified COI gene length of 500–600 bp with clear band visualization. BLAST analysis showed high genetic similarity with larvae accession numbers JF500465.1 and KC855282.1, closely resembling *S. Bkarnyi* and other larvae identified in Malaysia, including those by Tan (2012) and Siew et al. (2010). Larva KC855282 matched findings from Khoso et al. in Malaysia, which studied synanthropic flies—species living near humans and serving as potential pathogen vectors. Additionally, larva KF921296.1 shared genetic features with *Boettcherisca peregrina*, a forensic entomology species reported by Min Zhong et al. (2014), while larva JX861411.1 was similar to *Sarcophagidae* identified using mitochondrial COI in Korea by Yu Hoon Kim et al. (2014). The phylogenetic tree, constructed using MEGA X with the Neighbor-Joining algorithm, demonstrated taxonomic and systematic implications, confirming that the larvae belonged to the *Sarcophaga* genus, primarily *S. peregrina*, with close evolutionary ties to species from Malaysia, China, and South Korea. The paraphyletic grouping of *S. peregrina* aligned with findings by Tan et al., reinforcing that *Sarcophaga* flies predominantly inhabit tropical and temperate regions, particularly in Southeast Asia, but also extend to subtropical areas like China and Korea. The species' distribution spans tropical and subtropical zones within the Palaearctic, Oriental, and Oceanic regions, further supporting the phylogenetic relationships observed in this study.

Table 2. BLAST Results of Larval Samples from Bitung

BLAST Identification	Similarity Level (%)	Accession Number
S. Bkarnyi Pketam	97.60	JF500465.1
S. peregrina isolate PBS14	97.86	KC855282.1
S. peregrina mitochondrion complete genome	97.46	KF921296.1
S. peregrina Isolate Po3	97.46	JX861411.1
S. krathonmai voucher S-SWK-288	97.45	GU174023.1
S. peregrina COI gene partial	97.45	AF259509.1
S. peregrina isolate CSU150701CS5 COI	97.31	KY001833.1
S. peregrina isolate 25	97.34	KJ496793.1
S. peregrina isolate Po4	97.31	JX361412.1
S. peregrina isolate CSU130512CS8	97.31	KF037991.1

B. Identification of Bacteria in Larval Bodies

The bacterial infection in ophthalmomyiasis results from pathogenic bacterial growth in wounds caused by larval infestation. Diptera harbor various microorganisms at all life stages, especially in their digestive systems, with bacterial species differing from their environmental surroundings (Ravasan et al., 2020). Bacterial isolation was conducted on the larvae's digestive tract to eliminate contamination from external sources. Gram staining revealed mostly Gram-positive bacilli, along with cocci in irregular clusters, while biochemical tests identified *Staphylococcus sp.*, *Bacillus sp.*, and *Stenotrophomonas maltophilia*. Molecular identification using 16S rRNA sequencing confirmed bacterial variants including

B. cereus, *B. thuringiensis*, *B. paranthracis*, *Staphylococcus sciuri*, and *S. maltophilia*. Further analysis showed that the *S. peregrina* digestive tract harbored commensal bacteria from phyla *Proteobacteria*, *Bacteroidota*, and *Firmicutes* (Fengqin et al., 2024), with genera such as *Ignatzschineria*, *Providencia*, *Myroides*, and *Proteus* (Gupta et al., 2014). The predominant *Bacillus* species belong to *Firmicutes*, while *S. maltophilia* is part of *Proteobacteria*, a major Gram-negative pathogenic phylum. Comparisons using BLAST analysis showed strong genetic similarities to bacterial strains reported in previous studies from Sumatra, Sri Lanka, and Bangladesh, highlighting the heterogeneity of *Stenotrophomonas maltophilia* (Caylan, 2004).

Table 3. Bacterial Variants in Larvae

BLAST Identification	Similarity Level (%)	Accession Number
B. cereus	99.25	MG027666.1
S. sciuri strain CB212	100.00	MT527534.1
S. sciuri strain AA1	100.00	MT275460.1
S. sciuri strain CG1	100.00	MT072194.1
S. sciuri strain CT12	100.00	MT072175.1
S. maltophilia strain NCTC10498	100.00	CP049956.1
S. maltophilia strain S-14	100.00	MN732977.1
S. maltophilia strain PEG-173	100.00	CP040439.1
B. subtilis strain ACL103	100.00	CP110634.1
B. subtilis strain KO3	100.00	OP788114.1
B. subtilis strain KO1	100.00	OP788112.1
B. thuringiensis strain TG-5	99.91	CP110109.1

C. Identification of Bacteria on Wound Surface

Bacterial swabs from eyelid and corneal wounds revealed various species, some of which are commensals that became pathogenic, potentially leading to bacteremia. While culture remains the gold standard for identification, 16S rRNA PCR provides higher accuracy, especially for rare bacteria in minimal samples, such as intraocular infections. Gram staining of wound isolates identified Gram-negative bacilli, with biochemical tests confirming *Proteus sp.*, *Enterobacteria sp.*, and *Alcaligenes faecalis*, which secrete β-lactamases contributing to antibiotic resistance. 16S rRNA amplification showed 1500 bp bands with variations in thickness due to DNA concentration, with clearer bands indicating higher-quality extractions. Molecular sequencing identified *Proteus mirabilis* and *Enterobacter hormaechei*, both from *Proteobacteria*, with BLAST results showing high similarity to previously reported strains. *A. faecalis*, commonly found in soil and water, was also detected, with *P. mirabilis* strains matching isolates from previous studies in Shanghai, Singapore, and patients with wound infections (Al-Rubaeaee et al., 2023; Sophie et al., 2019).

Table 4. Bacterial Variants on the Eyelid

BLAST Identification	Similarity Level (%)	Accession Number
E. hormaechei strain BW	99.64	CP027111.1
E. hormaechei subsp steigerwaltii strain ME-1	99.84	CP041733.1
E. hormaechei strain 2013	99.64	CP031565.1
E. hormaechei isolate EC-T080	99.84	LS999206.1

E. hormaechei subs xiangfangensis strain OSUVMCKPO4-2	99.84	CP029246.1
E. hormaechei subsp charae	99.79	AJ853889.1
E. hormaechei strain AMS-38	99.79	CP051132.1
E. hormaechei strain BW	99.79	CP023569.1
E. hormaechei strain C44	99.79	CP042566.1
P. mirabilis strain CRPM10	100.00	CP043332.1
P. mirabilis strain ENT1301	100.00	CP044135.1
P. mirabilis strain MPE-5203	100.00	CP053685.1
P. mirabilis strain MPE-0027	100.00	CP053683.1
P. mirabilis strain JPM24	100.00	CP053894.1

Table 5. Bacterial Variants on the Cornea

BLAST Identification	Similarity Level (%)	Accession Number
A. faecalis strain BJD1	100.00	MT378145
A. faecalis strain OsEnb HZB H11	100.00	MN889404
A. faecalis strain MUB14	100.00	CP048039.1
A. faecalis strain SNH-K76	100.00	MN493925.1
P. mirabilis strain WTP140	99.36	MH396753.1
P. mirabilis strain MPE4059	99.36	CP053718.1
P. mirabilis strain MPE0027	99.36	CP053683.1
P. mirabilis strain MPE0346	99.36	CP053719.1
P. mirabilis strain MPE0767	99.36	CP045257.1
P. mirabilis strain JPM24	99.36	CP053894.1
P. mirabilis strain STP3	99.36	CP051260.1

Discussion

The identification of Sarcophaga peregrina (Diptera: Sarcophagidae) as the primary fly species responsible for ophthalmomyiasis in this study aligns with its feeding behavior, which requires nutrient-rich environments for larval survival. S. peregrina thrives in human-inhabited areas with abundant organic waste, particularly in chicken coops, where larvae feed on food scraps, broken eggs, and accumulated waste (Lipin et al., 2022). This species plays an essential role in decomposition within the ecosystem, functioning as a natural recycler.

With over 3,000 identified species worldwide, Sarcophagidae flies are predominantly found in tropical and subtropical regions, particularly in Southeast Asia (Wang et al., 2017). S. peregrina has been reported in Japan, Korea, China, India, Australia, and Africa, but it is most frequently observed in Indonesia and neighboring countries. The species thrives optimally at temperatures between 22–30°C, while lower temperatures extend the pupal stage (Lipin et al., 2022). Due to its frequent interaction with humans, S. peregrina has significant implications in public health as a myiasis-causing agent and in forensic entomology for estimating time of death.

Previous reports have identified multiple Sarcophaga species as ophthalmomyiasis agents, including S. crassipalpis, S. peregrina, S. haemorrhoidalis, and S. bullata. The lifecycle of S. peregrina consists of three developmental stages—larva, pupa, and adult fly—due to its ovoviviparous reproduction, where females deposit live larvae directly onto hosts or decaying organic matter (Szpila et al., 2015). The larvae undergo three instar stages, with third-instar larvae leaving the host to pupate in soil before emerging as adult flies (Shang et al., 2019).

The pathogenesis of ophthalmomyiasis results from both direct tissue damage caused by larval movement and secondary bacterial infections. The larvae invade deeper tissue layers, leading to complex tissue destruction and inflammation (Can et al., 2024). The severity of ophthalmomyiasis-related complications is heightened by bacterial colonization, which exacerbates inflammation and can lead to corneal ulcers, endophthalmitis, or permanent vision loss.

The variations in bacterial composition observed in this study can be attributed to environmental influences, larval feeding habits, and microbial adaptation mechanisms within S. peregrina. Bacterial strains isolated from the larval gut and wound surfaces indicate significant host-pathogen interactions, with Gram-negative bacteria being the dominant group associated with pathogenicity. Among the identified bacterial species, Proteus sp. accounted for 35%, followed by Enterobacter sp. (21%) and Alcaligenes faecalis (17%). These results align with previous studies identifying Proteus, Enterobacter, and Stenotrophomonas maltophilia as key pathogens associated with fly-borne infections (Gupta et al., 2014).

The bacterial diversity detected in S. peregrina larvae suggests that microbiota acquisition depends on habitat, feeding sources, and host contact. The presence of opportunistic pathogens such as Staphylococcus sciuri and Bacillus subtilis in the larval gut indicates that flies act as reservoirs for antimicrobial-resistant bacteria. Studies on fly microbiomes have confirmed that Sarcophagidae species frequently harbor Proteobacteria and Firmicutes, which dominate their digestive tracts (Fengqin et al., 2024). In comparison, studies in Thailand and Malaysia reported Coxiella and Rickettsia as the most prevalent pathogens in flies (Khalili et al., 2018; René-Martelle et al., 2017). This study, however, found a higher prevalence of Enterobacteriaceae, highlighting geographical and ecological differences in bacterial composition.

BLAST analysis confirmed that bacterial isolates from this study closely matched strains detected in clinical and environmental settings. P. mirabilis (100% similarity, accession CP043332.1) has been linked to nosocomial infections, while E. hormaechei (99.84% similarity, accession CP041733.1) is associated with hospital-acquired bacteremia (Tanim et al., 2021). The genetic similarity of bacterial isolates from Sumatra, Sri Lanka, and Bangladesh further supports the hypothesis that fly-associated infections contribute to regional public health concerns (Delfiani et al., 2020).

The ability of S. peregrina to thrive in tropical climates and livestock-dense areas increases the risk of ophthalmomyiasis, particularly in communities where livestock enclosures are near human residences. The species' ovoviviparous reproductive strategy enables rapid larval deposition on wounds, accelerating tissue destruction and secondary bacterial infections. Additionally, the infestation process is exacerbated by maggot-secreted enzymes, including maggot kinase (MK), which disrupts coagulation and delays wound healing. These biochemical interactions between larvae and bacterial pathogens explain the severe necrotic tissue damage and inflammatory response observed in ophthalmomyiasis cases.

The presence of multidrug-resistant bacteria in S. peregrina highlights the importance of early detection and intervention strategies to mitigate ophthalmomyiasis risks. Further research on bacterial virulence factors, antimicrobial peptides, and fly-associated disease transmission is essential to developing effective control measures. Given the increasing clinical significance of antimicrobial-resistant pathogens, studies on fly microbiomes and their implications for human health remain a critical area of scientific investigation.

CONCLUSION

This study identifies *Sarcophaga peregrina* as a key cause of ophthalmomyiasis, emphasizing its close association with human environments and rapid infestation capability. The presence of pathogenic bacteria in larvae, including *Stenotrophomonas maltophilia* and *Bacillus spp.*, along with *Alcaligenes faecalis*, *Proteus mirabilis*, and *Enterobacter hormaechei* on infected eye surfaces, suggests a significant role of secondary infections. Some commensal bacteria, like *Staphylococcus sciuri*, can become opportunistic pathogens. Ophthalmomyiasis can occur even in sterile conditions, highlighting the

importance of hygiene in prevention. Further research is needed to understand *S. peregrina*'s bacterial microbiome and host interactions for better disease control.

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