Original Article

Molecular Identification of Forensically Important Blowflies By DNA Barcoding to Effectively Determine Postmortem Interval

Sundus Mona,[1] Muhammad Zahid Bashir,[2] Abdul Ahad,[3] Aimal Nasrullah,[4] Muhammad Mubashir,[5] Manal Mubarak,[6] Mudassar Rashid[7]

Abstract

Objective: To identify blowflies by molecular method of DNA barcoding and measure post mortem interval by studying their life cycles and calculating time since death retrospectively.

Method: 200 larvae were collected from different cadavers from mortuaries of Lahore that were brought for autopsies from different parts of the city. After DNA extraction, CO1 region of 658bp was amplified using suitable primers. Sanger sequencing was done and all sequences were submitted to BLAST for molecular identification. These sequences were then submitted into BOLD for sequence comparison.

Results: Two species of blow flies were identified Chrysomya megacephala in 142 samples and Chrysomya rufifacies in 34 samples, showing predominance of blow flies feeding on human dead remains.

Conclusion: Molecular identification is better, effective, time saving and more accurate than morphological identification of insects feeding on human dead remains. Forensic entomology is an effective method to measure post mortem interval when human dead bodies are found after many days unidentified and putrefied.

Keywords: Postmortem Interval (PMI), DNA (Deoxyribonucleic Acid), DNA barcoding, CO1 (Cytochrome oxidase subunit 1), BLAST (Basic Local Alignment Search Tool, BOLD (Barcode of Life Database), blowflies

How to cite: *Mona S, Bashir MZ, Ahad A, Nasrullah A, Mubashir M, Mubarak M, Rashid M. Molecular Identification of Forensically Important Blowflies By DNA Barcoding to Effectively Determine Postmortem Interval. Esculapio - JSIMS 2022;18(04):248-252*

DOI: https://doi.org/10.51273/esc22.2518421

Introduction

Forensic entomology deals with use of insects to aid the legal investigations. The most important usage is the measurement of postmortem interval (PMI). Most common and important order of insects is Diptera and the most common Diptera are blow flies as they reach dead matter most initially. The eggs or larvae they hatch on dead bodies are picked by forensic entomologists to study their identification, life cycles and time of colonization to determine how long the body is dead.[1] When the postmortem interval gets prolonged and conventional methods become limited in its determination, then blowflies or Calliphoridae flies are evidence themselves, especially in criminal cases of unknown bodies.[2] Proper identification either morphological or molecular is extremely important whenever they are studied by forensic entomologists.[3] These flies lay eggs on moist parts of the dead bodies or directly deposit larvae which feed on the carrion and slowly mature into adult forms. Their growth cycle acts as natural biological clock that can be utilized to determine time since death as well as the habitat where bodies are found.[4]

Many a times flies infest bodies in domestic settings and investigators label such deaths as natural. Even in such cases, larvae, pupae, immature flies or adult ones should be preserved as their utilization afterwards can prove a crime even after cremation of such bodies.[5] As sometimes flies at larval stage look similar, their accurate

1. *Department of Forensic Medicine and Toxicology, Shalamar Medical and Dental College, Lahore*
2. *Department of Forensic Medicine and Toxicology, Shalamar Medical and Dental College, Lahore*
3-6. *Shalamar Medical and Dental College, Lahore*
7. *Department of Human Genetics and Molecular Biology, University of Health Sciences, Lahore*

Correspondence:
Dr. Sundus Mona, *Senior Demonstrator, Department of Forensic Medicine and Toxicology, Shalamar Medical and Dental College, Lahore, Pakistan E-mail drsundusmona61@gmail.com*

Submission Date: 05-08-2022
1st Revision Date: 08-11-2022
Acceptance Date: 01-12-2022

and proper identification is vital for solving criminal cases. For proper identification taxonomic keys are used and sometimes they can be unavailable. This can be a huge drawback of morphological identification especially when mature forms of insects are not available. Morphological keys identify adult forms of insects generally which may require to hire a specialized taxonomist who can identify these insects accurately.[6] If insects are identified imprecisely, it can change understanding of habitat and its entomofauna entirely. Currently our knowledge of taxonomy and morphological format of identification lacks especially regarding immature forms of insects. So, in order to link both genders of same insects and their true identification, molecular methods of identification are getting attention.[7] Molecular methods overcome the difficulties of morphological key utilization in the sense that they can work with all forms of same insect, whether immature or adult. They take lesser time to process and identify forensic insects; moreover results are more authentic.[6]

The field of molecular identification is relatively new and evolving. That includes DNA analysis and matching molecular sequences of same species which has diversified with time. Entomologists need to follow updated guidelines of entomofauna as various factors affect the timeline of colonization and succession of insects on the dead bodies. Not only identification but differentiation of these insects from other similar ones can reduce chances of error while determining time since death mainly relying on time of colonization and life cycles of identified insects.[8] DNA barcoding has emerged as a revolutionary taxonomic method which can be reliable for saving an organized record of identified DNA sequences of insects studied until now for systematic identification of forensically relevant insects. The two main objectives of this newly developed technique are insect identification and locating its species group. The newly discovered insects add to data of already identified sequence pool.[9]

For DNA barcoding, mt DNA is commonly used as it has high mutation rate that enhances prospects of producing species-specific markers of relevant insects and possesses both variable and conserved bits. Also it is easier to extract as compared to nuclear DNA. The best gene for barcoding is cytochrome oxidase 1 (CO1) gene. CO1 gene is conserved as protein coding region in all aerobes and is frequently used to identify the species which makes it perfect for tracing the phylogeny of similar insects.[10] CO1 is therefore widely utilized region of mt DNA to study population genetics and biodiversity which makes it an effective tool to identify forensically relevant insects universally.[11] Regarding the essential prerequisite of proper identifi-cation of forensic insects, molecular identification of forensically important insects is direly needed. As it is new emerging technique in Pakistan, it needs more atten-tion by law enforcement agencies.

Material and Methods

Sampling was purposive and 200 larvae were collected from five cadavers in putrefaction phase of decomposition with larval colonization found on bodies, from mortuaries. After approval obtained from the Ethical Committee of UHS, we took 40 larvae from each of the five cadavers from mortuaries of Lahore. The appropriate animal care, experimental protocols, and the recommendations of the International Public Health Service Guide for the Care and Use of Laboratory animals were followed. The larvae were collected with soft forceps put in absolute alcohol with labels for each sample and stored at 4˚C in UHS. Pupae and adult flies were excluded from the study. DNA extraction was done by phenol-chloroform method and quantified by nanodrop. CO1 658bp region was amplified by using LCO1490 as forward and HCO2198 as reverse primer after optimization. PCR mix was placed in thermo cycler (Applied Biosystems by life technologies, ProFlex PCR System model) by adjusting the conditions. Each PCR product was gel purified. The CO1 region of mtDNA was amplified by PCR. Gel Electrophoresis was done with Agarose gel. Clearest bands were selected for annealing the primers with desired DNA products. Sanger sequences methodology was adopted to sequence the Amplified PCR products. The DNA sequence analysis was done by ChromasPro Version 2.6 to edit and confirm the sequence electropherograms. Each sequence was submitted to BLAST (Basic Local Alignment Search Tool) to verify sequence similarity to previously identified CO1 dipteral sequences. These sequences were then submitted into BOLD (Barcode of Life Data Systems) for sequence comparison. DNA Barcode analysis was carried out by Nucleotide sequence divergences for dipteral insects using software MEGA 7. Similar sequence was picked from NCBI and compared to the results.

Results

DNA was extracted by organic method of phenol-chloroform method. It was quantified by nano drop. Primers were optimized and bands were observed in gel dock through computer software.

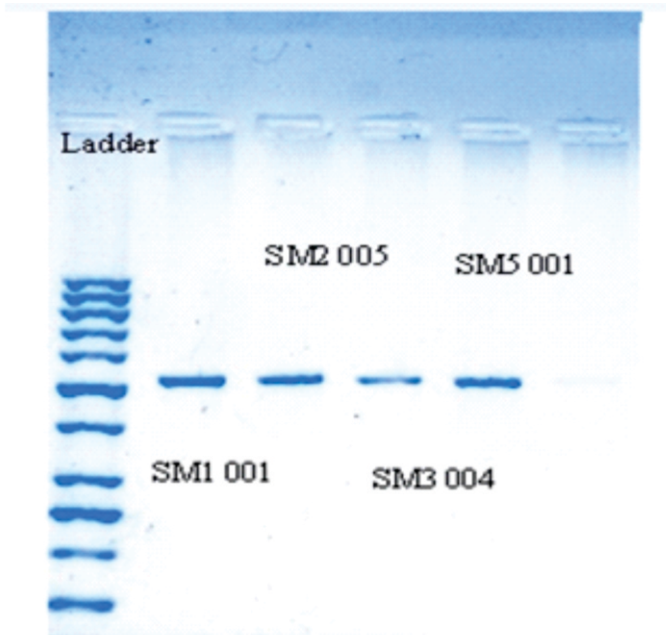

Fig-1: *PCR product in gel electrophoresis*

DNA Concentration of few extracted samples obtained from NanoDropTM

Sample 33 (Body 1) had 201.9ng/µl of nucleic acid
Sample 45 (Body 2) had 338.5ng/µl of nucleic acid
Sample 88 (Body 3) had 485.9ng/µl of nucleic acid
Sample 128 (Body 4) had 548.2ng/µl of nucleic acid
Sample 156 (Body 5) had 228.6ng/µl of nucleic acid

After submission of sequences to Blast, sequence similarity of each result sequence was verified and compared to previously identified CO1 dipteral sequences. These sequences were then submitted into BOLD (Barcode of Life Data Systems) where storage and preliminary data analysis was undertaken. Two (02) species were identified; Chrysomya megacephala in 142 samples and Chrysomya rufifacies in 34.

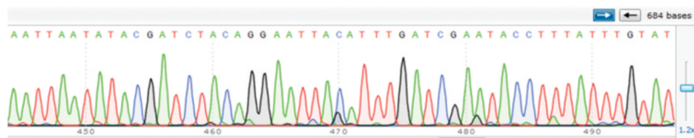

Fig-2: *Sequence clipping of Sample 1 (Chrysomya megacephala)*

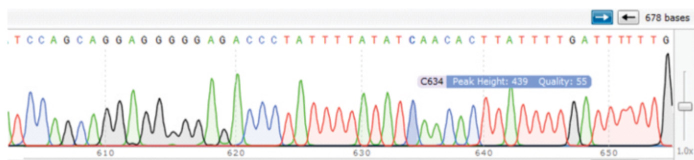

Fig-3: *Sequence clipping of Sample 187 (Chrysomya rufifacies)*

After sequences were received, their electropherograms were generated and the data quality checked. Fig. 2 & 3 indicates cutouts from sequences of the two collected species.

Table 1: *Similarity index for species identification using BLAST (Few samples)*

Query ID	Best ID	Search Database	Top% Similarity	Low% Similarity
Sample 1 Body 01	*Chrysomya megacephala*	COI species database	100	99.85
Sample 54 Body 02	*Chrysomya megacephala*	COI species database	100	99.81
Sample 98 Body 03	*Chrysomya megacephala*	COI species database	99.84	99.68
Sample 143 Body 04	*Chrysomya megacephala*	COI species database	99.85	99.65
Sample 187 Body 05	*Chrysomya rufifacies*	COI species database	99.87	99.16

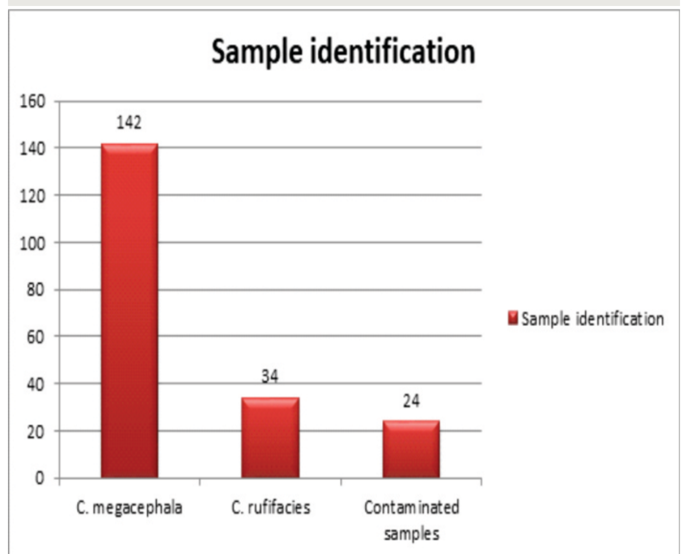

Table 2: *Abundance of flies in 200 samples*

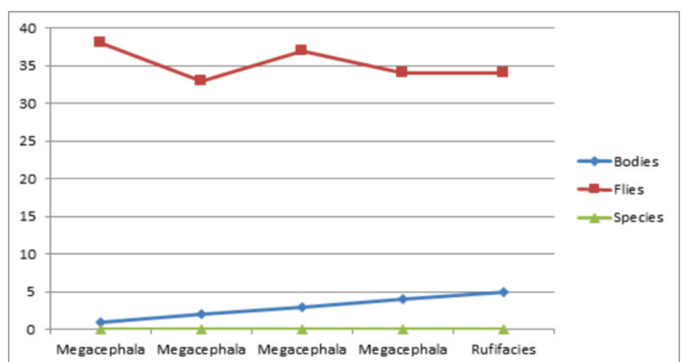

Table 3: *Species identified per body*

Discussion

Forensic entomology is a sporadically used science. After 72 hours of death, rate methods of determining port mortem interval hardly define an exact time since death, so as a concurrent method and circumstantial evidence; insects can tell time since death by their time of colonization, life cycles and genetic sequences fairly well. In our study we collected larval samples from human cadavers, all homicidal cases with identities unknown or amputated body parts and injuries pertaining to assault before death. These larval samples were preserved and analyzed by DNA extraction, PCR amplification of CO1 gene and after aligning sequences by MEGA software, the sequences were submitted in BLAST software to match with already stored sequences. These sequences were then submitted in BOLD (Barcode of Life Database). The sequences with 99 or 100% match were included in study, although 24 samples had to be discarded as they showed contamination by human DNA. Out of 176 samples that clearly identified insects, 142 samples belonged to Chrysomya megacephala while 34 samples showed Chrysomya rufifacies species. Chrysomya megacephala was discovered by larvae collected on four dead bodies, while Chrysomya rufifacies was found on only one.

The technique used for DNA barcoding was utilizing the 658-670bp of 5' end of mitochondrial CO1 gene. It is frequently used region for identification of forensic insects especially the blow flies shown in many studies.[12-17] Most frequent sequence was that of Chrysomya megacephala and its standard prototype sequences were available on National Center of Biotechnology Information (NCBI) and Barcode of Life databases (GenBank and BOLD), respectively.[13,14] In our study the most frequent blow fly was also C. megacephala which is consistent with study of Badenhorst et al., 2018 who found C. megacephala as globally distributed species, a most abundant one and most forensically relevant too. It is known as the earliest and dominant scavenger that colonizes dead bodies and the best entomological candidate to be utilized for determination of time since death.[18] Chrysomya megacephala is also abundant in Sub-continent and Middle East which makes its abundance in Pakistan usual, which is consistent with study of Akbarzadeh et al., in 2015.[19]

The second most abundant sequences we got were of Chrysomya rufifacies. It is a blowfly that is forensically relevant and fairly encountered on vertebrate dead carrions and commonly found in tropical climates with temperate weather like North and central Americas, the continent of Australia and Asia.[20] So, Chrysomya rufifacies (Macquart) which is a Calliphoridae is a common fly around the globe which is frequently found on human dead bodies.[21] It is also known as hairy maggot blowfly which causes human myasis and also found as an early colonizer on dead bodies.[22] It can be seen in all seasons as it adapts to all weather conditions and myasis cases among humans coincide with those of Chrysomya megacephala.[23] Its ability to cope in all seasons makes it abundant in both urban and mountainous zones.[24] The habits of C. Rufifacies are not well understood especially in their own habitats but it is still considered as an important forensic blowfly. Regarding their frequent discovery from under the dead bodies, sample collection from underneath dead bodies is very crucial and can be fruitful at the crime scene. [25]

After identification, the life cycles of insects are correlated with postmortem body changes and postmortem interval is calculated by adding life cycle duration of insects with time of colonization according to environmental differences. Postmortem interval is always measured retrospectively by calculating rate methods and then putrefaction timeline where insects' life cycles gives a clue of time spent after death. Though the study was limited and short, the results were promising. If the sample size was higher, more species may have been identified. Human contamination was another limitation of the study. We direly need trained forensic entomologists to assist in medicolegal framework regarding effective sampling at the crime scene and getting promising results to aid legal investigations.

Conclusion

Molecular identification is better, effective, time saving and more accurate method than morphological identification of insects feeding on human dead remains. Forensic entomology is an effective technique to measure post mortem interval when human dead bodies are found after many days unidentified and putrefied. Insect's species, habitat and life cycle determines place of death, time since death, time since colonization and levels of insect succession to aid in legal investigations.

Conflicts of interest *None*

Funding Source *None*

References

1. Langer SV, Kyle CJ, Illes M, Larkin S, Beresford DV. Urban and rural spatial delineations in blow fly species (Diptera: Calliphoridae) across Canada: implications for forensic entomology. Journal of Medical Entomology. 2019 Jun 27;56(4):927-35.
2. Singh R, Kumawat RK, Singh G, Jangir SS, Kushwaha P, Rana M. Forensic entomology: A novel approach in crime investigation. GSC Biological and Pharmaceutical Sciences. 2022;19(2):165-74.
3. Villet MH. History, accomplishments and challenges of forensic entomology in Africa. International dimensions and Frontiers in forensic entomology. Taylor & Francis, Boca Raton. 2015: 161-71.
4. Byrd, J.H.; Tomberlin, J. Forensic Entomology: The Utility of Arthropods in Legal Investigations, 3rd ed.; CRC Press: Boca Raton, FL, USA; Taylor & Francis Group: Boca Raton, FL, USA, 2019; p. 620.
5. Niederegger, S. Technical Note: Effects of Makeshift Storage in Different Liquors on Larvae of the Blowflies Calliphora vicina and Lucilia sericata (Diptera: Calliphoridae). Insects 2021, 12, 312.
6. Al-Mekhlafi FA, Alajmi RA, Almusawi Z, Abd Al GAlil FM, Kaur P, Al-Wadaan M, Al-Khalifa MS. A study of insect succession of forensic importance: Dipteran flies (diptera) in two different habitats of small rodents in Riyadh City, Saudi Arabia. Journal of King Saud University-Science. 2020 Oct 1;32(7):3111-8.
7. Grzywacz A, Jarmusz M, Walczak K, Skowronek R, Johnston NP, Szpila K. DNA barcoding identifies unknown females and larvae of Fannia R.-D.(Diptera: Fanniidae) from carrion succession experiment and case report. Insects. 2021 Apr 23;12(5):381.
8. Mona S, Khalid M, Jawad M, Noreen S, Rakha A. Forensic entomology: a comprehensive review. Advancements in Life Sciences. 2019 Feb 25;6(2):48-59.
9. DeSalle R, Goldstein P. Review and interpretation of trends in DNA barcoding. Frontiers in Ecology and Evolution. 2019 Sep 10;7:302.
10. Ghaffar HA, Moftah MZ, Favereaux A, Swidan M, Shalaby O, El Ramah S, Gamal R. Mitochondrial DNA-based identification of developmental stages and empty puparia of forensically important flies (Diptera) in Egypt. Journal of Forensic Science and Medicine. 2018 Jul 1;4(3):129.
11. Samuel MY. Molecular identification of house fly, Musca domestica L.(Diptera: Muscudae), using mitochondrial DNA partial genes cytochrome oxidase sub unit 1 (CO1) in Manado city. International Journal of Entomology Research. 2018;3.
12. Harvey ML, Mansell MW, Villet MH, Dadour IR. Molecular identification of some forensically important blowflies of southern Africa and Australia. Medical and Veterinary Entomology. 2003 Dec;17(4):363-9.
13. Harvey ML, Gaudieri S, Villet MH, Dadour IR. A global study of forensically significant calliphorids: implications for identification. Forensic science international. 2008 May 2;177(1):66-76.
14. Nelson LA, Wallman JF, Dowton M. Using COI barcodes to identify forensically and medically important blowflies. Medical and Veterinary Entomology. 2007 Mar;21(1):44-52.
15. Sonet G, Jordaens K, Braet Y, Bourguignon L, Dupont E, Backeljau T, De Meyer M, Desmyter S. Utility of GenBank and the Barcode of Life Data Systems (BOLD) for the identification of forensically important Diptera from Belgium and France. Zookeys. 2013(365):307.
16. Oliveira PV, Matos NS, Klippel AH, Oliveira-Costa J, Careta FD, Paneto GG. Using DNA barcodes to identify forensically important species of Diptera in Espírito Santo State, Brazil. Brazilian Archives of Biology and Technology. 2017;60.
17. Zajac BK, Sontigun N, Wannasan A, Verhoff MA, Sukontason K, Amendt J, Zehner R. Application of DNA barcoding for identifying forensically relevant Diptera from northern Thailand. Parasitology Research. 2016 Jun;115(6):2307-20.
18. Badenhorst R, Villet MH. The uses of Chrysomya megacephala (Fabricius, 1794)(Diptera: Calliphoridae) in forensic entomology. Forensic sciences research. 2018 Jan 2;3(1):2-15.
19. Akbarzadeh K, Wallman JF, Sulakova H, Szpila K. Species identification of Middle Eastern blowflies (Diptera: Calliphoridae) of forensic importance. Parasitology research. 2015 Apr;114(4):1463-72.
20. Bauer AM, Bauer A, Tomberlin JK. Effects of photoperiod on the development of forensically important blow fly Chrysomya rufifacies (Diptera: Calliphoridae). Journal of Medical Entomology. 2020 Sep 1;57(5): 1382-9.
21. Hu G, Wang Y, Sun Y, Zhang Y, Wang M, Wang J. Deve-

lopment of Chrysomya rufifacies (Diptera: Calliphoridae) at constant temperatures within its colony range in Yangtze River Delta Region of China. Journal of Medical Entomology. 2019 Sep 3;56(5):1215-24.
22. Bambaradeniya YT, Karunaratne WA, Tomberlin JK, Magni PA. Effect of type of tissue on the development of Chrysomya rufifacies (Diptera: Calliphoridae) in Sri Lanka. Journal of Medical Entomology. 2021 Jul; 58(4):1673-9.
23. Klong–klaew T, Sontigun N, Sanit S, Samerjai C, Sukontason K, Koehler PG, Pereira RM, Chareonviriyaphap T, Kurahashi H, Sukontason KL. Daily and seasonal prevalence of the blow fly Chrysomya rufifacies (Diptera: Calliphoridae) as revealed by semiautomatic trap collections in suburban Chiang Mai Province, northern Thailand. Florida Entomologist. 2018 Dec;101(4):617-22.
24. Moophayak K, Klong-Klaew T, Sukontason K, Kurahashi H, Tomberlin JK, Sukontason KL. Species composition of carrion blow flies in northern Thailand: altitude appraisal. Revista do Instituto de Medicina Tropical de São Paulo. 2014 Mar;56:179-82.
25. Ahmad A, Omar B. Effect of carcass model on maggot distribution and thermal generation of two forensically important blowfly species, Chrysomya megacephala (Fabricius) and Chrysomya rufifacies (Macquart). Egyptian Journal of Forensic Sciences. 2018 Dec;8(1):1-8.

Authors Contribution

SM: Conceptualization of Project
SM, MM: Data Collection
SM, MZB, AA, AN: Literature Search
MR: Statistical Analysis
SM, MZB, MM: Drafting, Revision
SM: Writing of Manuscript