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Morphological and molecular identification of the larvae causing myiasis in cattle

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Abstract A myiasis infestation occurs when dipterous larvae infest living vertebrates, includeing the humans and animals. Depending on where they live and how they interact with their host, dipterous larvae in mammals can feed on either living tissues or dead tissue, bodily fluids, or food that has been consumed. They can also cause a variety of infestations. The Caliphoridae family is considered the most important family causing myiasis, so the research's goal was to identify the species causing infestation in cows. Methods: Infested animals (50 cattle) were examined and larvae from each one were collected from each animal, then the species firstly studied and identified microscopically based on their the morphological and anatomical characteristics of larvae followed by study the effect of certain risk factors related to the infestation occur, then they were examined using PCR technique through amplifying the small subunit ribosomal RNA of chrysomya pseudolucilia (CPS) gene to confirm the diagnosis, also their genetic sequence and phylogenetic tree were analysis. Results: The results showed that all larval samples isolated from infected cows represented *Chrysooma bezziana* according to the morphological examination of these larvae. PCR examination and genetic sequence analysis of some samples (10) confirmed the species of fly diagnosed microscopically, and it was noted through studying the genetic tree of these samples that there is a similarity (95-97%) with one of the global isolates. Regarding the risk factors studied, it was noted that there is an effect of the age and sex of the animal on the infestation rate, and it was noted that its rates were affected by the site of infection on the body and the geographical location of the animal, and no significant effect was noted for the year months. Conclusion: From the results obtained in this study, we conclude that most cases of worms found *Chrysoma bezziana* causes cows, and it seems that it is still endemic in Iraq / Diwaniyah despite the therapeutic attempts, and that the local isolates are of external origin due to the great similarity with the global isolates.

Keywords: Calliphoridae, cattle, Chrysoma bezziana, PCR

Introduction Myiasis is adisease that is known to be caused by parasitic flies in their larval stages infecting human and animal tissues and natural openings. It is widespread and abundant throughout the world and is a pathological condition whereby live and dead tissue is infected by Dipteran fly larvae. It primarily spreads throughout Africa, South-East Asia, and the tropical and subtropical areas America (1). The word "myiasis" has two parts: "myia" (which means fly) and "sis," which means sickness (2).The main producers of cutaneous myiasis are the families Calliphoridae, Oestridae, and Sarcophagidae. Flies and maggots from the Calliphoridae family-which comprises both facultative and obligatory parasites-cause asignificant percentage of cases (85%–90%) of cutaneous myiasis (3) It is common knowledge that flies annoy and irritate animals with their biting and pinching sensations. In addition to their ability to lay their eggs on human flesh, their ability to penetrate and invade subcutaneous tissues as well as external body cavities, such as the ears, genital organs, nostrils, and orbits, as well as their ability to spread infectious diseases and penetrate living tissues deeply, can result in disfigurement, amputation, and in rare cases, even death (4). For five to ten weeks, larvae feed inside a subdermal area. When they are ready, they burrow out of the skin, fall to the ground, and pupate. After a month or so, adults emerge and repeat the cycle. (5). Bhola et al., (6)Pointed to that the invasion of flies that causes myiasis in vertebrate species is pandemic; nonetheless, it typically occurs in tropical and sub-tropical countries with warm, humid climates, poor housing infrastructure, a high prevalence of domestic animals, and poor hygiene a side from mosquitoes, there are over 86,000 different species of medical important flies (7).one such species is the screw-worm fly, *Chrysomya bezziana* , which is a member of the Calliphoridae family. Myiasis is a zoonotic disease that affects all vertebrates. In humans, the most common sites affected are skin wounds, eyes, noses, ears, guts, vaginas, and sporadically the mouth (8). Unlike other larvae or maggot infestations, Ch. bezziana can invade tissue even in the absence of skin scission or necrosis. It creates a hole and burrows deeply into the host's healthy live tissue, feeding like a screw (9).Given the significance of myiasis for human health, public health, and the extinction of wildlife, there is an urgent need to develop and carry out a systematic planned epidemiological survey to ascertain the prevalence of Ch. bezziana in domestic and wild animals and to develop an appropriate control and treatment plan to reduce the ongoing, irreversible, and financial losses caused by infestation of domestic animals, wild animals, and their owners in the myiasis-affected area(10 ,11).Due to the risky of myiasis in domestic animals specially in cow and because of little or absent of studies that deals with this disease in Iraq this study was design to determine of infestation rate of some Calliphoridae genera confirmed definite diagnosis by using molecular method.

Materials and methods

Ethical approval

The study project was approved (1890) in 28/08/2023 issued by the College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.

Samples collection

Fifty samples of larvae were collected from infested wounds of cows. The infested animals were of different ages (less than one year – more than seven years) and of both sexes. The process of collecting samples continued for nine months (September 2023 - May 2024), during which two visits per week were performed. The study covered different areas of Al-Diwaniyah province (location between latitudes 31.17 and 32.24 north, and longitudes 44.24 and 45.49 east).

Macroscopic Examination

All samples collected directly from animals. The larvae were collected by used a forceps then put in tubes or containers with warm water for about 30 seconds in order to prevents decay and maintains the natural color. After that larvae were converted in to caps with ethanol (70%) and transported to the parasitology laboratory which belongs to the College of Veterinary Medicine, University of Al-Qadisiyah for examination.

Molecular examination

DNA was extracted from 20 larval samples according to the kit manufacturer's instructions (Geneaid/Taiwan). The CPS gene was targeted using a specific primer (Shiz 3' TTKGCAATNAK YTGCATGTTRAA Shiz 5' CANGTGGCYGGAGAA TGGC) to amplify the extracted DNA by conventional PCR. PCR product of 10 samples were sequenced (AB13730XL by Macrogen Corporation – South Korea) and the fly bioinformatics and phylogenetic tree were studied by used the MEGA 11.2 version multiple alignment analysis tool.

Results

Morphological study

By using dissecting microscope, the larvae appear in a creamy white color, dark brown to black in color, the circular rows of spines had tapering, darker ends that recurved toward the body and ended in a point. Eleven rows are used to segment the larvae. The posterior end is bigger than the spine that surrounds the body, has two sharp, pointed protuberances, and is broader than the anterior end. (figure 1). The body of the third instar larvae showed with compound head, the young third instar larvae are whitish to cream colored, while mature larvae developing a pinkish coloration. There are heavy bands of dark, robust, thornlike spines are very prominent with single teeth, anterior spiracles, posterior spiracles are obvious. Second instar larvae each of the pair of posterior spiracles is surrounded by a heavily sclerotized peritreme, dark brown to blackish in color, which is incomplete or nearly so dorsally and ventrally, and has two slit-like spiracular openings (figure2). The third instar larva posterior spiracles are each with three spiracle slits straightened toward the open part at button of the surrounding heavily sclerotized peritreme (dark brown to blackish),(figure3). The spiracles in front appears as a hand-like in shape with 4-6 fingers like branches (lobes) lightly sclerotized, pale brown in color (Figure

4). The anterior end has pointed hocks "mouth hook" have no accessory sclerites (figure 5). There is other differentiated character related to the secondary tracheal trunks which appear in different degree (light – heavy) of pigmentation and in different extensions in different Calliphoridae species. The results of this study showed that they are colored from the twelfth segment to at least the tenth (Figure 6). According to the above morphological and anatomical characteristics, the results showed that all the collected larval samples represent the Chrysoma bezziana species.

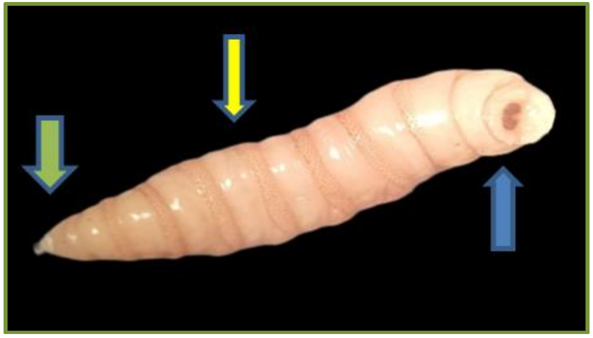

Figure 1: External morphology of Calliphoridae larva (green arrow = anterior pointed end, blue arrow = posterior end with spiracle, yellow arrows = bands of cuticular spines) (Dissecting microscope).

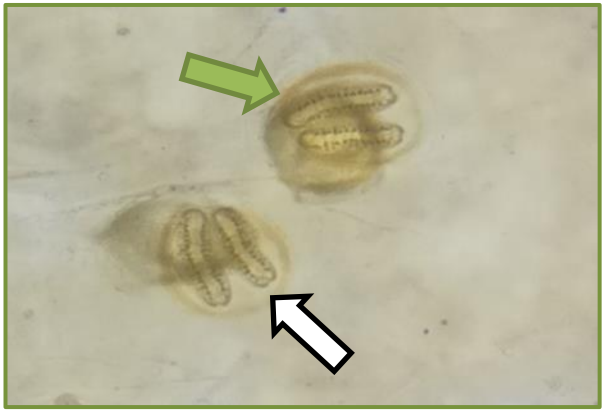

Figure 2: The posterior spiracle plates of Second instars (peritreme =green arrow, slit =white arrow) (x40).

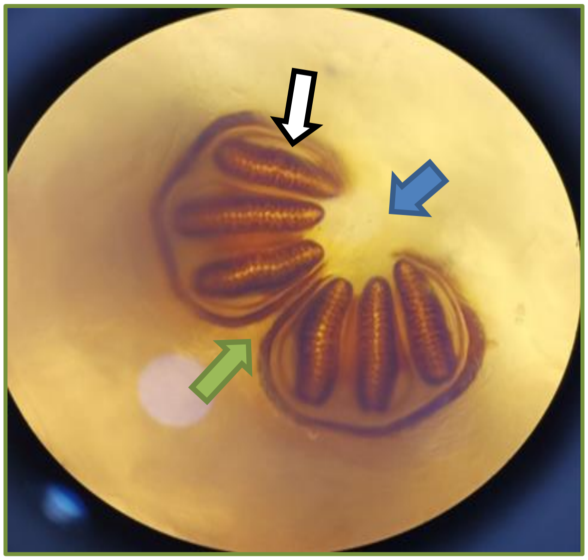

Figure 3: The posterior spiracle plates of third instars (peritreme =green arrow, peritreme opening =blue arrow, slit =white arrow) (x40).

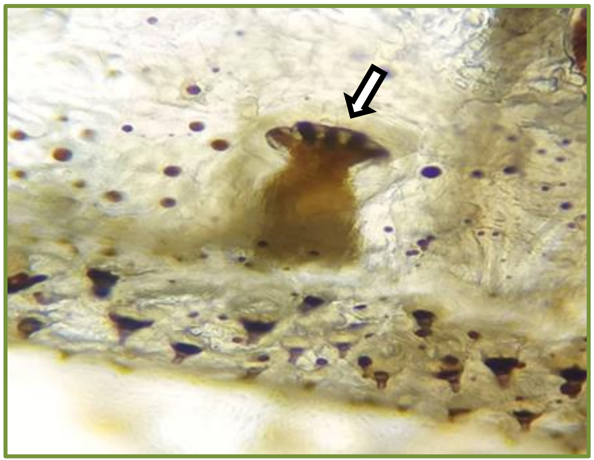

Figure 4: The anterior spiracles of Ch.bezziana with 4-6 lobes (x40)

Figure 5: "mouth hook" of Ch. bezziana (x10).

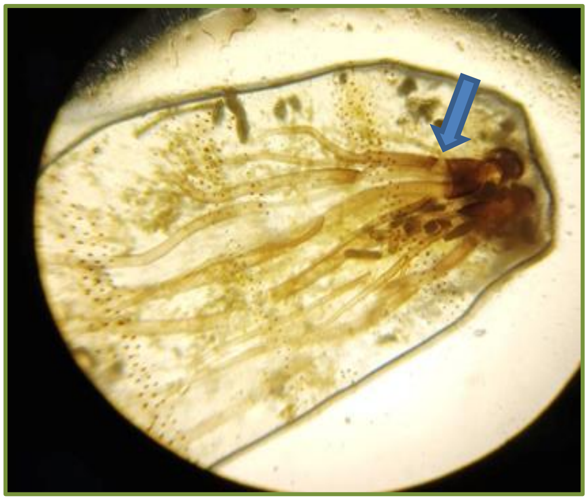

Figure 6: Secondary tracheal trunks of Ch. bezziana (x40).

Molecular study

PCR results confirmed that the amplicon size was 875 bp and that all the examined samples belong to the Caliphoridae family (Figure 7). The results of the genetic sequence analysis of the examined samples identified the species Chrysoma bezziana, which is consistent with the microscopic examination of the larvae. The isolates identified in this study were registered in the GeneBank under the accession numbers (PQ474796, PQ474797, PQ474798, PQ474799, PQ474800, PQ474801, PQ474802, PQ474803, PQ474804, PQ474805). The phylogenetic tree analysis showed identity of 95-97%, 100% query coverage and e-value of 0.00 with one of the subjects (hits) with the accession number ACN25121.

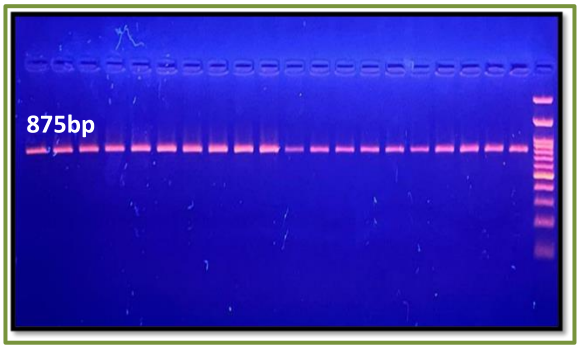

Figure (7): Agarose gel electrophoresis of cattle's larvae amplified DNA using small subunit ribosomal RNA gene. The first right lane represents the DNA marker ladder M100-3000 bp and all rest lanes showed positive small subunit ribosomal RNA gene in Calliphoridae species at 875 bp PCR product size.

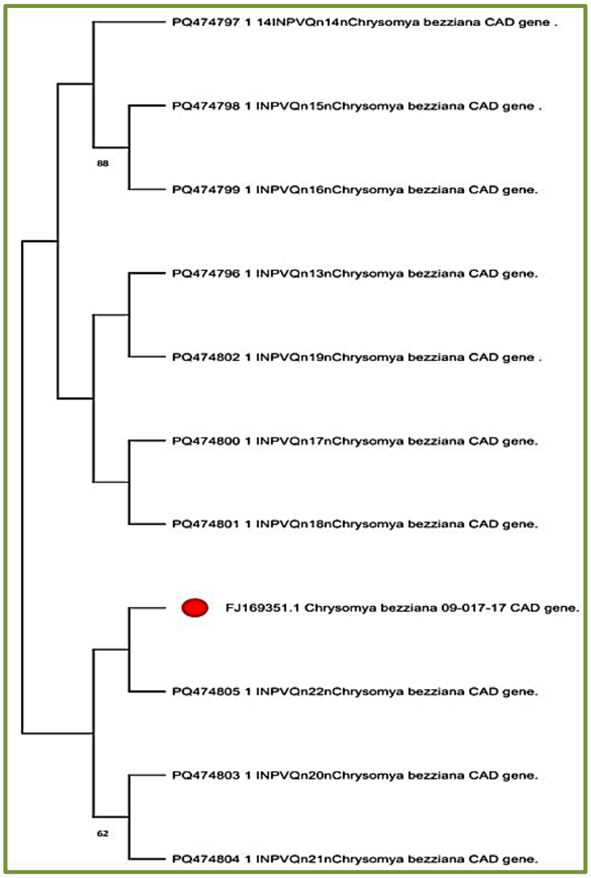

Figure 8: Phylogenetic tree analysis of cattle Chrysoma bezziana isolates.

Discussion

Screwworm has the body is covered with noticeable rings of spines that, when viewed under a microscope, look enormous and noticeable. with contrast to the majority of non-screwworm species, OWS spines are always single-pointed and thorn-like, and their front spiracles often have four to six branches (12,13,14,15). Three somewhat oval-shaped, straight slits pointing towards the peritreme's break are enclosed by the dark, incomplete ring that surrounds the posterior spiracles on the terminal segment (16,17) The structure of third instar posterior spircles considers as the most important characteristic feature used in differentiating among different Calliphoridae spp. In comparison with other previous Iraqi studies, that which recorded by (18) in Basra province / south of Iraq (19) . Notes on the first record of Ch. bezziana (Dipttera: Calliphoridae) from Iraq. This difference between this study and the rest may be due to the differences in numbers of examined animals, management, environmental conditions and stress factors (20).

The widely used method for identifying nucleic acids from various sources is polymerase chain reaction, or PCR (21). According to (22) this method has emerged as a helpful tool for identifying specimens with incomplete remains that lack morphological characteristics. In order to address this issue, forensic professionals have been using PCR technique for Forensic entomology has been using insect species identification since 1994. In order to do this, bug samples' DNA was isolated from their original source, often with the use of a commercial extraction kit. After that, the isolated DNA was amplified using a particular primer made for a particular gene. (23, 24) in previous study in Al-Diwaniyah province used a primers that target the 548 bp product size of the 16S rRNA gene. (25) had been used both genes of ITS2 and cytochrome b for all examined specimens. The difference in the use of gene type between the current study and the previous studies mentioned is due to the fact that the previous studies targeted the insect species Ch. bezziana directly, while in our current study the aim was to use a gene that can be characterized at the family level for the purpose of determining whether there are other species other than Ch. bezziana based on the work of the PCR product gene sequencing.(24),in his previous study in Diwaniyah found that the Ch.bezziana fly diagnosed by him was identical to some isolates in Australia and Brazil. Also (25), confirmed in her study conducted in Baghdad a great similarity between local isolates and global isolates that appeared in other studies in Malaysia, Indonesia and Oman. The lack of similarity between the isolates of the current study and other global isolates, with the exception of one isolate, imposed restrictions to reach its reference, perhaps due to the use of the CPS gene in identifying the insect species in Iraq.

Conclusion

We conclude that most cases of worms found Chrysoma bezziana causes cows, and it seems that it is still endemic in Iraq / Diwaniyah despite the therapeutic attempts, and that the local isolates are of external origin due to the great similarity with the global isolates.

Acknowledgments

Not applicable.

Conflicts of Interest

The authors declare there is no conflict of interest.

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