ijlpr 2023; doi 10.22376/ijlpr.2023.13.4.L158-L166

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Detection of X-Chromosome by PCR Assay

Detection of X-Chromosome Inversions in *Anopheles Pharoensis* Collected from Egypt and Sudan by PCR Assay

Najlaa Siddig Nasir[1], Fatima Fadul Ali[1], Fatima M. Mohamed[1], Mohamed Osman Elamin[2], Ali M. Alshehri[2], Hatim M. Badri[2], Hatim A. Natto[2], Mashael Alfaifi[2], and Ahmed A. Osman[3]

[1] *Alzaiem Alazhari University- Sudan.*

[2] *Faculty of Public Health and Health Informatics, Umm Al-Qura University. KSA*

[3] *Kassala University, Sudan*

Abstract: Globally, the WHO estimated 241 million malaria cases with 627000 deaths in Africa in 2020. Studying inversions in the X-chromosomes could detect behavioral changes and efficiency of transmission of Malaria in *Anopheles phronesis* species. These changes may lead to shifting from becoming a major vector of Malaria after the inability to transmit Malaria. The knowledge of the malaria transmission pattern of *Anopheles pharoensis* in Sudan needs to be improved. We aimed to detect the presence of any inversions or mutations in *Anopheles pharoensis* species collected from Egypt and Sudan and to undergo a Polymerase Chain Reaction (PCR) technique to detect mutations or X-chromosome inversions in the Sudanese and Egyptian strains of *Anopheles pharoensis* also to compare between the X-chromosome of the two species. It is an experimental, analytical study that aimed to detect inversions in the X –chromosomes of *Anopheles phronesis* collected from Egypt (Faiyoum Governate) and Sudan (Khartoum, Gezira, and Sennar states) of Sudanese Mosquito species. From the gel electrophoresis and PCR assay, inversions were detected by the 100Bp PCR products using primers. Only very few revealed bands from 75 Egyptian strains were detected by bands. The study revealed that *Anopheles pharoensis* species collected from Egypt have X- chromosome inversions. Inversions were absent in the Sudanese strains. The study also reacted to the fact that *Anopheles phronesis* could be a sibling species or a species complex, but further studies are needed to prove this. From the results and observations of this investigation, it was recommended to undergo further genetic studies on Anopheles species in Sudan and use of PCR technique in the classification.

Keywords: *Anopheles Pharoensis*; Egypt; PCR; Sudan; X-chromosomes Inversions.

***Corresponding Author**

Mohamed Osman Elamin , Faculty of Public Health and Health Informatics, Umm Al-Qura University. KSA

Received On 18 February, 2023

Revised On 8 April, 2023

Accepted On 29 April, 2023

Published On 1 July, 2023

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Najlaa Siddig Nasir, Fatima Fadul Ali, Fatima M. Mohamed, Mohamed Osman Elamin, Ali M. Alshehri, Hatim M. Badri, Hatim A. Natto, Mashael Alfaifi and Ahmed A. Osman , Detection of X-Chromosome Inversions in Anopheles Pharoensis Collected from Egypt and Sudan by PCR Assay.(2023).Int. J. Life Sci. Pharma Res.13(4), L158-L166 http://dx.doi.org/10.22376/ijlpr.2023.13.4.L158-L166

1. INTRODUCTION

Anopheles mosquitoes are important for their role in malaria transmission in many countries. They are found in the tropics and subtropics of Africa, South America, and Asia. There are more than 430 species of Anopheles in the World, of which 30 to 40 can transmit human Malaria. Most of the 40 species favor feeding on humans more than animals; thus, they are usually found to be close to human dwellings[1]. Malaria transmission depends on temperature, humidity, and rainfall. At temperatures below 20°C, the Plasmodium falciparum cannot develop or be transmitted [2]. Anopheles larvae have varying habitats for breeding. They can breed in broken water pipes, open tins, poor drains, market gardens, tires, and pools at construction sites. All available landscapes in the city can be breeding places for Anopheles mosquitoes [3].

1.1. Biology of Anopheles pharoensis in Egypt

Anopheles pharoensis in Egypt prefers clean, shallow, stagnant water with a thick growth of vegetation[4]. It has been found to breed in drains, irrigation canals (mosques), near rice fields, and burrow pits containing weeds and reeds[4]. *Anopheles phronesis* breeds at moderate temperatures of 21.1 ºC(min) and 36.6ºC(max) and favors oxygen content of 7.32 mg/liter at 30º C[5]. Studies in Faiyoum Governate revealed that *Anopheles pharoensis* has seasonal biting activity extending from May to December, reaching a peak in November[6]. The biting activity of *Anopheles phronesis* starts after sunset (8.00 pm) until midnight and may continue biting until the early hours of the morning[7]. The length of the gonotrophic cycle of Anopheles *pharoensis* was 6.14 days. The female may survive 9-11 days at 25.7ºC after taking an infective blood meal to transmit *Plasmodium vivax* and *Plasmodium falciparum*[8]. *Anopheles phronesis* was observed to favor feeding on animals and was also attracted to humans[9]. Twelve Anopheles species were observed in Egypt, but only five of them are vectors of Malaria: *Anopheles phronesis* (the most important species in Egypt), *Anopheles sergenti, Anopheles multicolor, Anopheles stephensi,* and *Anopheles superpictus*[10]. Studies were carried out in Faiyoum Governate, Nile Valley Eastern, Western desert, and the red sea at different seasons. Results revealed that *Anopheles pharoesis* was the most important species in Egypt's Aswan, Faiyoum, and new valley districts [10]. Our visit to Egypt was of great importance for this Research.

1.2. Biology of Anopheles pharoensis in Sudan

Lewis (1944) observed hundreds of records of *Anopheles pharoensis* from Central and Southern Sudan. It is common among the Blue and White Niles in the rainy season and at Kadaru, Zeidabrahad, Hawata, Faras, Sennar, and Jebalawlia. It is an important species in the Gezira irrigated area, where it causes a great deal of discomfort by biting humans[11]. *Anopheles pharoensis* prefers to rest by day in vegetation and breeds in Gezira on the banks of irrigation canals, as observed by Evans (1938) [11]. Studying X-chromosome inversions is important in identifying species complexes or sibling species in Anopheles. Inversions were located in the X-chromosomes of many Anopheles species, thus separating one Anopheles species into many groups. On Studying *Anopheles gambiae* X-chromosomes, 5 chromosomal forms were observed: Bamako, Savanna, Mopti, forest, and Bissau[12]. Polymerase Chain Reaction (PCR)Molecular Genetics can be used to distinguish between Anopheles species complexes. It is an accurate tool for identifying members of *Anopheles gambiae* complexes. It is a quick and simple method that needs less expertise [13]. Coulibaly et al. (2007) developed a PCR diagnostic assay that detected differentiation in arm arrangements in the X-Chromosome and thus differentiated between different forms in a species. Four primers, Prfow +PF and D1+D1N, were used to detect inversions and breakpoints in the X-chromosome of *Anopheles gambiae* forms [14]. Similar primer pairs (Prfow +Pr) and (D1 + D1N) were applied in this Research for both Sudanese and Egyptian *Anopheles pharoensis*. The *Anopheles pharoensis* samples collected from Sudan showed no bands, while those collected from Egypt revealed the expected clear bands at ~ 100Bp in the inverted Band. A study in southern Africa revealed that studying the behavior and chromosomal inversions of *Anopheles pharoensis* suggested that *Anopheles pharoensis* may be a species complex[15]. It is similar to the fact that this research has reacted. Further studies are needed to prove this. In this study, we aimed to detect the presence of any inversions or mutations in *Anopheles pharoensis* species collected from Egypt and Sudan by application of PCR assay. There is little knowledge about *Anopheles pharoensis* and its relation with malaria transmission in Sudan. This mosquito successfully transmitted Malaria in Egypt but failed to do so in Sudan [10`11]. Important questions are needed about the transmission pattern of Sudanese strains of Anopheles pharoensis. This research has successfully answered these questions. By lack of knowledge of the malaria transmission pattern of *Anopheles* pharoensis, this mosquito could become a main vector of Malaria in Sudan without realizing it. The results of this research will help prevent future malaria transmission by *Anopheles pharoensis*.

2. MATERIALS AND METHODS

The experimental, analytical study was conducted in Egypt and Sudan in the fourth larval stage. Samples were collected. Five hundred samples were obtained from Faiyoum Governate (Sinnuris Centre), Egypt. Another five hundred samples were obtained from Gezira state (Wad Elshafie village), Sennar state, and Khartoum state (Soba), Sudan.

2.1. Materials for PCR technique

We prepared Eppendruf tubes/PCR tubes-White 0.5 tips-blue tips, yellow tips, four racks, micropipettes, aluminum foil, wrapping foil, and a PCR machine to conduct the procedures. DNA-extracted materials from Anopheles mosquitoes were prepared according to the standards. We used 4 primers along with MgCl2-Dntps-Taq, Polymerase-Distilled water, PCR buffer solution Centrifugation, Vortex (shaker), Heater, Gel tray, comb, Falcon Tube-Electrophoresis apparatus, Agaros powder, Bromophenyl blue dye, Ethidium bromide, Electrophoresis buffer (running buffer), ULV machine, marker pen, Absolute ethanol, and DNA ladder(100bp).

2.2. Materials for DNA extraction

We prepared the samples of *Anopheles pharoensis* larvae (50 samples from Egypt + 50 samples from Sudan) along with vortex (Shaker), centrifugation, water bath, TRIS buffer, Lysis buffer, 500ml STE, 2.5M of 10mg stock solution of Proteinase, 25ml of 20% SDS solution (sodium dodecyl sulfate), incubator, CI solution (Chloroform + iso + amyl alcohol at ratios 24:1), 500M PCI solution (Phenol chloroform + isoamyl alcohol at ratios: 25:24:1), 45ml of 2M NaCl, Eppendorf tubes, yellow tips, blue tips, white tips, 4 racks, wrapping foil, gel documentation, and UVITEC machine.

2.3. Methods

Collection of Larvae: For the collection of larvae, the dipping WHO standard method (WHO, 2013) [16] was performed to determine the presence of larval stages of *Anopheles pharoensis* species. They were emptied into a dish, counted, and transferred to a laboratory to be stored in Carnoy's fixative and placed in a freezer until experimental work. *Polymerase chain reaction technique:* We extracted DNA from *Anopheles pharoensis* by putting 100 larvae in the fourth stage in 100 (1.5m) Eppendorf tubes.500µl STE (0.1 M NACL,0.5M TRIS HCL, and 0.001M EDTA) was added to the larvae.2.5M of 10pk stock solution of Proteinase K were added.25ml of 20% SDS solution (Sodium dodecyl sulfate). The solution was incubated at 37°C overnight.500M PCI solution (Phenol chloroform + iso-amyl alcohol at ratios 25:24:1). The solution was again incubated at room temperature for 5 minutes. Samples were centrifuged at 7000rpm for 5 minutes. The aqueous layer was removed carefully with a micropipette and transferred into a clean tube. The aqueous layer was re-extracted with PCI.CI solution was added (Chloroform and iso-amyl alcohol in a ratio of 24:1). The solution was incubated at room temp for 3 minutes. Then, it was centrifuged for 3 min. at 7000rpm.The aqueous layer was removed with a micropipette and transferred to a clean tube. The aqueous layer was again re-extracted with PCI.45ml of 2M NaCl, and double the same volume of absolute cold ethanol was added to precipitate the DNA. Ethanol was decanted, and the pellet was dried. The pellet was re-suspended in 20ml of 1 TE buffer (0.01ml TRIS HCL, PH 8-0.001mlEDTA) [17]. According to Santolamazza et al. (2004). *PCR conditions:* The PCR machine was run under the following conditions:94°C for 2 minutes.40 cycles of 94 °C for 20 seconds.55°C for 15 seconds.72°C for 20 seconds.72°C for 5 minutes and a 4°C hold. PCR products were separated on 1.5% agarose gel stained with Ethidium bromide according to the technique of Coulibaly (2007) [14]. *Preparation of the PCR mixture:* For each sample, a mixture was prepared using PCR buffer 2.5ul, Taq polymerase 0.2ul, DNTPs 1ul, MgCl2 3ul, and Distilled water1103ul. *Primers:* We used primer 1 plus primer 2 (mixed) 3ul (1.5+1.5). In the second experiment, we mixed primer 3 and primer 4 (Table 1).

Table 1. The distribution of the primers.

Oligo name	Accession number in gene bank
P4 fow (primer 1)	gb/Ef015881.1
P4Re (primer 2)	gb/Ef015880.1
D1N (primer 3)	gb/Ef015881.1
D1 (primer 4)	gb/Ef015881.1

Primers were ordered from Vivanties Technologies –Malaysia (Coulibaly et al.,2007). For 20 samples, the above solutions were multiplied by 20 to give the exact volume of the mixture poured into each of the 20 PCR tubes. After putting the mixture in each PCR tube, the DNA was put in each PCR tube of volume 4ul. Vortexed the final mixture, and the PCR tubes were put inside the PCR machine for 1 hour and 30 minutes. Then, the PCR tubes were removed from the PCR machine and, in the end, left in the incubator overnight at 37C, according to Coulibaly et al. (2007). *Gel Electrophoresis:* We used 0.2gm agarose powder to TBE (18ml of water to 2ml 10TBE). The mixture was heated in a water bath until the gel dissolved. Then, 0.5 ml of Ethidium bromide was added to the gel solution. The agarose gel was poured into a horizontal electrophoresis apparatus. The comb was put in the gel and left until the gel solidified. Next, the TBE solution was poured into the gel apparatus. Then we mixed 5ul of each PCR product with 3ul of loading buffer (glycerol, bromophenol blue, and water in a ratio 1:1:8). A marker DNA ladder of 1.5ul was loaded to determine the amplified DNA size. The gel electrophoresis apparatus was connected to a power supply at a voltage of 85V and ran for 30 minutes. When the run was complete, the gel was taken and observed under ultraviolet light to determine the amplification of the PCR product. The selected gels were photographed using a gel documentation UVITEC machine, according to Badawi (2002)[18].

2.4. Statistical analysis

The collected data was cleaned and validated for accuracy and completeness before statistical analysis. Then, we collected data and entered it into the Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). Finally, we used descriptive statistical methods to describe the socio-demographic variables and reported them as frequency tables and graphs.

3. RESULTS

PCR in Anopheles phronesis species: Two primers (D1 +D 1 N) and (Prfow + Pr) were used to detect inversions in the X-chromosomes of Sudanese and Egyptian *Anopheles phronesis* samples. The products were easily distinguished by gel electrophoresis. In addition, Egyptian samples were amplified at 100bp product, and the Sudanese products showed no bands in the gel electrophoresis. *Sudanese samples:* In all three PCR tests, no bands were detected in the Sudanese strains of *Anopheles pharoensis* (Figure 1).

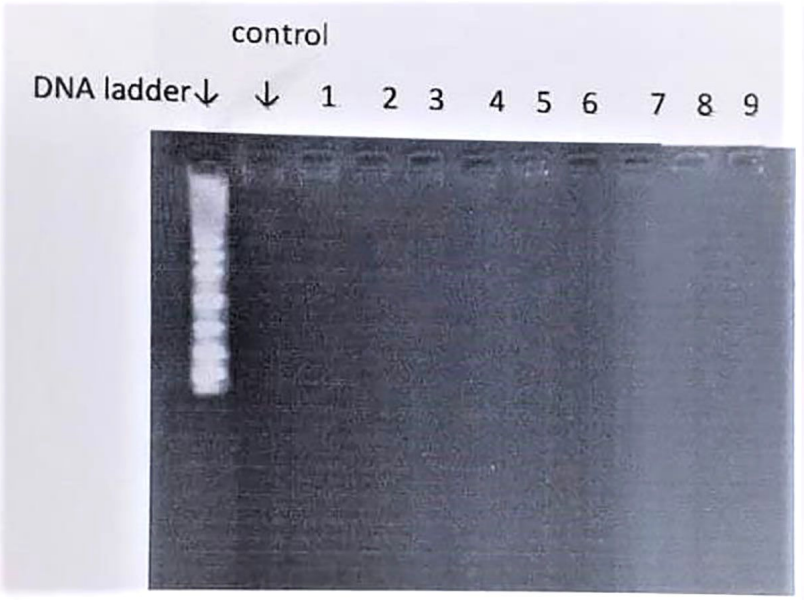

Fig 1. Gel Electrophoresis PCR product from Sudanese *Anopheles Pharoensis*

(Figure 1) The gel electrophoresis PCR product from Sudanese Anopheles Pharoensis, a PCR ladder, is clear with no bands detected.

Egyptian samples: Inversions were recognized by the appearance of bands in the gel electrophoresis (Figure 2) and (Figure 3). A triple test was undergone. In the first test, 7 samples out of 50 depicted bands. In the second test, 4 samples out of 50 depicted bands. In the third test, 10 samples out of 50 depicted bands. Using a DNA ladder, bands were recognized at ~ 100 Bp from the PCR.

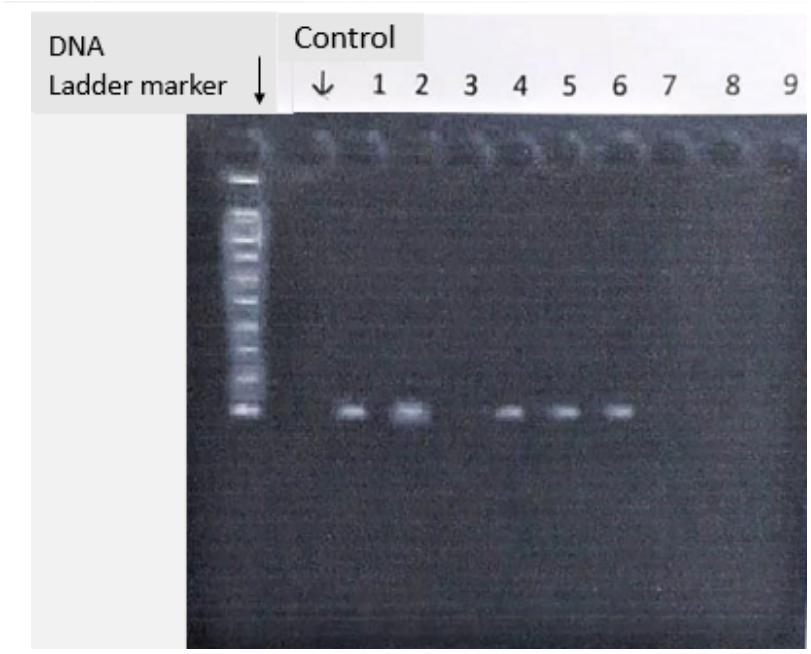

Fig 2. Gel Electrophoresis PCR product from Egyptian *Anopheles Pharoensis*.

(Figure 2) describes the gel electrophoresis PCR product from Egyptian Anopheles Pharoensis, and it shows the PCR ladder is clear (One Band was detected at 5 samples at ~ 100 Bp).

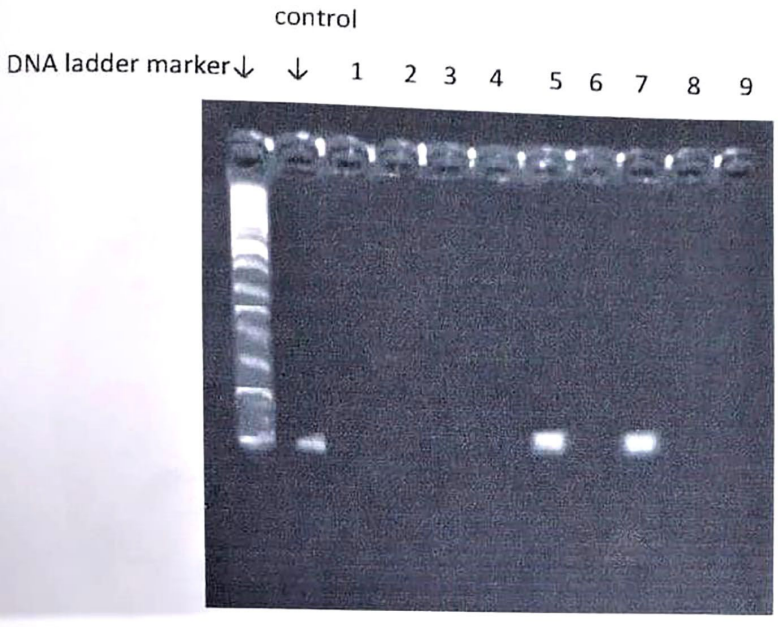

Fig 3. Gel Electrophoresis PCR product for Egyptian *Anopheles Pharoensis*.

In (Figure 3) it is clear that the gel electrophoresis PCR product for Egyptian Anopheles Pharoensis had one Band detected in 3 samples at ~ 100 Bp.

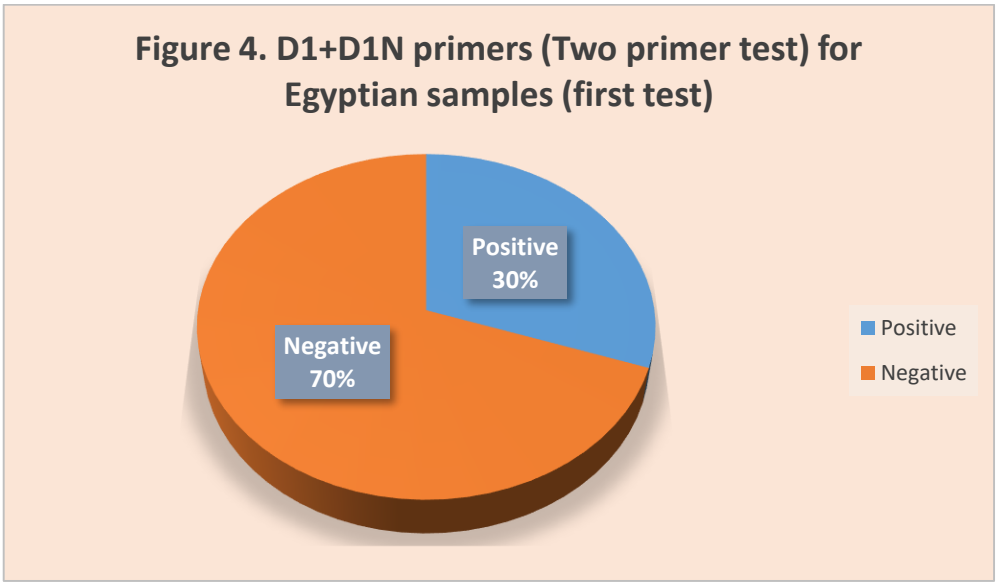

Fig 4. Two Primer tests for Egyptian *Anopheles pharoensis* first test.

(Figure 4) is showing D1+D1N primers (Two primer tests) for Egyptian samples (First test). There were 70% negative and 30% positive.

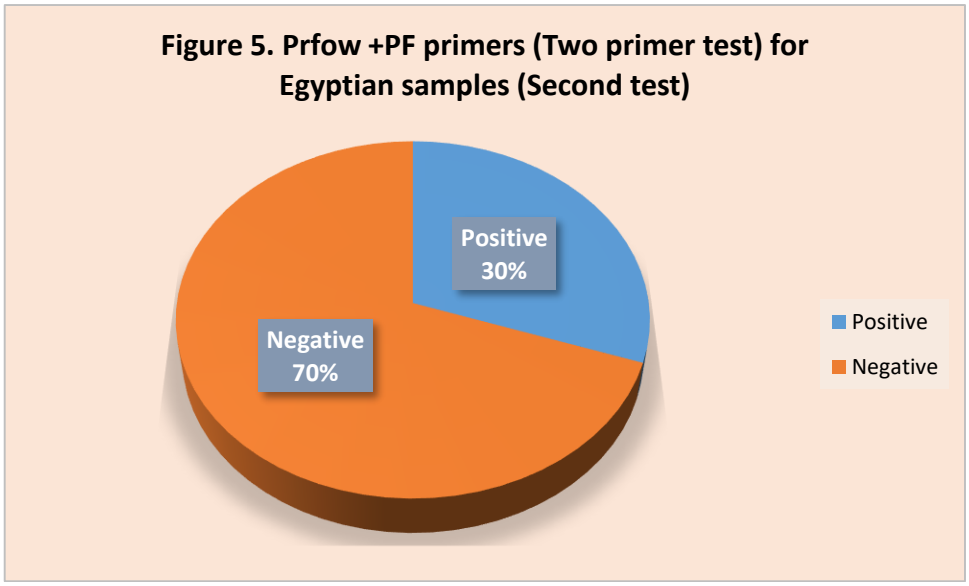

Fig 5. Two Primer test for Egyptian *Anopheles pharoensis* second test.

In (Figure 5) there are Prfow + PF primers (Two primer tests) for Egyptian samples (Second test), and they had70% negative and 30% positive for the second test.

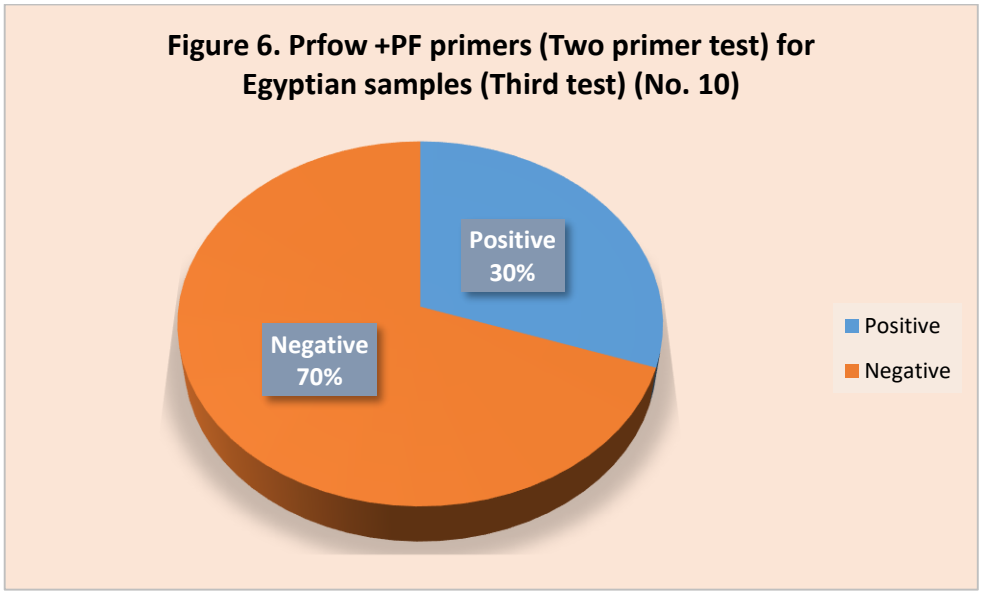

Fig 6. Two Primer tests for Egyptian *Anopheles* pharoensis third test.

In (Figure 6) we conducted Prfow +PF primers (Two primer tests) for Egyptian samples (Third test) (No. 10), and we found that there were 70% negative and 30% positive.

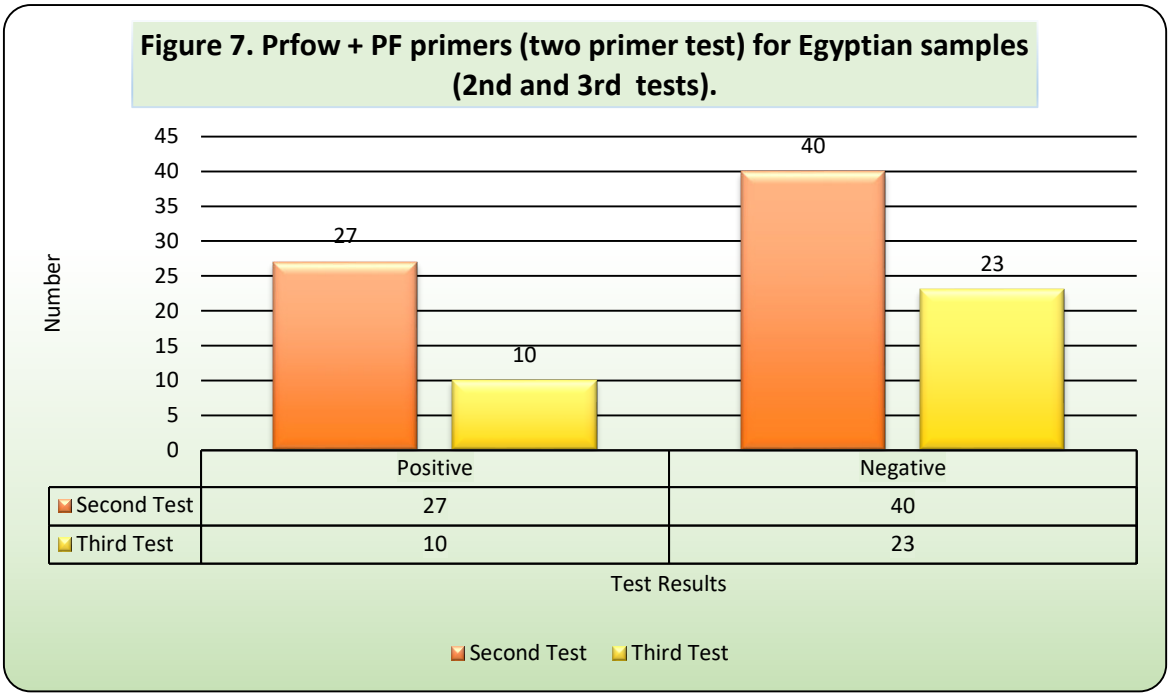

Fig 7. Prfow + PF primers (two primer tests) for Egyptian samples (2nd and 3rd).

In (Figure 7), we conducted shows Prfow + PF primers (two primer tests) for Egyptian samples (2nd and 3rd tests). And we found 27% positive and 40% negative in the second test. And in the third test, there were 10% positive and 23% negative.

4. DISCUSSION

Agarose gel electrophoresis is a reliable method for separating PCR products and comparing samples following a PCR reaction, as observed by Wittmeier and Humme, 2022.Gel electrophoresis was applied with PCR assay in this Research, and different results were observed for Sudanese *Anopheles pharoensis* and Egyptian *Anopheles pharoensis*[19]. A study by Akejuetal showed that out of 550 *Anopheles gambiae* complex species, 530 were found positive, and 20 were negative. Molecular analysis confirmed that 78.3% were *Anopheles gambiae*s. The DNA bands of this species were identified on the gel image at 390Bp, and 17.64% were found. *Anopheles arabiens is* identified at 315Bp on the gel image showing molecular variation. Our study detected bands for Egyptian *Anopheles pharoensis* at 100Bp in gel electrophoresis. In contrast, no bands were detected in the Sudanese species showing molecular variation between the two *Anopheles pharoensis* species [20]. Coulibaly et al. (2007) developed a PCR diagnostic assay that detected differentiation in arm arrangements in the X-Chromosome and thus differentiated between different forms in a species. Four primers, Prfow +PF and D1+D1N were used to detect inversions and breakpoints in the X-chromosome of Anopheles gambiae forms. Inversions were detected by clear bands at 250Bp and ~500Bp in the gel electrophoresis of the PCR product. Similar primer pairs (Prfow +Pr) and (D1 + D1N) were applied in this study for both Sudanese and Egyptian *Anopheles pharoensis*. The *Anopheles pharoensis* samples collected from Sudan showed no bands, while those collected from Egypt revealed the expected clear bands at ~100Bp in the inverted Band. The two primers detected inversions in the arm arrangement of the X-chromosome of the *Anopheles pharoensis* species. Of the Seventy-five *Anophelespharoensis* Egyptian strains detected, only very few revealed bands similar to the results of the PCR assay by Coulibaly et al. (2007) [14]. Molecular characterization of *the Anopheles gambiae* complex was studied in Sudan and the Republic of Southern Sudan by A.M. Hamza et al. (2008); The molecular investigation predicts the existence of two species within the *Anopheles gambiae* complex [21]. This finding is similar to the findings of our study, which has reached a similar fact that *Anopheles pharoensis* collected from Egypt and Sudan could be sister species or species complex. A study in southern Africa (2015) revealed that by studying the behavior and chromosomal inversions of Anopheles *pharoensis*, Anopheles *pharoensis* might be a species complex[15]. It is similar to the fact that this Research has shown that *Anopheles phronesis* collected from Egypt and Sudan may be sister species or species complexes. Further studies are needed to prove this. Garros et al. (2004) presented a multiplex PCR using 11 primers for identifying species complexes in *Anopheles funestus*[22].This method could be applied in future research to identify species complexes in the *Anopheles pharoensis* (Theobald) group of mosquitoes. Further research is needed to prove that *Anopheles pharoensis*is a species group. A study by Barron et al. (2018) on a new species in *the Anopheles gambiae* complex, *Anopheles fontenillei*, recently discovered in a forested area of Gabon, central Africa.Results placed the next taxon in the phylogenic tree with *Anopheles gambiae* complex. The study detected introgression with Anopheles *gambiae* and *Anopheles colluzzii* of genes directly involved in vectorial capacity[23]. Our study revealed a direct relationship between chromosome inversions in *the Anopheles pharoensis* Egyptian sample and its vectorial capacity showing interference of genes in vectorial capacity, which agrees with the results of Barron et al. Naumenko, 2020, Studied the inversions in X-chromosomes to identify species complexes or sibling species in Anopheles. Inversions were located in the X-chromosomes of many Anopheles species, thus separating one Anopheles species into many groups. On Studying *Anopheles gambiae* X-chromosomes, 5 chromosomal forms were observed: Bamako, Savanna, Mopti, forest, and Bissau[12]. Our study has come to the fact that *Anopheles pharoensis* could be a species complex similar to the findings of this study, but further studies are needed to prove this fact. Also, inversions were found in X-chromosomes, similar to our study's findings where

inversions were found in X-chromosomes of *Anopheles pharonesis* Egyptian strains. A study by Mashair Sir El Katimetal in Sudan (2021) revealed that *Anopheles arabiensis* was grouped into two population clusters: four and two populations. The Kassala state species showed high genetic differentiation from the other collected populations, including a population located in the same state, which may show signs of species complexes in *Anopheles arabiensis*[24], similar to the findings of our study, which has reached the fact that *Anopheles pharoensis* from Sudan and Egypt may be sister species or species complexes. A study by Lanzaro and Yoosook to develop a molecular diagnosis for chromosomal forms of *Anopheles gambiae* M and S. The study led to a suggestion that *Anopheles gambiae* is an early stage of speciation or species complex[25], similar to our suggestion in this study where *Anopheles pharoensis* could be a species complex. Another study by Yoosook Lee et al. observed that inversions in the M and S molecular forms of *Anopheles gambiae* were located in the X-chromosomes of *Anopheles gambiae*. The study concluded that genes located on X chromosomes might be the major force driving speciation between these chromosomal forms of *Anopheles gambiae*[26].In our study, we have reached similar results that X chromosome inversions have a strong relationship with speciation and vector transmission of disease and maybe the main reason why *Anopheles pharoensis* collected from Egypt and Sudan may be sister species or species complexes and also a reason that Egyptian strains are vectors and Sudanese strains are not(due to absence of the inversion in the X chromosome). A study revealed that many species complexes include members that make significantly different contributions to malaria transmission, such as blood meal preference, and differ in their biology and involvement in disease transmission[27]. This finding agrees with our study's findings, where *Anopheles pharoensis* collected from Sudan and Egypt shows the differences in the pattern of disease transmission and X-chromosome inversions, thus suggesting that they may be species complexes. Studies suggest that a comparison of morphological and molecular identification of anopheles species determined that species composition, host preference, and plasmodium infection showed incorrect results when using morphological identification alone. A study recognized 8 Anopheles species while 18 species were identified from molecular analysis, and the 18 species, 12, were not thought to be vectors and were found to carry plasmodium by PCR [28]. Further morphological and genetical studies on *Anopheles pharoensis* in Sudan are needed to prove whether it is a vector of Malaria or not. Petrarca et al. suggested that from more than 40 Anopheles samples collected from Sudan (2000), *Anopheles arabiensis* showed more inversions in the West than in the East African population. Sudan populations showed similarities with those from westwards than eastwards of the great rift valley[29].Further study is needed to prove the relationship between *Anopheles pharoensis* inversions from Egypt to Sudan and the direction of the increase of these inversions. A study by Sharma et al. (2020) suggested differences in the size and structure of X chromosomes and pointed to a possible role of DNA in speciation. *Anopheles gambiae* and *Anopheles colluzzi* shared variations in the heterochromatin band on the X chromosome. The study suggested that an inversion in the X chromosome may cause this genetic polymorphism. Our study observed inversions in the X chromosome of *Anopheles pharoensis* Egyptian strains and found that inversions may play an important role in speciation in *Anopheles phronesis* strains[30].

5. CONCLUSION

Anopheles pharoensis is an important Malaria vector in Egypt, especially in Faiyoum Governate. In Sudan, *Anopheles pharoensis* is not encountered in Malaria transmission in any part of Sudan. The PCR assay detected inversions in the X-chromosome of *Anopheles phronesis* collected from Egypt and Sudan. In addition, the appearance of bands in the gel electrophoresis detected inversions. Bands were detected in Egyptian strains, while no bands were detected in Sudanese strains.

6. RECOMMENDATIONS

We recommend the application of PCR assay and molecular biology tools to classify Anopheles species in Sudan. Moreover, we recommend using molecular biology tools to test and treat mutations in different Anopheles species. Further studies are needed to be conducted on the effect of different environmental factors on the genetics of *Anopheles pharoensis* as well as the possibility of the role of *Anopheles pharoensis* in disease transmission.

7. AUTHORS CONTRIBUTION STATEMENT

Najlaa Nasir, Fatima Ali, and Fatima Mohamed did the concept and methodology. Najlaa Nasir, Fatima Ali, and Fatima Mohamed did the validation and formal analysis. Najlaa Nasir, Fatima Ali, and Fatima Mohamed did the reagent preparation and data curation. The original draft was prepared by Najlaa Nasir, Mohamed Elamin, Ali Alshehri, Hatim Badri, Hatim Natto, Mashael Alfaifi, and Ahmed Osman. The writing and reviewing were done by Mohamed Elamin, Ali Alshehri, Hatim Badri, Hatim Natto, Mashael Alfaifi, and Ahmed Osman. All authors have read and agreed to the published version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

9. REFERENCES

1. Global vector hub; 2020. Global Vector Hub [cited Feb 10, 2023]. Available from: https://globalvectorhub.tghn.org/.
2. CDC. CDC – parasites – Malaria [online]; 2021. Available from: https://www.cdc.gov/parasites/malaria/index.html#:~:text=In%202020%20an%20estimated%20241 [cited 4/20/2023].
3. Mattah PAD, Futagbi G, Amekudzi LK, Mattah MM, de Souza DK, Kartey-Attipoe WD, et al. Diversity in breeding sites and distribution of Anopheles mosquitoes in selected urban areas of southern Ghana. Parasit Vectors. 2017;10(1):25. doi: 10.1186/s13071-016-1941-3, PMID 28086941.
4. Kenawy MA, Gad A. Elsaid, S.and A., Merdan(1982).Field studies on Anopheline mosquito larvae in Egypt(Diptera: Culicidae). J Egypt Public Health Assoc;57(5-6):541-62.
5. Soliman AA, Rifaat MA, Ibrahim MTH. Biology of Egyptian Anophilines (biological activity of *Anopheles pharoensis* in nature). Cairo, Egypt: Ain Shams University; 1967.

6. Kenawy M, Elsaid S, J, C, Bier. Anopheles population dynamics in two Malaria endemic villages in Faiyoum Governate Egypt. Journal Am Mosq Control Assoc. 1986;2(2):158-63.
7. Soliman AA, 1979. Entomological and parasitological studies about Malaria and its responsible vectors in Egypt.Annual report no.2 office of naval research contract no.14-77.G.O.044.
8. Kenawy MA. Development and survival of Anopheles pharoensis and An. Multicolor from Faiyum, Egypt. J Am Mosq Control Assoc. 1991;7(4):551-5. PMID 1787399.
9. Morsy TA, Elkadery AA, Salama MMI, Sabry AH, IMA, Elsharkawy. Studies on the bionomics and vector competence of Adult Anopheline Mosquitoes in FaiyoumGovernate, Egypt. Journal Egypt Soc Parasitol. 1995;25(1):213-44.
10. Wassim NM. Secondary structure and sequence of ITS2-rDNA of the Egyptian malaria vector Anopheles phronesis (Theobald). J Egypt Soc Parasitol. 2014;44(1):197-204. doi: 10.12816/0006459, PMID 24961025.
11. El Safi SH, Haridi AM. Field trial of the insect growth regulator, Dimilin, to control Anopheles pharoensis in Gezira, Sudan. J Am Mosq Control Assoc. 1986;2(3):374-5. PMID 3148687.
12. Naumenko AN, Karagodin DA, Yurchenko AA, Moskaev AV, Martin OI, Baricheva EM et al. Chromosome and Genome Divergence between the Cryptic Eurasian Malaria Vector-Species Anopheles message and Anopheles Daciae. Genes. 2020;11(2):165. doi: 10.3390/genes11020165, PMID 32033356.
13. Van Rensburg AJ, Hunt RH, Koekemoer LL, Coetzee M, Shiff CJ, Minjas J. The polymerase chain reaction method is a tool for identifying members of the Anopheles gambiae complex (Diptera: Culicidae) in northeastern Tanzania. J Am Mosq Control Assoc. 1996;12(2 Pt 1):271-4. PMID 8827604.
14. Coulibaly MB, Pombi M, Caputo B, Nwakanma D, Jawara M, Konate L et al. PCR-based karyotyping of Anopheles gambiae inversion 2Rj identifies the Bamako chromosomal form. Malar J. 2007;6:133. doi: 10.1186/1475-2875-6-133, PMID 17908310.
15. Norris LC, Douglas E. Norris; 2015Phylogeny of Anopheline in Southern Africa based on nuclear and mitochondrial genes. Journal of the Society for vector ecology,40(1):16-27.
16. WHO. Larval source management: an extra measure for malaria vector control [online]; 2013. Available from: https://www.who.int/publications/i/item/9789241505604 [cited 4/20/2023].
17. Santolamazza F, Della Torre A, Caccone A. Short report: A new polymerase chain reaction-restriction fragment length polymorphism method to identify Anopheles arabiensis from An. Gambiae and its two molecular forms from degraded DNA templates or museum samples. Am J Trop Med Hyg. 2004;70(6):604-6. doi 10.4269/atm.2004.70.604, PMID 15210999.
18. Badawi NA [MSc thesis]. University of Khartoum; 2002." Genetic characterization of Housefly Musca domestica.
19. Wittmeier, Humme. Agarose gel electrophoresis to assess PCR product yield: comparison with spectrometry fluorometry and qPCR. Bioteqchniques vol. Vol. 72(4); 2022.
20. Akeju AV, Titus A. Ollusi and Iyabo A. Simon-oke. Sci Rep. 2022. Molecular identification and wing variation among malaria vectors in Akure north local government area, Nigeria;7674:(2022C)12.
21. Hamza AM, Abukashawa SA MA.2014. Molecular characterization of mosquitoes of anopheles gambaiespecies complex from Sudan and the Republic of Southern Sudan. Journal of Mosquito Research 4(1).
22. Garros C, Koekemoer LL, Coetzee M, Coosemans M, S, Manguin. A single multiplex assay to identify major Malaria vectors within the African Anopheles funestus and the original Anopheles minimus group. Am J Trop Med Hyg. 2004;7(6):583-90.
23. Barron MaiteG, Christophepaupy, Nilrahola, Ousmanakoneella MF. Ngangue,Theodela,Wilson Bahun, Marcopombi, Pierekengne, Carlocostantini, Fredricsimard, Josefagonzalez, Diegoayala. Vectorial Evol Anopheles Gambiae Complex. 2018 .Digitalcsic. es.
24. Mash air Sir Elka Tim, Zairijaal, sumia Abukashawa, Sitiazizah Mohammed Norp. Malar J 19. 2021 Population genetics of Anopheles arabiensis the primary vector in the republic of Sudan; 469:20 (1).
25. Gregory C. lanzaro and yoosook lee, 2016. Speciation in Anopheles gambiae is the distribution of genetic polymorphism and patterns of reproductive isolation among natural populations—Anopheles mosquitos' book edited by Sylviemanguin.
26. Yoosooklee. Travisclier, Michelle R Sanford, Clare D marsden, Abdelrhman Efofana, Anthony J cornel, Gregory Clanzaro, (2013). Chromosome inversions genomic differentiation and speciation in the African malaria mosquito anopheles gambiae. PLOS ONE. 2013;8(3):e57887.
27. Frank H. collins, Lunakamau, Hilary A. Ranson and john M.vulule. Bull World Health Organ. 2000 molecular entomology and malaria control;78(12).
28. Lobo NF. Brandy St. Laurent and Frank H. Vol. 5. Collins; 2015.Unexpected diversity of anopheles species in eastern Zambia implications for evaluating vector behavior and interventions using molecular tools. Science report. p. 17952.
29. V.Petrarca,A.D.Nugud,M.A.Ahmed,A.M.Haridi,M.A.Deco,Mcolluzzi. Cytogenetics of the Anopheles gambiae complex in Sudan with special reference to an arabiensis: relationships with east and west African populations. Med Vet Entomol. 2000,14(2);2000:149-64.
30. A.sharma, Timoshevskiy VA, sharakhova mV, sharakhov iV. Nicholas. Kinney. Genes. 2020. Structural variation of the X chromosome heterochromatin in the Anopheles gambiae complex;11(3):327.