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Expression of TAg 5 in Relation to Presence of Trypanosome Parasites in Tsetse Flies Captured Kagarko LGA, Kaduna State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author ASU designed the study, participated in sample collection, interpretation of results and corrected the manuscript. Author AIK also participated in interpreting results and correction of manuscript. Author MSA collected samples, handled literature searches and wrote manuscript. All authors read and approved the final copy of manuscript.

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Original Research Article

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ABSTRACT

Tsetse antigen 5 has an accession number of AF259957 and full length transcript of 926 bpconcealed by its complementary DNA without the poly (A) tail. It forms constituent of Tsetse saliva and is therefore injected into the mammalian host during Tsetse feeding and is also known to promote Trypanosome establishment in the host by modulating host's immune response. This research studied the expression rate of Tsetse Antigen 5 using molecular tools and also the relationship between expression of Tsetse Antigen 5 and presence of *Trypanosoma* parasites in field captured flies in Kagarko Local Government Area, Kaduna state, Nigeria. During the study, fourty seven (47) Tsetse flies were caught within the period of four (4) months. Only thirty-eight (38) of the fourty seven (47) captured flies were dissected as nine died. Thus, percentage mortality of (19.15%). Dissection was done using the procedure described by the FAO Training manual for Tsetse control personnel and identification of Trypanosomes was done by direct observation of

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dissected organs under a microscope. RNA extraction was carried out using Trizol plus RNA extraction kit. Further to that, complementary DNA was synthesized from the extracted RNA which was used for reverse transcription PCR (RT-PCR) using gene specific primers. 4 (10.53%) of the 38 Tsetse flies examined, had expression of Tsetse antigen 5 and also had the presence of *Trypanosoma* parasites. The expression of Tsetse antigen 5 in field captured flies infected with *Trypanosoma* parasites showed a positive relationship between presence of *Trypanosoma* parasites and the expression of Tsetse antigen 5 in field captured Tsetse. The expression of Tsetse antigen 5 is associated with that of Trypanosome infection in the captured flies corroborating thegene's role in aiding successful transmission of Trypanosomes from vector to host.

Keywords: Tsetse flies; tsetse antigen 5; trypanosoma; parasites; Kagarko LGA; Kaduna.

1. INTRODUCTION

Tsetse antigen 5 (TAg 5) has an accession number of AF259957 [1] in the GenBank and full length transcript of nine hundred and twenty-six base pair concealed by its complementary DNA (cDNA) without the poly (A) tail [2]. The gene has two hundred and fifty-nine (259) amino acid residues on its open reading frame (ORF) that encodes for a protein of 28.9KDa and an iso-electric point of 8.27 [1]. TAg5 has a non-polar end enclosed in its N-terminus that has some signal peptide characteristics [1].The presence of the transcript peculiar to the gene is observed in all developmental stages of Tsetse fly but it is however principally expressed in salivary glands of the fly. In addition to the salivary gland, it is also present in proventriculus and mid-gut [3]. The expression of the gene differs within different species of Tsetse fly [4] and with sex of the fly with male adult flies having more genes than female[1]. The gene happens to be a homologue of antigen 5 string venom with its presume products very much related to that of Agr I and Agr II complementary DNAs from Drosophillia and IuloAg 5 from Lutzomia [3]. Molecules expressed in the Tsetse salivary gland, which include Tsetse antigen 5 likely play both direct and indirect roles in the growth, maturation and transmission of trypanosomes. It is also one of the proteins that is believed to hypothetically triggers mechanisms which affects the localization and establishment of blood streams forms of trypanosomes [5] and the presence of trypanosoma parasite greatly affect genes which encodes most saliva proteins in the salivary gland [6].

Tsetse antigen 5 is immunogenic and also forms constituent of Tsetse saliva, therefore injected into the mammalian host during Tsetse feeding and is known to promote Trypanosome establishment in the host by modulating host's immune response [7]. This research was designed to study the expression of Tsetse Antigen 5 using molecular tools and also to determine if the expression of the gene is related to the presence of parasites in field captured flies in the study area.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Kagarko Local Government Area of Kaduna State, Nigeria (co-ordinates 9°27´0" North, 7°41´0"East) which covers an area of 1,864 km^2 with a population of 240,943 [8]. The study location was picked due to reported cases of the disease outbreak in some part of the area [9] and also scarcity of information on the presence of TAg 5 and its relationship with presence of *Trypanosoma* parasites in flies captured from Kagarko Local Government Area, Kaduna, Nigeria.

2.2 Sampling of Tsetse Flies

Sampling was done for a period of four (4) months in accordance with the method of Challier and Larvessier [10] as cited by Waber et al. [11]. Ten (10) standard biconical traps were set 100m apart across the three (3) sampling sites (River Dogo, Kubacha Forest Reserve and River Babuwa). Traps were baited with cow urine and acetone [12] to attract the flies and improve catch were placed in grazing passages of animals and river side where animals pause to drink water. Metal poles were greased with oil to stop insects from bugging the traps. The location of each trap was recorded using a GPS (Etrex 10 Garmin) and were allowed to stay at sampling sites for a day before harvesting.

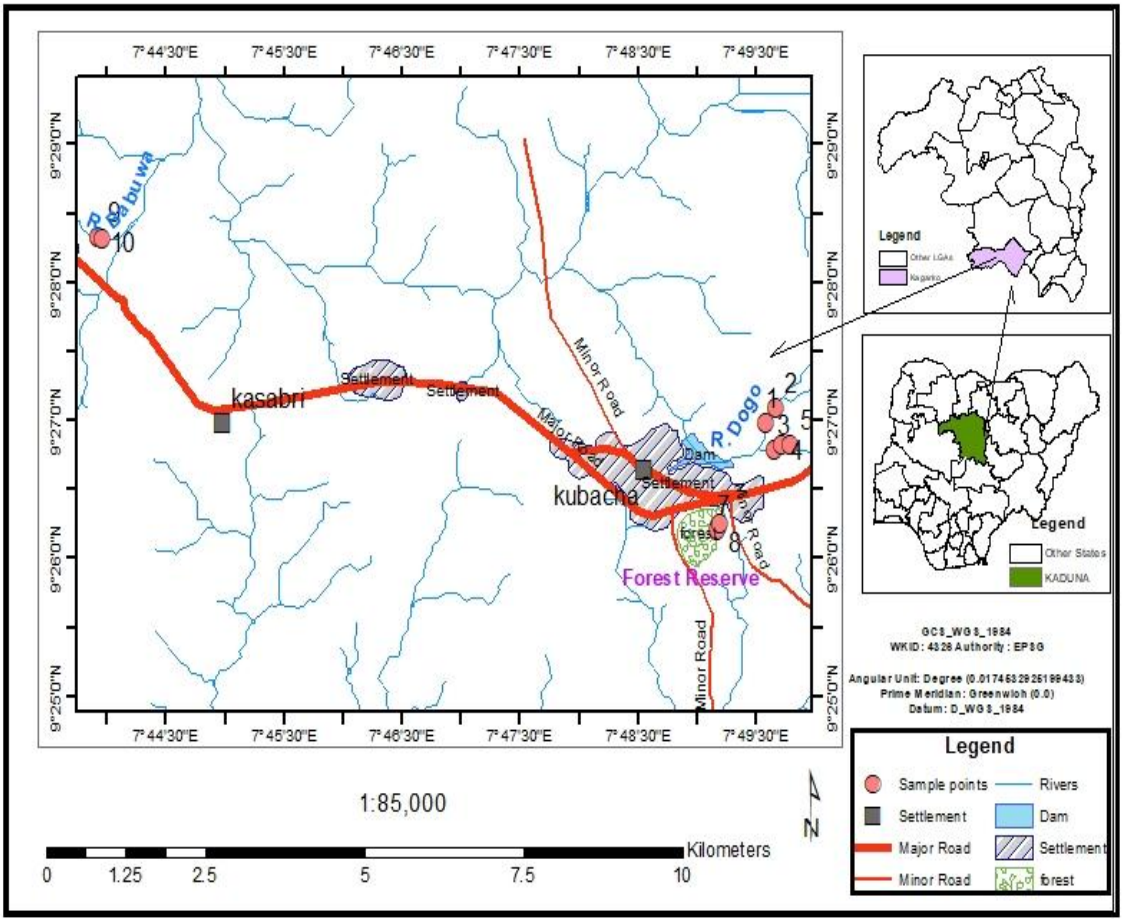

Fig. 1. Map of Kagarko LGA showing sampling points

2.3 Identification and of Sorting of Files

Trapped flies were identified, counted and arranged in order of species and sex by certain morphological features. This was possible by using identification key described by Leak et al. [13] as cited by Wama et al. [14]. Distinguishing features were looked for on the mouth parts (Proboscis), wings, antenna and eyes for Tsetse identification. Differentiation based on species was done by observing the sizes, colours found on Tsetse abdomen and nature of clasper found on male genital armature. Male flies were differentiated from the female on the basis of their abdomen. The male fly possesses on its abdomen the hypogeum which is missing on the abdomen of a female Tsetse.

2.4 Dissection of Flies

Flies were kept in a petri dish under dissecting microscope and saline solution was added in drops. After removing the wings and legs, the mouth part was detached and its different parts were disjoined. The salivary glands and the midguts were also carefully removed and placed between glass slide and cover slip. All these organs were viewed under a microscope (Mg x 400) for the presence of parasites. This procedure was earlier described by the FAO Training manual for Tsetse control personnel [15] cited by Mulugeta [16].

2.5 Identification of Trypanosomes

The usual technique of microscopy was used in which a direct observation of dissected organs under a microscope was employed Lloyd and Johnson [17] cited by Okoh et al. [18]. Hence, proboscis, salivary glands and midguts were examined. The parasites were identified based on their locations. The parasites found only in the proboscis were recognized as *T. vivax* while those found in both proboscis and midguts were

termed *T. congolense*. Parasites associated with mid guts and salivary glands were identified as *T. brucei* and those restricted to the midguts were regarded as un-developed *T. brucei* or *T. congolense.*

2.6 Preservation and Transportation of Dissected Flies

Dissected flies were labelled indicating trypanosome status, species, sex and date of collection, and kept in iced condition before transported to the laboratory for molecular procedures.

2.7 RNA Extraction

Trizol plus RNA extraction / purification kit (2016) was used for the extraction of RNA as described by the manufacturer's guide. 1ml of trizol reagent was added to a blended fly to make a solution. The lysate was centrifuged at 12,000 rpm for 5 minutes at a temperature of 4 – 10^{o}C. A fresh tube was used to collect the supernatant and incubated for 5 minutes in order to achieve a full separation of the nucleo-protein complexes. Chloroform (0.2 ml) was added to the tube covered and incubated for two 2 to 3 minutes after which it was centrifuged at 12,000 rpm for 15 minutes at 4°C. This resulted in separation of mixture into a lower red phenol-chloroform, an interphase and a colourless upper aqueous phase. The colourless upper aqueous phase containing the RNA was collected in a new tube and to it 70% ethanol of equal volume was added and mixed by vortexing. After mixing, all visible precipitates were discarded from the tube.

A spin cartridge attached to a collection tube was used to collect about 700µl of the sample which was centrifuged for 15 seconds at 12,000 rpm. The flow through was thrown away and the spin cartridge was replaced into the same collection tube. These processes of collecting the sample, centrifuging and dispensing of flow through were repeated until whole sample was processed and in order to increases sharpness of RNA, on column DNase treatment was performed.

700µl of wash buffer 1 was added to the spin cartridge and centrifuged for 15 seconds. The flow through was thrown out and spin cartridge was replaced back into the sample collection tube. 500µl of wash buffer II was added to spin cartridge and centrifuged for 15 seconds at 12,000xg. Flow through was thrown out and spin cartridge was re-placed back into the collection tube and the process of centrifuging was repeated as well as dispending flow through and addition of 500µl of wash buffer II. Centrifuging was carried out for a minute at 12,000xg to dry the membrane. The spin cartridge was attached to a recovery tube this time and RNase free water was added to the spin cartridge at the middle. Addition was done in 100ml for 3 times which at the end were collected in a single tube. It was incubated for a period of 60 seconds and centrifuged for about 120 seconds at a speed greater than 12,000xg. The spin cartridge was thrown away whereas the total RNA was collected in the recovery tube. Now the total RNA precipitates formed a white gel-like pellet at the bottom of the recovery tube.

2.8 Complementary DNA (cDNA) Synthesis

Synthesis of complementary DNA was done using super Script™ IV First-Strand Synthesis System (2017) as instructed by the manufacturer. To the extracted RNA, 1µl of gene specific reverse primer and 1µl of mixed dNTPs was added. DEPC treated water 13µl was added, mixed appropriately and centrifuged. Furthermore, the mixture was heated for 5 minutes at a temperature of 65^{o}C before incubated one ice of for the shortest period of 60 seconds.

The 5x SSIV buffer was vortexed and centrifuged for a while to mix very well. 4µl of the buffer was fetched and combined with 1µl of DDT, one 1µl of ribonuclease inhibitor and to it one 1µl of the SSIV reverse transcriptase enzyme was added. The mixture was covered in a tube and blended before been centrifuged for a while. Now the 2 mixtures above (Annealed primer to template RNA and prepared reverse transcription (RT) mix) were combined together, incubated at 50-55^{o}C for a period of 10 minutes. It was further inactivated by incubating at eighty degrees centigrade 80^{o}C for 10 minutes. RNA was cleared out by the addition of one 1µl E. coli RNase H, and incubated for 20 minutes at a temperature of 37^{o}C.

Reverse transcription reaction (cDNA) was used immediately for reverse transcription polymerase chain reaction (RT-PCR).

2.9 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Gene specific primers were used as described by [1] for amplification of TAg 5. Primer sequences are as follows;

Forward: 5´-ATGGCCACTATGAAATGG- 3´
Reverse: 3´-CAGAAGCGGCAACCC- 5´

The RT - PCR was performed according to the protocol of Super Script™ IV RT-PCR reactions (2017) described as follows; The addition of one microliter (1µl) of gene specific primers of both forward and reverse direction into PCR tubes containing (37.8µl) of DEPC-treated water, five microliter (5µl) of PCR buffer, two microliter (2µl) of $MgSO_4$, one microliter (1µl) of the dNTP mix, two microliter (2µl) cDNA and (0.2µl) of DNA polymerase enzyme all referred to as PCR amplification mix. The PCR amplification mix was blended mildly and centrifuged for a while. The thermal cycler was pre-heated up to a temperature of ninety-four (94^oC) before loading of PCR tubes. The PCR amplification was achieved via the following conditions.

An initial denaturation was performed which lasted for one hundred and twenty (120) seconds at a temperature of 94^oC. The three (3) PCR steps denaturation, annealing and extension were performed under the following set of conditions;

A denaturation step was performed for fifteen (15) seconds at a temperature of 94^oC while annealing ran for a period of thirty (30) seconds at a temperature of 55^oC and extension was done for sixty (60) seconds at a temperature of 68^oC. These three (3) steps were maintained for thirty- five (35) PCR cycles and were finally held at 4^oC for a period of considerate time.

2.10 Gel Electrophoresis

A mixture of RT-PCR products and the loading dye were subjected to electrophoresis in 1.5% agarose stained with ethidium bromide following the protocol of Addgene as described by Lee et al. [19]. The two ends of the electrophoresis tank (i.e. positive and negative) were plugged to a power source and allowed to run for fourty (40) minutes in order to determine various sizes of resulting PCR fragments. Results were viewed and documented after removal of the gel. A UV trans-illuminator with the quantity one software was used to view the bands. A hundred base pair ladder was set and bands were seen at the five hundred and twenty base pair ladder on lanes 2,7,10 and 11 respectively.

3. RESULTS

Bands from the Gel electrophoresis, indicated that cDNA of TAg 5 was expressed in four (4) out of the thirty-eight (38) dissected flies shown in Fig. 2. Furthermore, three (3) that is (7.89%) of all flies that expressed the gene were male flies while only (1) that is (2.63%) was a female fly. Thus, giving an overall expression rate of (10.53%) as presented in Fig. 3.

All the four flies that had cDNA of TAg 5 present were also found with *Trypanosoma* parasites depicted in Fig. 4.

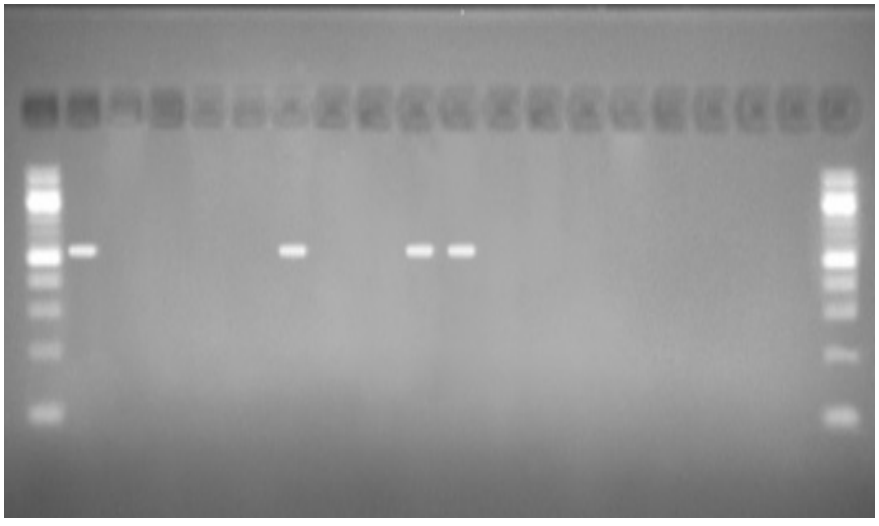

Fig. 2. An agarose gel picture showing expression of TAg 5 cDNA in captured flies from Kagarko LGA, Kaduna State, Nigeria

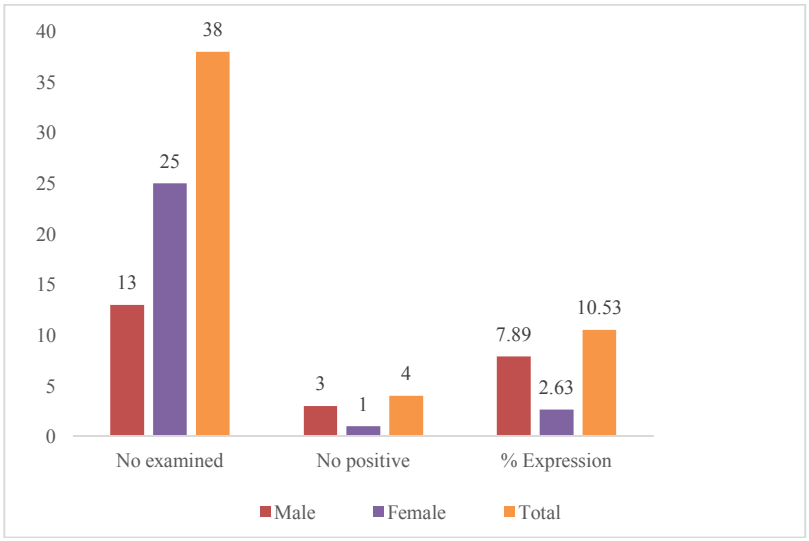

Fig. 3. The number of flies examined, number positive and the expression of Tsetse antigen 5 based on sex of flies caught among the three sampling sites within Kagarko Local Government Area, Kaduna, Nigeria

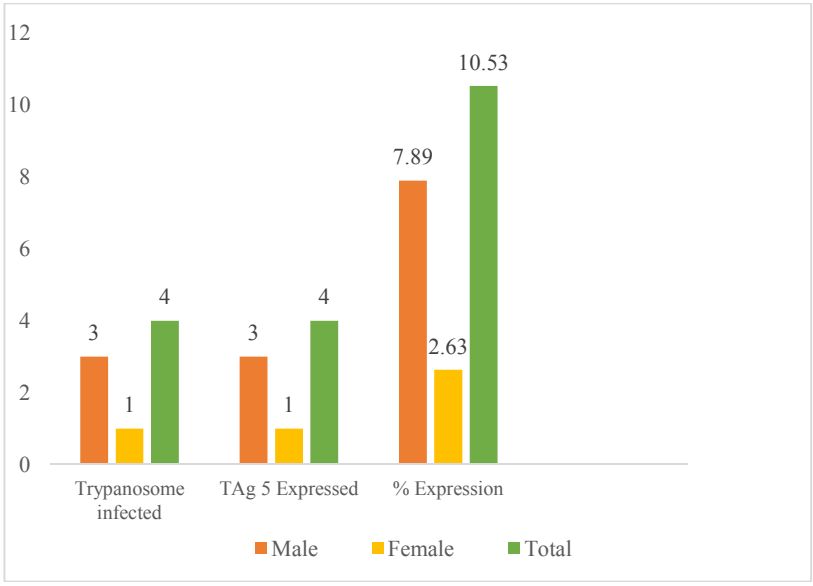

Fig. 4. The relationship between the presence of Trypanosomes and expression of Tsetse antigen 5 among the three sampling sites within Kagarko Local Government Area, Kaduna, Nigeria

4. DISCUSSION

cDNA of Tsetse antigen 5 was found in four of the thirty-eight (38) examined flies giving an expression rate of (10.53%). The expression of the gene is low and this is associated with differences in species as higher expression of TAg 5 was observed in members of other groups of *Glossina morsitans*. This finding agrees with a study conducted by Li in USA who reported having a significantly less expression of TAg 5 in members of the group *Glossina palpalis palpalis* [1] and the work of Haddow et al. [5]. The presence of parasite from both salivary gland and mid gut of flies found in this studies is in agreement with previous studies conducted where Parasites were recovered from both organs in trypanosome infested Tsetse [20].

Similarly Studies conducted by Guy et al. [21] showed the presence of Tag 5antigeneinTsetse flies Saliva.

Although, 3 of the flies representing (7.89%)that expressed the gene were male and only 1(2.63%) of the flies was a female, this study indicated that TAg 5 expression has no statistically significant association with sex of flies however, this might be is associated with the type of flies used. Insects reared in the laboratory were used in previous works compared to field captured flies used in this work. This disagrees with a study carried out in USA by [1] who reported having higher expression of TAg 5 in male flies.

The expression of TAg5 among captured flies showed a statistically significant association with the presence of Trypanosomes. Again this is attributed to the type of flies used as previously mentioned.

This result does not agree with those of previous studies who reported having a down regulation of TAg 5 in parasitise tissues [1,22,23].

5. CONCLUSION

The present study concludes that there is expression of TAg 5 (10.53%) in flies captured from Kagarko LGA, Kaduna state, Nigeria and that the expression of Tsetse antigen 5 is associated with that of Trypanosome infection corroborating the gene's role in aiding successful transmission of Trypanosomes from vector to host.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard written ethical permission has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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