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Research Article

Insecticide Resistance Profile and Detoxification Enzyme Activities of *Anopheles* Mosquitoes from Three Different Breeding Habitats in Sudan Savannah Region of Jigawa State, Nigeria

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ABSTRACT

Malaria is a disease of global public health concern. The disease has its highest global burden and mortality in Africa while Nigeria has up to 25% of global malaria burden. Prevention is achievable through targeting mosquito vectors with insecticides. A primary concern in the global malaria control is resistance to insecticides which affects vector control interventions. It is therefore significant to know mosquitoes' insecticide resistance profile. This study is aimed at determining insecticide resistance profile and enzyme activities of Anopheles mosquitoes from three different breeding habitats. Larval samples were collected during rainy season (August, September and October, 2019) from Hadejia and Dutse towns of Jigawa State. Larvae were reared to adults in the insectary and morphologically identified. WHO insecticide bioassay was conducted using permethrin 0.75%, deltamethrin 0.05%, bendiocarb 0.1%, and dichlorodiphenyltrichloroethane 4%. About 100 samples (20 – 25 per test per four replicates) were used for each tested insecticide. Results showed preponderance of *An. gambiae* complex in the three study sites. High resistant levels according to WHO protocol with mortality less than 90% were recorded for all the tested insecticides, suggesting resistances in the Anopheles mosquitoes. Highest monooxygenase, glutathione-s-transferases and esterases activities were recorded in agricultural site compared to other sites. These findings can be attributed to differences in activities that occur in the study areas. Findings of this study can serve as threat to vector control measures in the study sites.

Keywords: *Permethrin, deltamethrin, bendiocarb, Dichlorodiphenyltrichloroethane*

INTRODUCTION

Malaria is a disease of immense global public health concern. The disease has its highest global burden and mortality records in Africa. Malaria in the African continent usually affects children below the age of five and pregnant women. Malaria negatively affects the African economy by contributing to poverty, lower productivity etc. (WHO, 2017). Countries in Sub-Saharan Africa have continuously recorded high global malaria cases. There was an increase in number of cases from 228 million in 2018 to 229 million in 2019. About 94% of global malaria cases and deaths in 2019 were recorded in Africa (WHO 2021). Greater than half of all the global malaria cases come from six countries including; Nigeria (25%), Democratic Republic of Congo (12%) and Uganda having (5%) (WHO, 2019). In Nigeria, 76% and 24% of the population live in high and low malaria transmission areas respectively (UPMI, 2020). Prevalence of malaria in children under the age of five in Nigeria is 23%, although this differs among regions (WHO, 2019). Malaria can cause anaemia which can result in maternal mortality. It can also result in complications like high placental plasmodia burden, fetal complications, low birth weight and new born death (Erhabor *et al.,* 2010; Jenavine *et al.,* 2015). Mortality caused by malaria have remarkably reduced annually as a result of the scale-up of long-lasting insecticidal nets (LLINs) (Strode *et al.,* 2014) and implementation of indoor residual spraying (IRS) (Mnzava *et al.,* 2015). This progress in some areas has been affected, with an increase of 2 million cases from 2016 to 2017 (WHO, 2018).

A key control strategy against major mosquito-borne diseases such as malaria, dengue, yellow fever, etc., involves targeting mosquito vectors to stop the transmission of these diseases (Mulla, 1994). This is due to the strong relationship that exists between vectors and transmission of pathogens to humans and other vertebrates (Wilke and Marrelli, 2015). Preventing infection, prompt diagnosis and effective treatment are measures used to control malaria and its complications (Kalilani- Phiri *et al.,* 2011). Vector control strategies involving insecticide-based measures are effective against major mosquito-borne diseases (Niyang *et al.,* 2018). Resistance to insecticides remains a primary concern in global malaria control, it has potential implications to the effectiveness of available vector control interventions and it is a rising problem among *Anopheles* vector populations (Stica *et al.,* 2019; Safiyanu *et al.,* 2019), where vector control primarily relies on insecticide-based measures (Camara *et al.,* 2018). From available records of the 80 malaria-endemic countries for year 2010 onwards, 68 countries recorded reduced susceptibility to at least one insecticide among vector populations, while 57 countries recorded resistance to two or more chemical classes of insecticides (WHO, 2018). Knowledge of mosquitoes' insecticide resistance is of primary importance in the control of resistance to insecticides (Safiyanu *et al.,* 2019). Close monitoring of insecticide resistance in disease vectors can assist in observing resistance changes over a period of time in a given site or changes between distinct sites (WHO, 2017). Global plan for insecticide resistance management indicates increase threat caused by insecticide resistance and calls for immediate measures to have suitable resistance management strategies against malaria vectors in order to have continued effectiveness of the available control interventions (WHO, 2012). Indiscriminate use of insecticides can result in rising insecticide resistance that can make use of insecticides ineffective and limiting the available options for malaria control (WHO, 1998). A study by Safiyanu *et al* observed insecticide resistance in both residential and irrigation sites of their study area and concluded that the resistance found may be due to increase activities of detoxifying enzymes, induced by uncontrolled use of insecticides and agrochemicals that were used against malaria vector and other flying insects in the study sites (Safiyanu *et al.,* 2017).

Long-term use of insecticides in agricultural practices to control pests and disease vectors has led to the selection of resistance in numerous insect species (WHO, 2013). The broad use of dichlorodiphenyltrichloroethane (DDT) in the 1950 – 1960s, followed by the current rise in use of pyrethroids in LLINs, and the widespread use of the same insecticides in agricultural practices have resulted in the development of resistance in mosquito populations globally (Djouaka *et al.,* 2008). Malaria control strategies make use of insecticides to eliminate the disease vector. As the efforts in controlling the disease intensifies, so is the rise in selection pressure to insecticides by malaria vector to develop resistance to the available insecticides. The strength and distribution of this resistance has dramatically risen in recent years and now threatens the success of malaria control programmes (Ranson and Lissenden, 2016). Insecticide resistance primarily involves various genetic modifications (point mutations in coding regions, transcriptional changes, and changes in gene amplification). These genetic modifications favour increase in rates of insecticide detoxification (metabolic detoxification), or decrease the sensitivity of target proteins (target site insensitivity) (Raymond *et al.,* 1998; Hemingway *et al.,* 2002).

Increased activity of detoxification enzymes has been shown to cause insecticide resistance by breaking down the insecticide before it reaches its target sites of action and/or decreasing the sensitivity of the target sites (voltage-gated sodium channel) for pyrethroids (Nwane *et al.,* 2009) and binding site (acetylcholinesterase) for carbamate (Weill *et al.,* 2004). Esterase activity is connected to an increase in the relevant gene (Hemingway *et al.,* 1998). Glutathione-S-transferases (GSTs), a dimeric protein family, play an important role in the breakdown of organophosphates and organochlorines (Clark, 1990). Earlier research found significant monooxygenase, GST, and esterase activity in pesticide resistant vectors, suggesting that enzymes play a role in insecticide resistance owing to unregulated insecticide administration, which leads to pressure selection (Alhassan *et al.,* 2015; Safiyanu *et al.,* 2017).

In some studies, the esterase activity of resistant vectors was found to be higher (Yang *et al.,* 2004; Wu *et al.,* 2004). Esterases are enzymes that can break down ester bonds in pyrethroids, carbamates, and organophosphates. Esterases hydrolyze ester bonds to break down synthetic pyrethroids, carbamate, and organophosphate (Crow *et al.*, 2007). Insects' cytochrome p450 monooxygenase breaks down foreign chemicals like pesticides, which can lead to insecticide resistance (Wen *et al.*, 2003).

Improved knowledge of recent resistance levels and its underlying causes is important in designing proper management strategies and to limit future selection for insecticide resistance (Stica *et al.*, 2019). This study analyses the susceptibility profile, and levels of detoxifying enzymes in *Anopheles* mosquitoes from the Sudan savannah region of Jigawa State, Nigeria.

MATERIALS AND METHODS

The study was carried out in Sudan savannah region of Jigawa State. Three sites were studied; agricultural and industrial sites from Hadejia town (Latitude: 12°44′98″N, Longitude: 10°04′44″E) and residential site from Dutse town (Latitude: 11°75′62″N, Longitude: 9°33′90″E). The State has a total land area of approximately 22,410 km^2 and a density of 251.7 per km^2. It has coordinates of: 120 00^1 N 90 45^1 E between latitudes 11.00^0 N to 13.00^0 N and longitudes 8.00^0 E to 10.15^0 E (JGS, 2015). Occupation of inhabitants include: fishing, rice farming and establishment of irrigation based activities

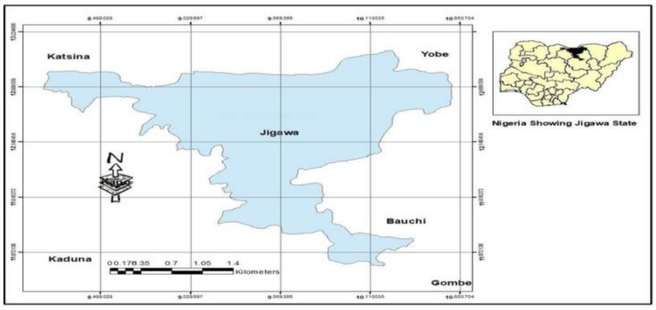

Figure 1. Coordinates of Sampled Study Site (Abdulkadir *et al*., 2019)

Anopheles larval collection

The larvae were collected from their natural breeding sites at different points in the study sites. Samples were collected during rainy season (August, September and October, 2019).

Anopheles mosquito larvae were cluster sampled from randomly selected water bodies on vegetation farms, which were commonly used for irrigation (agricultural sites). Also, at industrial and residential sites, *Anopheles* mosquito larvae were sampled from randomly selected chocked gutters, waterlogged, marshy/swampy areas around each study site. These sampling sites were found highly polluted with organic materials. The larvae were identified based on presence of head having mouth brushes, segmented abdomen with thorax. Also, presence of respiratory siphon and position parallel to the water surface to breathe through spiracles on the abdomen were checked. A dipper was used to obtain *Anopheles* larvae after screening for its presence. The larvae were transferred along with breeding waters to the holding containers before searching for more. This procedure was done several times until significant numbers of larvae were obtained for each period of the study, according to method of Robert *et al.*, (2002).

Rearing of *Anopheles* larvae to adults in the insectary

The larvae in their breeding site water were transferred into white plastic containers. The mouth of the container was covered with an untreated mosquito net (Service, 1993). Larvae collected were transported to the insectary (Bayero University, Kano, Nigeria) with the following conditions: temperature 25°C to 33°C, humidity 70% to 80%, and a 12-hour day/night cycles, according to Das *et al.* (2007). Larvae were fed with Tetramin™ baby fish food (about 10 g dissolved in 50 ml) once daily until adults emerge. The pupae were separated and placed in mosquito net cages until adults emerged. Adults were fed with 10% sucrose solution using cotton wool (Service, 1993).

Identification of species by morphological species identification keys

Anopheles species were identified morphologically by standard keys for identification (Gillies and Coetzee, 1987). Characteristics unique to all *Anopheles* mosquitoes were screened using a Zeiss ×10 light microscope. The identification checked dark spot at the upper margins of the wings that is common to all *Anopheles*. The number of segmentations and palpis elongation were also checked. The legs were considered for the presence of speckles on the third preapical dark area on vein 1, a pale interruption and tarsi 1-4 with conspicuous pale bands which were common features for *Anopheles gambiae*.

WHO insecticide bioassay tests

Mosquitoes’ insecticides diagnostic kit was used to establish susceptibility and resistant status. Insecticide susceptibility bioassays were performed using 2- to 4-day-old adults that emerged, they were randomly selected from pool and used for insecticide bioassays as described by (Cuamba *et al.*, 2010; Wondji *et al.*, 2012) using the WHO protocol (WHO, 1998) under room temperature ranging from 25°C to 31°C and relative humidity of 65% to 80%. The following insecticides were tested: 0.75% permethrin (type I pyrethroid), 0.05% deltamethrin (type II pyrethroid), 0.1%bendiocarb (carbamate), and 4% DDT (organochlorine).

For each insecticide, about 100 adult mosquitoes were divided into batches of 20 – 25 active mosquitoes per test per four replicates and were exposed to insecticides treated paper for 1 hour, the effect of paper treated with only carrier oils were assayed in parallel as control. The knock down rate was recorded after each 15 minutes as 15min, 30min, 45min and 60min in succession for 1 hour. At exactly 1-hour post exposure, the mosquitoes were transferred back to the holding tubes for 24 hours during which the number of dead mosquitoes, temperature and humidity were recorded, they were fed on glucose using 10% glucose solution soaked on pad of cotton wool placed on mesh-screen end of the holding tubes. Mosquitoes were considered dead or knocked down when immobile or not able to fly. After the bioassay, mosquitoes were transferred to appropriately labelled tubes having a lid for airtight locking. Mortality rates between 98 - 100% indicated full susceptibility, 90 -97% indicated possible resistance and less than 90% was considered as resistant to the tested insecticides (WHO, 1998). Percentage mortality was recorded by counting the number of dead and alive mosquitoes.

Enzymes analyses

Mosquito samples were analysed for monooxygenases, glutathione -s- transferases and esterases. Single mosquito was individually homogenized with glass rod in 150µl ice cold distilled water and was centrifuged at 13000g for 2 minutes. Monooxygenases were obtained by method of Borgdon *et al*. (1998). Monooxygenase catalyze the reduction of hydrogen peroxide and oxidation of tetramethylbenzedine to give water and oxidized blue colour tetramethylbenzidine at 630nm. Crude estimate of the amount of monooxygenases present were determined using a standard curve of cytochrome c**.** Glutathione -s- transferases (GST) were analysed by method of Habig *et al.* (1974). GST catalyse the conjugation of glutathione and chloro 2, 4 dinitrobenzene (CDNB) to form 2- choro-4- nitrophenyl glutathione at 340nm. Esterases were analysed by a spectrophotometric method according to Faiz *et al*. (2007). Esterases hydrolyse paranitrophenylacetate to acetate and produce a yellow colour paranitrophenol at 405nm.

RESULTS AND DISCUSSION

The species composition of adult *Anopheles* mosquitoes from the study sites based on morphological species identification in table 1 showed abundance of *An. Gambiae* complex in the three study sites with presence of *An. pharoensis*, which is a secondary malarial vector seen in residential site of the study. In Africa, the primary mosquito species that transmit malaria parasites are mainly from *Anopheles gambiae* complex and *Anopheles funestus* (Coetzee *et al.*, 2013). Abundance of one of the primary mosquito species in all the three study sites can result in increased malaria infection in these study sites.

Table 1. Species Composition of Adult *Anopheles* Mosquitoes from the Three Study Sites in Sudan Savannah, Northwestern Nigeria Based on Morphological Species Identification

Study Sites	*Anopheles* Species	
	An. Gambiaes complex	*An. Pharoensis*
Agricultural	100%	-
Industrial	100%	-
Residential	96%	4%

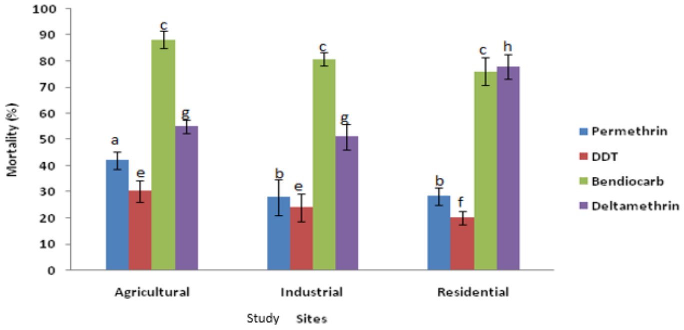

Figure 2. Recorded Mortality of *Anopheles* Mosquitoes Collected in Rainy Season from the Three Study Sites After 24 Hours Exposure.

Super scripts: Bars bearing different letters and representing the same insecticide (having the same colour) across different sites are statistically different *($p<0.05$)*

Table 2. Knockdown Time (KT_{50}) of *Anopheles* Mosquitoes' Insecticide Bioassay to Permethrin 0.75%, Deltamethrin 0.05%, DDT 4% and Bendiocarb 0.1% for *Anopheles* Mosquitoes Obtained in Rainy Season from the Three Study Sites.

Insecticides	Agricultural site		Industrial site		Residential site	
	KT_{50}(min)	95% C.I	KT_{50}(min)	95% C.I	KT_{50}(min)	95% C.I
Permethrin	181.05^a	30.34 -392.44	185.12^a	52.31 - 317.93	206.38^a	93.53 - 319.22
Deltamethrin	74.43^a	49.02 - 99.85	124.51^b	85.49 -334.50	58.36^a	16.46 - 90.27
DDT	306.85^a	232.79-380.91	245.43^b	175.52 -415.34	146.09^c	83.06 - 179.12
Bendiocarb	56.49^a	42.75 - 70.23	43.01^a	15.52 - 70.23	68.84^a	21.72 - 115.95

Super scripts: values bearing different letters along a row (across the sites) are statistically different *($p<0.05$).*

KT_{50}: Knockdown Time in minutes, C.I: Confidential Interval

Table 3. Monooxygenase Activities in *Anopheles* Mosquitoes Tested from Agricultural, Industrial and Residential Sites

Variables	Monooxygenase Enzyme Activity REA (mol/hour/µg)	Regression Coefficient	Standard error of Mean	p-value
Sites				
Agricultural	0.45 ± 0.31^{a}	Ref		
Industrial	0.29 ± 0.08^{b}	-0.20	0.28	0.000
Residential	0.22 ± 0.14^{b}	-0.26	0.28	0.000

Table 4. Glutathione-s-transferase Activities in *Anopheles* Mosquitoes Tested from Agricultural, Industrial and Residential Sites

Variables	GST Activity ($\times 10^{-9}$) EA (mol/min/mg)	Regression Coefficient	Standard error of Mean	p-value
Sites				
Agricultural	8.85 ± 3.30^{a}	Ref		
Industrial	7.10 ± 1.37^{b}	1.75×10^{-9}	6.24×10^{-10}	0.006
Residential	8.55 ± 3.30^{a}	2.99×10^{-10}	6.24×10^{-10}	0.633

Table 5. Esterases Activities in *Anopheles* Mosquitoes Tested from Agricultural, Industrial and Residential Sites

Variables	Esterase Activity ($\times 10^{-9}$) EA(mol/min/mg)	Regression Coefficient	Standard error of Mean	p-value
Sites				
Agricultural	5.03 ± 3.10^{a}	Ref		
Industrial	3.10 ± 0.76^{b}	-1.93×10^{-9}	5.00×10^{-10}	0.000
Residential	3.35 ± 1.76^{b}	-1.68×10^{-9}	5.00×10^{-10}	0.001

Resistance of *Anopheles gambiaes. l.* mosquitoes to the tested insecticides from pyrethroid, carbamate and organochlorine classes of insecticides were observed in all the three study sites from Sudan savannah region of Jigawa State. These sites were agricultural, industrial and residential. The following insecticides were tested permethrin (0.75%), deltamethrin (0.05%), DDT (4%), and bendiocarb (0.1%). Also, recorded mortalities of *Anopheles* mosquitoes after 24 hours exposure indicated high knockdown effect on adult *Anopheles* mosquitoes by the tested insecticidal papers.

Knockdown time (KT$_{50}$) of *Anopheles* mosquitoes' insecticide bioassay obtained in rainy season from the three study sites in table 2 showed residential site having the highest KT$_{50}$ in minutes for permethrin and bendiocarb compared to other sites (agricultural and residential) though statistically not significant. Agricultural site had the highest KT$_{50}$ for DDT compared to other sites, which was statistically significant to other sites. This result indicates strong resistance to DDT in the agricultural site. Industrial site showed statistically significant highest KT$_{50}$ for deltamethrin relative to other sites. KT$_{50}$ obtained in this study revealed high level of resistance to the tested insecticides in the study sites. High level of KT$_{50}$ in mosquitoes was reported to serve as an indicator of primary impact for phenotypic resistance to pyrethroids (Boussougou-Sambe *et al.*, 2018). In present study, WHO bioassay revealed high level of resistance to all tested insecticides. This can be attributed to local selective pressure due to excessive use of insecticides for agricultural purposes and for indoor residual spraying as was reported by Ononamadu *et al.* (2020), a study carried out in the same region as that of present study but from different sites.

Earlier studies in the same region as that of present study confirmed knockdown effects of insecticidal papers against *Anopheles* mosquitoes with strong emergence of resistance to insecticides (Ibrahim *et al.*, 2014; Alhassan *et al.*, 2015; Safiyanu *et al*, 2016; Safiyanu *et al*, 2017; Ibrahim *et al.*, 2019 and Ononamadu *et al.*, 2020). This widespread resistance observed in *Anopheles gambiaes. l.* mosquitoes being a major malaria vector, needs further investigations.

Alhassan *et al.* and Safiyanu *et al.* in their researches implicated prior exposure of *Anopheles* mosquitoes to insecticides through uncontrolled use of insecticides in residential areas for control of mosquito vectors and other insects. Also, indiscriminate use of agrochemicals for pest control in the agricultural areas was reported to induce selection pressure (Alhassan *et al.*, 2015; Safiyanu *et al.*, 2016; Safiyanu *et al.*, 2017).

Adult *Anopheles* mosquitoes used for this study were resistant to permethrin, deltamethrin, DDT and bendiocarb. Reports from previous studies have confirmed resistance of *Anopheles* mosquitoes to permethrin (Kemabonta *et al.*, 2013; Awolola *et al.*, 2014), deltamethrin (Awolola *et al.*, 2014; Oduola *et al.*, 2012), DDT (Oduola *et al.*, 2010; Oduola *et al.*, 2012) and bendiocarb (Ibrahim *et al.*, 2013; Safiyanu *et al.*, 2016). All tested insecticides in this study recorded less than 90% mortality which indicates resistance according to WHO protocol (WHO, 1998). These observations have great implications in vector control measures applied by malaria control programmes. This resistance can affect efficacy of vector control measures and could result in failure of Indoor Residual Spraying (IRS) and Insecticide Treated Nets (ITNs) based control interventions.

Monooxygenases (P450s) are significant in xenobiotic detoxification and/or activation; hence overexpression of P450 genes can affect the pharmacological/toxicological activities of xenobiotics including insecticides. This concept has been linked to increased metabolism of insecticides and also aid in development of resistance to insecticides (Liu *et al.*, 2015). Increased total glutathione S-transferase and esterase activities were reported to cause possible resistance to insecticides (Serebrov *et al.*, 2006). Reyes et al. (2009) observed high activities of detoxifying enzymes (mixed-function oxidases (MFO), glutathione-S-transferases (GST), and esterases (EST)) were also related to insecticide resistance. Insects depend on cytochrome P450-, glutathione-*S*-transferase- and esterase- dependent detoxification systems (Russell *et al.*, 2011).

Detoxification of insecticides in insects/pests is a typical defense that relies heavily on detoxification enzymes including monooxygenases (e.g., cytochrome P-450 monooxygenases), hydrolases (e.g., esterases), and transferases (glutathione-S-transferase) (Khan *et al.*, 2020). In this study, highest monooxygenases and esterases activities were recorded in agricultural site which are statistically significant when compared to the industrial and residential sites' readings. For glutathione -s- transferase, highest activity was also recorded in agricultural site but the difference was only statistically significant when compared to industrial site. Overproduction of enzymes in insects/pests has the potential to develop resistance to insecticides. Insecticides are broken down before they reach and bind to target locations due to overproduction of particular enzymes (Khan *et al.*, 2020). These findings agree with that of several earlier studies, as reported by Reid and McKenzie (2016). In a descriptive assessment, they found that increased pesticide resistance was linked to agricultural insecticide usage in 23 of 25 relevant publications across Africa (Reid and McKenzie, 2016).

CONCLUSION

Abundance of *An. gambiae* complex as one of the primary mosquito species that transmit malaria parasites in Africa was observed in all the three study sites. Results obtained from this study suggested resistance of the *Anopheles* mosquitoes to the classes of insecticides tested namely: pyrethroids (permethrin and deltamethrin), organochlorine (DDT) and carbamate (bendiocarb). Highest detoxification enzyme activities were recorded in agricultural site. These findings point to the fact that there is a growing resistance to the commonly used insecticides and this can result in increased malaria infection in the study areas. This also threatens vector control measures applied in malaria control programmes. Hence need for prompt intervention in the study areas in terms of insecticide resistance management strategies so as to curtail the effect and progression of these observed resistances.

AUTHORS' CONTRIBUTIONS

AM drafted the initial manuscript, carried out the lab work. AJA made contributions to design and conception of the work. CJO analysed and interpreted data. NL made contributions to the conception of the work. SAB revised the manuscript. AAI designed the study and revised the manuscript. All the authors gave approval for the publication of the final version and agreed to be accountable in all aspect of the article.

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None

CONFLICT OF INTEREST

The authors declare no conflict of Interest.

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