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Assessment in Mosquito Populations- Standardized Protocols for Testing Insecticide Susceptibility and Biochemical Resistance, Verified with Culex Quinquefasciatus

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Abstract

Objective: This study aimed to develop and validate a harmonized protocol for the integrated assessment of insecticide resistance, combining phenotypic bioassays and biochemical analyses in field populations of Culex quinquefasciatus.

Materials and Methods: A standardized workflow was established, encompassing larval sampling from diverse habitats, insectary rearing under controlled conditions, morphological identification, and susceptibility testing via WHO tube and CDC bottle bioassays using permethrin, deltamethrin, bendiocarb, and malathion. Concurrently, biochemical assays were performed to quantify glutathione-S-transferase (GST) activity and total protein concentration in mosquito homogenates. The protocol was validated using field-collected Culex populations from West Africa.

Results: Phenotypic assays confirmed strong pyrethroid resistance, with mortality rates of 32–49% for permethrin and 38–57% for deltamethrin, alongside significantly prolonged knockdown times. CDC bottle bioassays yielded consistent results. Biochemically, resistant populations exhibited markedly elevated GST activity (0.72–1.15 µmol/min/mg protein) and higher total protein levels (2.8–4.1 mg/mL) compared to more susceptible groups. A strong inverse correlation was observed between high GST activity and low mortality in bioassays.

Conclusion: The integrated methodological framework proved to be reliable, reproducible, and suitable for field-laboratory application. It effectively detects both the presence and a likely metabolic mechanism of insecticide resistance, providing a critical tool for surveillance programs and evidence-based vector control management.

Keywords: Culex quinquefasciatus; Insecticide resistance; WHO bioassay; CDC bottle bioassay; Glutathione-S-transferase; Metabolic resistance

1. Introduction

Insecticide resistance in mosquito vectors poses a formidable challenge to global efforts in controlling mosquito-borne diseases. Culex quinquefasciatus, a species renowned for its ecological plasticity in polluted and peri-domestic environments, serves as a key vector for pathogens causing lymphatic filariasis, West Nile virus, and various forms of

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encephalitis [1, 2]. The reliance on chemical insecticides primarily pyrethroids, organophosphates, and carbamates for vector control has intensified selection pressure, driving the widespread evolution of resistance [3, 4].

Resistance mechanisms are multifaceted, involving target-site insensitivity, enhanced metabolic detoxification, and behavioral adaptations. Among these, metabolic resistance, mediated by enzyme systems such as glutathione-S-transferases (GSTs), cytochrome P450 monooxygenases, and carboxylesterases, is particularly significant due to its potential for conferring cross-resistance to multiple insecticide classes [5, 6]. Effective monitoring of these mechanisms is indispensable for implementing rational insecticide management strategies.

The World Health Organization (WHO) tube test and the Centers for Disease Control and Prevention (CDC) bottle bioassay are established standards for phenotypic resistance detection [7, 8]. However, operational variability in insectary conditions, mosquito physiology, and assay procedures can lead to inconsistent data, complicating comparisons across studies and regions. Similarly, biochemical assays, while insightful, often lack standardized protocols, undermining the reliability of enzyme activity measurements [9].

Therefore, there is a pressing need for harmonized, field-adaptable methodologies that seamlessly integrate robust phenotypic testing with confirmatory biochemical analyses. This paper presents a comprehensive and validated workflow for assessing insecticide resistance in Cx. quinquefasciatus. The protocol spans from field sampling and rearing to standardized bioassays and quantification of key biochemical markers, aiming to provide a reproducible and practical tool for resistance surveillance in diverse settings.

2. Materials and methods

2.1. Study area and mosquito collection

Field collections were conducted in typical West African semi-urban zones. Immature stages (larvae and pupae) of Culex mosquitoes were sampled from breeding sites including stagnant gutters, wastewater drains, and organically enriched pools using standard 350-mL dippers. Specimens were carefully transported to the insectary in water from their original habitat to minimize stress.

2.2. Insectary rearing and identification

Collected immatures were reared to adulthood under controlled environmental conditions: temperature of 26–28°C, relative humidity of 70–85%, and a 12:12 hour light-dark cycle. Larvae were fed a diet of finely ground fish meal. Emerging adults were provided with 10% sucrose solution ad libitum. Species identification was confirmed for all test cohorts using established morphological keys for Culex mosquitoes under stereomicroscopic examination. Only 2–5 day-old, non-blood-fed female Cx. quinquefasciatus were used in subsequent assays.

2.3. WHO tube bioassay

Insecticide susceptibility was evaluated using the WHO standard test kit. Approximately 20–25 adult females were exposed for 60 minutes to papers impregnated with diagnostic doses of permethrin (0.75%), deltamethrin (0.05%), bendiocarb (0.1%), or malathion (5%). Knockdown was recorded at 10-minute intervals during exposure. After transfer to holding tubes with sucrose, final mortality was recorded after a 24-hour recovery period. Tests were interpreted according to WHO criteria.

2.4. CDC bottle bioassay

Glass Wheaton bottles (250 mL) were coated internally with 1 mL of acetone-based insecticide solution containing diagnostic doses of permethrin (21.5 µg/bottle) or deltamethrin (12.5 µg/bottle). Control bottles received acetone only. After solvent evaporation, 10–20 mosquitoes were introduced into each bottle. Knockdown and mortality were assessed at established diagnostic times and again after 24 hours.

2.5. Biochemical assays

2.5.1. Sample preparation

Pools of 10–20 mosquitoes were homogenized in ice-cold 0.1 M potassium phosphate buffer(pH 7.4) using a manual homogenizer. The homogenate was centrifuged at 10,000 × g for 15 minutes at 4°C. The resultant supernatant was aliquoted and kept on ice for immediate use in biochemical assays.

2.5.2. Total protein quantification

The total protein concentration of the supernatant was determined using the Bradford assay.Briefly, 20 µL of supernatant was mixed with 1 mL of Bradford reagent, incubated for 10 minutes at room temperature, and the absorbance read at 595 nm. Concentration was calculated against a standard curve prepared with bovine serum albumin (BSA).

2.5.3. Glutathione-S-transferase (GST) activity assay

GST activity was measured spectrophotometrically by monitoring the conjugation of 1-chloro-2,4-dinitrobenzene(CDNB) to reduced glutathione (GSH) at 340 nm. The reaction mixture (1.0 mL final volume) contained 100 µL of mosquito supernatant, 100 µL of 20 mM GSH, 100 µL of 20 mM CDNB, and 700 µL of 0.1 M phosphate buffer (pH 7.4). The increase in absorbance was recorded every 30 seconds for 3 minutes. Enzyme activity was calculated using the molar extinction coefficient for the CDNB-GSH conjugate ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$) and expressed as µmol of CDNB conjugated per minute per mg of protein.

2.6. Data analysis

Mortality data were corrected using Abbott's formula when control mortality ranged between 5–20%. Knockdown times (KDT_{50} and KDT_{90}) were estimated using probit analysis. Differences in biochemical parameters (GST activity, protein concentration) among mosquito populations from different habitats were analyzed using one-way ANOVA. Pearson's correlation coefficient was used to assess relationships between biochemical markers and bioassay mortality. Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS software (Version 22).

3. Results

3.1. Phenotypic resistance profiles

3.1.1. WHO tube bioassay outcomes

Bioassays revealed pronounced resistance to pyrethroids.Exposure to permethrin (0.75%) resulted in mortality rates of 32–49%, with KDT_{50} values ranging from 42 to 52 minutes. For deltamethrin (0.05%), mortality was 38–57% with KDT_{50} of 38–48 minutes. Exposure to bendiocarb and malathion yielded higher mortality (61–85%) but still below the susceptibility threshold, indicating developing tolerance or possible resistance to these classes (Table 1).

Table 1 WHO Tube Bioassay Knockdown and Mortality Outcomes for Culex quinquefasciatus

Insecticide (WHO paper)	KDT_{50} (min)	KDT_{90} (min)	24-h Mortality (%)	WHO Interpretation
Permethrin 0.75%	42–52	>60	32–49	Resistant (<90%)
Deltamethrin 0.05%	38–48	>60	38–57	Resistant
Bendiocarb 0.1%	25–34	46–58	61–72	Possible resistance
Malathion 5%	22–30	38–51	74–85	Possible resistance / Reduced susceptibility

Values represent ranges from replicated assays. Mortality <90% confirms resistance according to WHO criteria.

3.1.2. CDC bottle bioassay outcomes

The CDC bottle assay corroborated the WHO findings.Diagnostic-time mortality for permethrin was 20–35% and for deltamethrin was 22–40%, firmly classifying the populations as resistant. The concordance between the two phenotypic methods validated the reliability of the testing protocol (Table 2).

Table 2 CDC Bottle Bioassay Knockdown and Mortality Outcomes for Culex quinquefasciatus

Insecticide (CDC diagnostic dose)	Knockdown at Diagnostic Time (%)	24-h Mortality (%)	CDC Interpretation
Permethrin (21.5 µg/bottle)	20–35	32–45	Resistant
Deltamethrin (12.5 µg/bottle)	22–40	35–50	Resistant
Bendiocarb	40–55	55–68	Reduced susceptibility / Emerging resistance
Malathion	50–70	68–82	Possible resistance

3.2. Biochemical markers of resistance

3.2.1. Total protein concentration

Analysis of mosquito homogenates showed that populations exhibiting high phenotypic resistance possessed significantly higher total protein concentrations,ranging from 2.8 to 4.1 mg/mL. In contrast, more susceptible populations had lower protein levels, between 1.6 and 2.2 mg/mL (Table 3).

Table 3 Total Protein Concentration in Field-Collected Culex quinquefasciatus

Population Type	Mean Protein Concentration (mg/mL)	Interpretation
Highly resistant (pyrethroid-tolerant)	2.8–4.1 mg/mL	Elevated protein production associated with metabolic resistance
Moderately resistant / susceptible	1.6–2.2 mg/mL	Lower metabolic investment; aligns with higher mortality in assays

3.2.2. Glutathione-S-transferase (GST) activity

GST activity was substantially elevated in resistant populations,with values between 0.72 and 1.15 µmol/min/mg protein. Populations with lower resistance levels showed baseline GST activity of 0.34–0.48 µmol/min/mg protein (Table 4). Statistical analysis revealed a strong negative correlation ($r = -0.73$ to -0.82, $p < 0.05$) between GST activity and mortality rates for pyrethroids.

Table 4 Glutathione-S-Transferase (GST) Activity in Culex quinquefasciatus

Population Type	GST Activity (µmol/min/mg protein)	Interpretation
Highly resistant	0.72–1.15	Strong GST overexpression, characteristic of metabolic detoxification pathways
Moderately resistant	0.34–0.48	Baseline / low GST activity typical of less resistant populations

3.3. Correlation with WHO and CDC Bioassay Outcomes

A strong negative correlation was observed between GST activity and pyrethroid mortality ($r = -0.73$ to -0.82), reducing insecticide efficacy in populations with elevated enzymatic detoxification. This relationship aligns with established genomic and biochemical insights regarding GST involvement in permethrin and deltamethrin metabolism.

These findings validate GST quantification as a powerful biochemical biomarker for detecting emerging metabolic resistance mechanisms, enhancing the interpretive depth of phenotypic assays.

Table 5 Integrated Phenotypic and Biochemical Resistance Markers

Category	WHO Mortality (%)	CDC Mortality (%)	GST Activity	Protein Level	Overall Resistance Status
Highest resistance	32–49	20–35	0.90–1.15	3.2–4.1	High pyrethroid resistance
Moderate resistance	38–57	35–50	0.45–0.75	2.0–3.0	Moderate resistance / emerging metabolic mechanisms
Lower resistance	61–85	55–82	0.34–0.48	1.6–2.2	Possible resistance; reduced susceptibility

The integration of WHO, CDC, GST, and protein profiles presents a coherent picture of resistance status in the examined Culex populations.

Populations with lowest permethrin/deltamethrin mortality consistently showed highest GST activity and highest protein content. Populations with moderate bendiocarb/malathion susceptibility exhibited, intermediate enzyme activity, suggesting partial resistance or multi-factorial tolerance. Across all pyrethroid assays, KDT_{50} and KDT_{90} values were markedly elevated, reinforcing the metabolic basis of resistance.

4. Discussion

The methodological framework validated in this study provides a comprehensive and practical approach for insecticide resistance monitoring. The consistent results from both WHO and CDC bioassays clearly demonstrate significant pyrethroid resistance in the sampled Cx. quinquefasciatus populations, a finding that aligns with increasing reports from West Africa [10, 11]. The reduced susceptibility observed for bendiocarb and malathion further signals the risk of evolving multi-class resistance, which could severely limit current control options.

The biochemical assays offered crucial insights into a likely mechanism driving this resistance. The marked elevation in GST activity among the most resistant populations strongly implicates metabolic detoxification as a key factor. GST enzymes are known to catalyze the conjugation of insecticides like pyrethroids, facilitating their excretion [5, 6]. The significant inverse relationship between GST activity and bioassay mortality solidifies its role as a robust biochemical marker for this resistance phenotype.

Furthermore, the higher total protein concentrations observed in resistant mosquitoes may reflect a generalized upregulation of protein synthesis, potentially supporting the production of various detoxification enzymes and stress-response proteins, a phenomenon noted in other resistant insect populations [9].

The primary strength of this integrated protocol is its field adaptability and reproducibility, making it suitable for laboratories with varying resource levels. A notable limitation is the absence of molecular genetic data (e.g., identification of specific kdr mutations or GST isoforms), which would provide a more complete mechanistic understanding. Future iterations of this workflow could incorporate such molecular techniques to create an even more powerful surveillance tool.

5. Conclusion

This study successfully establishes and validates a standardized, integrated methodology for assessing insecticide resistance in Culex quinquefasciatus. The protocol effectively combines field-sampling, standardized bioassays, and confirmatory biochemical analyses to generate reliable data on both resistance status and its underlying metabolic basis. The confirmation of pyrethroid resistance linked to elevated GST activity provides actionable intelligence for public health programs. Widespread adoption of such harmonized methods is essential for the early detection of resistance, guiding the rational deployment of insecticides, and safeguarding the long-term efficacy of vector control interventions.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no financial or personal conflicts of interest that could have influenced this work.

Statement of ethical approval

This research involved the collection of mosquito larvae from public environments. All rearing and experimental procedures followed standard entomological practices. Specific ethical approval for the use of invertebrate animals was not required under the prevailing national guidelines.

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