# Oral susceptibility to ivermectin is over fifty times greater in a wild population of *Anopheles albimanus* mosquitoes from Belize than the STECLA laboratory reference strain of *A. albimanus*

**Staci M. Dreyer[1], Kelsey J. Morin[1], Marla Magaña[2], Marie Pott[2], Nicole L. Achee[2,3], John P. Grieco[2,3] Jefferson A. Vaughan[1*]**

[1]University of North Dakota, Grand Forks, ND 58202, USA, [2]Belize Vector and Ecology Center, Orange Walk Town, Belize, [3]University of Notre Dame, South Bend, IN, 46556, USA

* Corresponding author: Jefferson A. Vaughan, jefferson.vaughan@und.edu

## Abstract

**Background:** The STECLA strain of *Anopheles albimanus* Wiedemann has been in continuous colony for many years and is the reference strain on which genomic studies for the species are based. Recently, the STECLA strain was demonstrated to be much less susceptible to ivermectin ingested in a blood meal ($LC_{50}$ of 1468 ng/ml) than all other *Anopheles* species tested to-date ($LC_{50}$ values range from 7 – 56 ng/ml). The ability of *A. albimanus* to survive ingestion of ivermectin at concentrations far beyond that typically found in the blood of ivermectin-treated people or livestock (*i.e.*, 30 – 70 ng/ml) could invalidate the use of ivermectin as a malaria vector control strategy in areas where *A. albimanus* is a primary vector.

**Methods:** To investigate this, we captured host-seeking *A. albimanus* in northern Belize and conducted membrane feeding bioassays of ivermectin, using the same methods as described earlier with the STECLA strain.

**Results:** Field-collected *A. albimanus* in Belize were 55 times more susceptible to ingested ivermectin than were the STECLA reference strain. Oral susceptible to ivermectin in wild *A. albimanus* from Belize ($LC_{50}$ of 26 ng/ml) was equivalent to that of other *Anopheles* species tested.

**Conclusion:** Contrary to our initial assessments using a highly inbred strain of mosquito, we show that ivermectin treatment of livestock could reduce *A. albimanus* populations in areas of Central America and the Caribbean where malaria transmission may occur. Toxicity screening of ivermectin and other systemic parasiticides for malaria control should examine wild populations of the vector species being targeted.



**Keywords:** *Anopheles albimanus*, ivermectin, STECLA, Belize

## Background

Ivermectin has long been an important drug for treating livestock against parasitic nematodes and arthropods (*e.g.*, ticks) and more recently, for treating humans against filarial nematodes that cause lymphatic filariasis and onchocerciasis. Ivermectin has also gained importance in the global effort to eliminate malaria because of its potential to reduce malaria vector populations [1]. When ingested by *Anopheles* mosquitoes at concentrations normally found in the plasma of treated people or livestock, ivermectin has been shown to reduce the survivorship and fecundity of almost every *Anopheles* species in which the drug has been tested [2-15]. The one exception has been the Central American vector, *An. albimanus*. In recent laboratory studies [16], we reported that the dose required to kill 50% (*i.e.*, the $IC_{50}$) of *An. albimanus* mosquitoes ($IC_{50}$=1468 ng/ml) was so much higher than the maximum concentration of ivermectin typically found in the sera of treated humans or cattle (*i.e.*, 30 – 70 ng/ml [1, 17-19]) that as a malaria control strategy, ivermectin would be useless against this mosquito species. The following year we conducted a pilot trial with cattle in northern Belize [20]. One of the animals was injected with a commercial formulation of ivermectin to serve as an extra 'negative control'. Unexpectedly, locally-captured *A. albimanus* that fed on the ivermectin-injected animal experienced significantly higher mortality than did mosquitoes fed on untreated cattle. It appeared that wild-caught *A. albimanus* mosquitoes from northern Belize were more susceptible to ingested ivermectin than were the laboratory strain of *A. albimanus* mosquitoes that we had obtained from BEI Resources (Manassas, VA USA) – *i.e.*, the STECLA strain, which is the reference strain used for many studies, including a recent physical genome map for the species [21]. Here, we report the acute oral susceptibility to ivermectin of *A. albimanus* wild-caught in Belize (hereafter referred as *A. albimanus* BELIZE) and compare it with that of the *A. albimanus* STECLA strain, as well as with other *Anopheles* species that have been similarly tested.

## Methods

**Mosquitoes**. Host-seeking mosquitoes were collected during nighttime human landing catches in San Roman Rio Hondo, Orange Walk District, Belize. Mosquitoes were transported to the Belize Vector and Ecology Center laboratory in Orange Walk Town, Belize. *Anopheles albimanus* mosquitoes were distinguished from other anopheline species based on the characteristic banding pattern on the hind tarsi [22]. After identification, *A. albimanus* BELIZE were transferred into smaller (*ca*. 0.5 liter) cylindrical plastic cages with mesh tops at a density of 15-30 mosquitoes. Mosquitoes were maintained at 26 °C with access to 8% honey solution ad libitum.

**Membrane Feeding**. Stock solutions of ivermectin (2 mg ivermectin per 1 ml dimethyl sulfoxide) were prepared at the University of North Dakota, frozen, and transported by air to Belize City and by automobile to Belize Vector and Ecology Center, Orange Walk Town, Belize (approximately 1 hour drive). Stock solutions were diluted in water to make initial starting concentrations. Final ivermectin concentrations were then prepared by adding appropriate volumes of human blood to a final volume of 8 ml. The control group received blood with no additives. Blood mixtures were kept warm prior to feeding. Natural ham collagen, pre-soaked in distilled water, was used as the material through which mosquitoes probed and fed. The collagen was affixed to glass membrane feeders with rubber bands, feeders were connected to one another with rubber tubing, and warm water (37°C) was circulated through the feeders. Membrane feeders were then placed on individual cages containing 15 to 30 wild-caught mosquitoes and the pre-warmed blood mixtures were pipetted into the feeders. Mosquitoes were allowed 90 minutes to feed in darkness. Afterwards, unfed mosquitoes were removed. Engorged mosquitoes were maintained at 26 °C with access to 8% honey solution ad libitum. Cages were checked every day and dead mosquitoes were counted and removed. After four days, surviving mosquitoes were counted.

**Data Analysis.** Mosquito mortalities observed within experimental groups were adjusted for mortality that occurred within corresponding control groups using Abbott's formula [23]. Only experimental trials having control mortalities less than 20% were used for further data analyses. Log-probit analyses were conducted on the corrected percent moralities to estimate $LC_{50}$ values (Minitab Inc., State College PA, USA). Mosquito survivorship was analyzed with a Kaplan-Meier survival analysis and Log-rank Mantel-Cox test (GraphPad Software, La Jolla CA USA). A 0.05 level of significance was used throughout.

## Results

A total 352 fully engorged mosquitoes over five separate feeding trials were used to determine the acute oral toxicity of ivermectin for *A. albimanus* BELIZE, collected in the field from northern Belize. The estimated average membrane-feeding rate was 31.5%. Post-feeding mosquito mortality was protracted and occurred over a period of several days after ingestion of treated blood (Fig. 1), as reported for other *Anopheles* species ingesting ivermectin. The $LC_{50}$ (lower and upper 95% confidence intervals) at day 4 post-feeding was 26.4 ng/ml (13.7 – 51.0); over 55-fold higher than that reported for the STECLA laboratory reference strain of *A. albimanus* ($LC_{50}$ = 1468 ng/ml) using the same methodologies [16].

## Discussion

With the notable exception of the *A. albimanus* STECLA strain, all *Anopheles* species tested thus far using membrane-feeding techniques, have $LC_{50}$ values (*i.e.*, 7 to 56 ng/ml, Table 1) well within the typical peak plasma concentrations of ivermectin reported for humans and livestock (*e.g.*, 30 – 70 ng/ml) following standard drug administration at approved doses. Thus, all *Anopheles* species examined to date are theoretically susceptible to population reduction *via* targeted administration of ivermectin to humans and livestock. To the best of our knowledge, this is the first study to quantify oral susceptibility to

ivermectin in a field population of *Anopheles* using the membrane feeding bioassay technique. Previous studies using this standardized technique have relied on laboratory strains of mosquitoes that have been in continuous colony for many years. Not surprisingly, there was more heterogeneity in the response to ingested ivermectin with the Belize field population, as indicated by wider confidence intervals around the $LC_{50}$ value than observed in colonized mosquitoes (Table 1). Similarly, there was a flatter slope in the dose-response curve of wild *A. albimanus* BELIZE than observed for the STECLA strain of *A. albimanus* and for laboratory strains of *A. stephensi* STE2 and *A. arabiensis* DONGOLA (Table 2). Greater heterogeneity in the response to ivermectin by a free-roaming population may have resulted both from testing mosquitoes of unknown age and physiological condition and to the greater overall genetic diversity inherent in field populations versus inbred laboratory strains. Importantly, the findings that different populations of *A. albimanus* (BELIZE versus STECLA) vary widely in their susceptibilities to ivermectin and that the response to ivermectin in a wild population is more heterogenous than in laboratory populations suggest that the development of ivermectin-resistant populations of *A. albimanus* in nature is possible.

## Conclusion

This study illustrates the importance of including wild-caught indigenous populations of vectors (as opposed to sole reliance on laboratory strains) during *in vitro* toxicological screening of ivermectin and other systemic parasiticides. By screening wild populations of a targeted vector species, investigators may know better what to expect in field trials that involve treating entire herds of livestock.

126 **List of Abbreviations**

127 $IC_{50}$: concentration of ivermectin required to kill 50% of treated mosquitoes.

128

129 # Declarations

130 **Ethical approval and consent to participate**

131 Not applicable.

132

133 **Consent for publication**

134 Not applicable.

135

136 **Availability of data and materials**

137 The data analyzed during this study are available on request from the corresponding author.

138

139 **Competing interests**

140 The authors declare that they have no competing interests.

141

142

## Funding

This study was funded by Grant R21AI119771 from the National Institute of Allergy and Infectious Diseases, NIH, USA. The funding agency had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Authors' contributions

Conceived and designed the study: SMD and JAV. Conducted the laboratory work: SMD, KJM, MM, and MP. Conducted data analysis and wrote the manuscript: SMD and JAV. Provided logistical and infrastructure support from the Belize Vector Ecology Center: NLA and JPG. All authors read and approved the final manuscript.

## Acknowledgments

The authors wish to thank the mosquito collectors in San Roman who assisting in collecting the mosquitoes used in this trial. The *Anopheles albimanus* STECLA strain was obtained as eggs (*i.e.*, MRA-126, contributed by Mark Q. Benedict) from the BEI Resources Manassas, VA, funded in part by the National Institute of Allergy & Infectious Diseases, National Institute of Health, USA.

## Author details

[1]University of North Dakota, Grand Forks, ND 58202, USA, [2]Belize Vector and Ecology Center, Orange Walk Town, Belize, [3]University of Notre Dame, South Bend, IN, 46556, USA

## References


1. Billingsley P, Binka F, Chaccour C, Foy B, Gold, Gonzalez-Silva SM, *et a*l. A roadmap for the development of ivermectin as a complementary malaria vector control tool. Amer J Trop Med Hyg. 2020;102:3-24.

2. Fritz ML, Siegert PY, Walker E.D., Bayoh MN, Vulule JR, Miller JR. Toxicity of bloodmeals from ivermectin-treated cattle to *Anopheles gambiae s.1.* Ann Trop Med Parasitol. 2009;103:539–547.

3. Chaccour CJ, Lines J, Whitty CJM. Effect of ivermectin on *Anopheles gambiae* mosquitoes fed on humans: the potential of oral insecticides in malaria control. J Infect Dis. 2010;202:113–6.

4. Sylla M, Kobylinski KC, Gray M, Chapman PL, Sarr MD, Rasgon JL, *et al*. Mass drug administration of ivermectin in south-eastern Senegal reduces the survivorship of wild-caught, blood fed malaria vectors. Malar J. 2010;9: 365.

5. Kobylinski KC, Deus KM, Butters MP, Hongyu T, Gray M, Marques da Silva MI, *et al*. The effect of oral anthelmintics on the survivorship and re-feeding frequency of anthropophilic mosquito disease vectors. Acta Trop. 2010;116: 119–26.

6. Kobylinski KC, Sylla M, Chapman PL, Sarr MD, and Foy BD. Short Report: Ivermectin mass drug administration to humans disrupts malaria parasite transmission in Senealese villages. Am J Trop Med Hyg. 2011;85: 3–5.

7. Fritz ML, Walker ED, Miller JR. Lethal and sublethal effects of avermectin/milbemycin parasiticides on the African malaria vector, *Anopheles arabiensis*. J Med Entomol. 2012;49:326–31.

8. Naz S, Maqbool A, Ahmad M, Anjum AA. Efficacy of ivermectin for control of zoophilic malaria vectors in Pakistan. Pak J Zool. 2013;45:1585–91.

9. Alout H, Krajacich BJ, Meyers JI, Grubaugh ND, Brackney DE, Kobylinski KC, *et al*. Evaluation of ivermectin mass drug administration for malaria transmission control across different West African environments. Malar J. 2014;13: 417.

10. Poché RM, Burruss D, Polyakova L, Poché DM, Garlapati RB. Treatment of livestock with systemic insecticides for control of *Anopheles arabiensis* in western Kenya. Malar J. 2015;14:351.

11. Sampaio VS, Beltrán TP, Kobylinski KC, Melo GC, Lima JBP, Silva SGM, *et al.* Filling gaps on ivermectin knowledge: effects on the survival and reproduction of *Anopheles aquasalis*, a Latin American malaria vector. Malar J. 2016;15:491.

12. Lyimo IN, Kessy ST, Mbina KF, Daraja, AA, Mnyone LL. Ivermectin-treated cattle reduces blood digestion, egg production and survival of a free-living population of *Anopheles arabiensis* under semi-field condition in south-eastern Tanzania. Malar J. 2017;16: 239.

13. Kobylinski KC, Ubalee R, Ponlawat A, Nitatsukprasert C, Phasomkulsolsil S, Wattanakul T, *et al*. Ivermectin susceptibility and sporontocidal effect in Greater Mekong Subregion *Anopheles*. Malar J. 2017;16:280.

14. Kobylinski KC, Escobedo-Vargas KS, López-Sifuentes VM, Durand S, Smith, ES, Baldeviano GC, *et al*. Ivermectin susceptibility, sporontocidal effect, and inhibition of time to re-feed in the Amazonian malaria vector *Anopheles darlingi*. Malar J. 2017;16:474.

15. Chaccour C J, Ngha'Bi K, Abizanda G, Irigoyen Barrio A, Aldaz A, Okumu F, *et al*. Targeting cattle for malaria elimination: Marked reduction of *Anopheles arabiensis* survival for over six months using a slow-release ivermectin implant formulation. Parasites Vectors 2018;11:1–9.

16. Dreyer SM, Morin KJ, Vaughan JA. Differential susceptibilities of *Anopheles albimanus* and *Anopheles stephensi* mosquitoes to ivermectin. Malar J. 2018;17:148.

17. Lanusse C, Lifschitz A, Virkel G, Alvarez L, Sánchez S, Sutra JF, *et al*. Comparative plasma disposition kinetics of ivermectin, moxidectin and doramectin in cattle. J Vet.Pharmacol Ther. 1997;20:91–9.

18. Lifschitz A, Sallovitz J, Imperiale F, Pis A, Jauregui Lorda J, Lanusse C. Pharmacokinetic evaluation of four ivermectin generic formulations in calves. Vet.Parasitol. 2004;119:247–57.

19. Ndong TB, Kane Y, Ba MA, Sane I, Sutra JF, Alvinerie M. Pharmacokinetics of ivermectin in zebu Gobra (*Bos indicus*). Vet Parasitol. 2005;128:169–73.

20. Dreyer SM, Leiva D, Magaña M, Pott M, Kay J, Cruz A, *et al*. Fipronil and ivermectin treatment of cattle reduced the survival and ovarian development of field-collected *Anopheles albimanus* in a pilot trial conducted in northern Belize. Malar J. 2019;18:296.

21. Artemov GN, Peery AN, Jiang X, Tu Z, Stegniy VN, Sharakhova MV, *et al.* The physical genome mapping of *Anopheles albimanus* corrected scaffold misassemblies and identified interarm rearrangements in genus *Anopheles*. G3 (Bethseda) 2017;7:155-64.

22. Wilkerson RC, Strickman D, Litwak TR. Illustrated key to the female anopheline mosquitoes of Central America and Mexico. J Am Mosq Control Assoc. 1990;6:7–34.

23. Abbott WS. Abbott's formula a method of computing the effectiveness of an insecticide. J Econ Entomol. 1925;18:265–7.

**Table 1** Acute oral toxicities to ivermectin for *Anopheles* species using *in vitro* membrane feeding techniques, ranked according to susceptibility.

| ***Anopheles* Species** | **Mosquito Strain & History*** | **Mortality Assessment Period (day)** | **N** | **Oral $LC_{50}$ (95% CL)** | **Reference** |
|---|---|---|---|---|---|
| *stephensi* | STE2; Long-standing | 4 | 573 | 7 (5, 9) | [16] |
| *arabiensis* | DONGOLA; Long-standing | 9 | 515 | 8 (6, 10) | [7] |
| *minimus* | ARIMS; Long-standing | 7 | 2376 | 16 (12, 19) | [13] |
| *gambiae s.s.* | KISUMU; Long-standing | 9 | Not reported | $20 \pm 3$ | [2] |
| *gambiae s.s.* | G3; Long-standing | 5 | 2013 | 22 (18, 27) | [5] |
| *albimanus* | BELIZE; Field-collected | 4 | 352 | 26 (14, 51) | Present Study |
| *campestris* | ARIMS; Long-standing | 7 | 2786 | 26 (22, 30) | [13] |
| *sawadwongporni* | ARIMS; Long-standing | 7 | 1446 | 27 (25, 29) | [13] |
| *darlingi* | NAMRU-6; Recent | 7 | 6161 | 43 (37, 49) | [14] |
| *aquasalis* | FMT-HVD; Long-standing | 5 | 1415 | 47 (45, 49) | [11] |
| *dirus* | ARIMS; Long-standing | 7 | 5029 | 56 (52, 59) | [13] |
| *albimanus* | STECLA; Long-standing | 4 | 582 | 1468 (1153, 1965) | [16] |

* 'Long-standing' is defined as more than 5 years of continuous colony prior to testing. 'Recent' is defined as two to three years in colony prior to testing.

231

232 **Table 2** Regression parameters describing the dose-response of various *Anopheles* species and strains to

233 ingested ivermectin.

| Species/Strain | $LC_{50}$ | N | df | Intercept | Slope | Reference |
|---|---|---|---|---|---|---|
| *A. albimanus* BELIZE | 26.4 | 352 | 5 | -1.1 | 0.78 | Present study |
| *A. albimanus* STECLA | 1468.0 | 582 | 5 | -4.5 | 1.41 | [16] |
| *A. stephensi* STE2 | 7.0 | 573 | 5 | -1.2 | 1.37 | [16] |
| *A. arabiensis* DONGOLA | 7.9 | 518 | 5 | -2.2 | 1.06 | [7] |

234

235

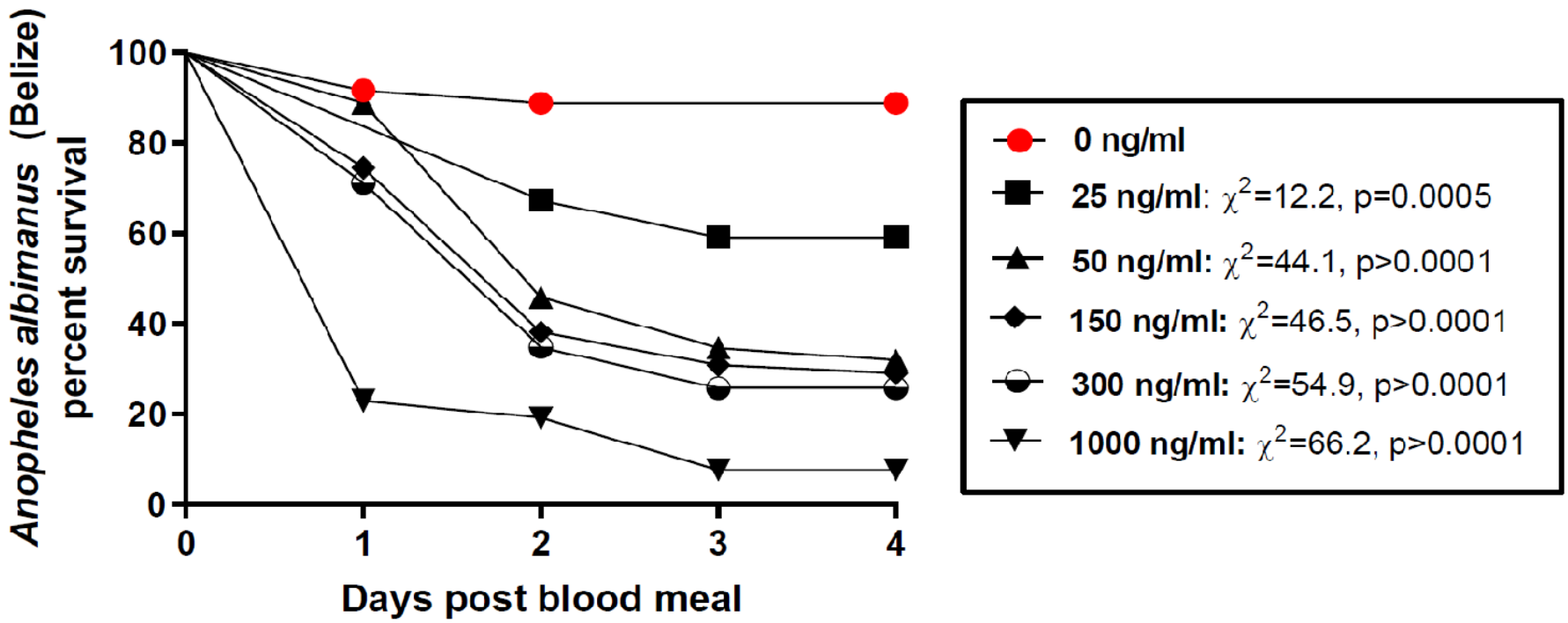


**Fig. 1.** Kaplan-Meier daily proportion of surviving *Anopheles albimanus* BELIZE after ingesting ivermectin at various concentrations.