RESEARCH

Insecticide resistance in *Anopheles arabiensis* from Ethiopia (2012–2016): a nationwide study for insecticide resistance monitoring

Louisa A. Messenger[1*], Josephat Shililu[2], Seth R. Irish[1], Gedeon Yohannes Anshebo[2],
Alemayehu Getachew Tesfaye[2], Yemane Ye-Ebiyo[2], Sheleme Chibsa[3], Dereje Dengela[4],
Gunawardena Dissanayake[3], Estifanos Kebede[5], Endalew Zemene[5], Abebe Asale[5], Mekonnen Yohannes[6],
Hiwot Solomon Taffese[7], Kristen George[8], Christen Fornadel[8], Aklilu Seyoum[4], Robert A. Wirtz[1]
and Delenasaw Yewhalaw[5,9]

Abstract

Background: Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) remain the cornerstones of malaria vector control. However, the development of insecticide resistance and its implications for operational failure of preventative strategies are of concern. The aim of this study was to characterize insecticide resistance among *Anopheles arabiensis* populations in Ethiopia and describe temporal and spatial patterns of resistance between 2012 and 2016.

Methods: Between 2012 and 2016, resistance status of *An. arabiensis* was assessed annually during the long rainy seasons in study sites from seven of the nine regions in Ethiopia. Insecticide resistance levels were measured with WHO susceptibility tests and CDC bottle bioassays using insecticides from four chemical classes (organochlorines, pyrethroids, organophosphates and carbamates), with minor variations in insecticides tested and assays conducted between years. In selected sites, CDC synergist assays were performed by pre-exposing mosquitoes to piperonyl butoxide (PBO). In 2015 and 2016, mosquitoes from DDT and deltamethrin bioassays were randomly selected, identified to species-level and screened for knockdown resistance (*kdr*) by PCR.

Results: Intense resistance to DDT and pyrethroids was pervasive across Ethiopia, consistent with historic use of DDT for IRS and concomitant increases in insecticide-treated net coverage over the last 15 years. Longitudinal resistance trends to malathion, bendiocarb, propoxur and pirimiphos-methyl corresponded to shifts in the national insecticide policy. By 2016, resistance to the latter two insecticides had emerged, with the potential to jeopardize future long-term effectiveness of vector control activities in these areas. Between 2015 and 2016, the West African (L1014F) *kdr* allele was detected in 74.1% (n = 686/926) of specimens, with frequencies ranging from 31 to 100% and 33 to 100% in survivors from DDT and deltamethrin bioassays, respectively. Restoration of mosquito susceptibility, following pre-exposure to PBO, along with a lack of association between *kdr* allele frequency and *An. arabiensis* mortality rate, both indicate metabolic and target-site mutation mechanisms are contributing to insecticide resistance.

---

*Correspondence: ndx1@cdc.gov
[1] Entomology Branch, Centers for Disease Control and Prevention, 1600
Clifton Road, Atlanta, GA 30329-4027, USA
Full list of author information is available at the end of the article

Messenger *et al. Malar J* (2017) 16:469

Page 2 of 14

Conclusions: Data generated by this study will strengthen the National Malaria Control Programme's insecticide resistance management strategy to safeguard continued efficacy of IRS and other malaria control methods in Ethiopia.

Keywords: Insecticide resistance, *Anopheles arabiensis*, Resistance mechanisms, Intensity assays, Malaria, *kdr*, Ethiopia

Background

Despite the scaling-up of key diagnostic, treatment and preventative measures, malaria remains a considerable public health problem in Ethiopia, with over 50.6 million (60% of the total population) at significant risk [1]. Transmission of *Plasmodium falciparum* and *Plasmodium vivax* is highly heterogeneous and unstable across the country, concentrated in lowland and highland fringe areas [2]. Unlike other sub-Saharan countries, where malaria morbidity and mortality mainly impacts young children, in Ethiopia, low levels of immunity predispose many individuals to clinically severe malaria and epidemics among all age groups. As part of the National Malaria Strategic Plan (2014–2020), vector control by the National Malaria Control Programme (NMCP), with support from the President's Malaria Initiative (PMI) and the Global Fund, is based on indoor residual spraying (IRS) and universal coverage campaigns of long-lasting insecticidal nets (LLINs) [1–4].

IRS was first implemented in Ethiopia in 1959 and continues to play a prominent role in malaria control. LLIN coverage has been scaled up since 2005, resulting in over 64 million nets distributed by 2014 [2]. However, the long-term effectiveness of both strategies is currently under threat from widespread emergence of insecticide resistance in the principal malaria vector, *Anopheles arabiensis* [3]. To date, in Ethiopia, *An. arabiensis* has developed resistance against insecticides belonging to all four chemical classes approved for IRS, including DDT (organochlorine), malathion (organophosphate), bendiocarb and propoxur (carbamates) and alpha-cypermethrin, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin and permethrin (pyrethroids) [5–14]. The West African *kdr* (L1014F) mutation has been reported in *An. arabiensis* populations at high frequencies [9, 14, 15] and pre-exposure of *An. arabiensis* to piperonyl butoxide (PBO) significantly increased vector susceptibility to deltamethrin and permethrin [12], suggesting both metabolic and target-site mutation mechanisms are responsible for insecticide resistance. Historically, DDT, and to a lesser extent, malathion were used for IRS in Ethiopia [12]. In 2010, vector control by the NMCP, with support from PMI, discontinued DDT spraying in favour of deltamethrin, which was used initially in combination with bendiocarb from 2011, before being superseded exclusively by bendiocarb and propoxur in 2013. In 2015, in response to incipient resistance, PMI-supported IRS activities were based on bendiocarb in 28 districts (and focal pirimiphos-methyl application in 8 districts) and in 2016, pirimiphos-methyl replaced bendiocarb in all PMI-supported districts [2] (Fig. 1 and Additional file 1: Table S1).

Considering only a limited number of alternate insecticides are available for public health use, the aim of this study was to characterize contemporary nationwide insecticide resistance in *An. arabiensis* populations and describe longitudinal trends in resistance between 2012 and 2016, to strengthen the NMCP's insecticide resistance monitoring and management strategy and safeguard continued efficacy of IRS in Ethiopia [16].
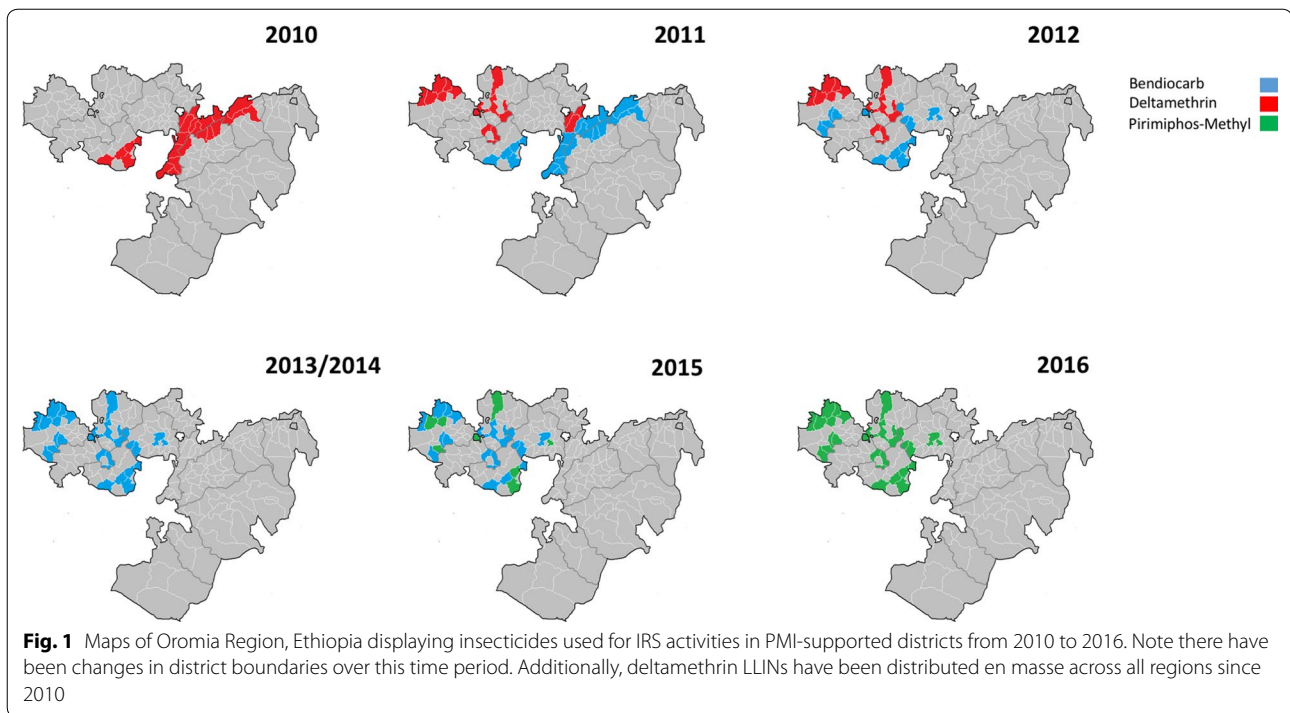
Methods

Study sites

Data are from study sites in seven of the nine regions [Afar, Amhara, Benishangul Gumuz, Gambela, Oromia, Southern Nations, Nationalities, and Peoples' Region (SNNPR), and Tigray] in Ethiopia between 2012 and 2016.

Susceptibility tests

For all susceptibility tests, mosquito larvae were collected by dipping from a range of breeding sites in each study area and reared to adults under standard insectary conditions (temperature 25 ± 2 °C, relative humidity 80 ± 10%). Bioassays were conducted annually during the long rainy season (June–September). WHO tube tests and CDC bottle bioassays were used to determine susceptibility levels of *Anopheles gambiae* s.l. populations (henceforth *An. arabiensis*) to different insecticides, with minor variations in insecticides tested and assays conducted between years (Additional file 1: Table S2). From 2014 onwards, CDC resistance intensity assays were undertaken and synergist assays were also performed by pre-exposing mosquitoes to PBO in selected sites. For all assays, care was taken during storage and field transportation of insecticide-impregnated papers and technical grade insecticide stock solutions to reduce heat exposure and minimize potential loss of efficacy.

WHO susceptibility tests

World Health Organization (WHO) susceptibility tests for the following eleven insecticides with diagnostic doses (alpha-cypermethrin (0.05%), bendiocarb (0.1%),

Messenger *et al. Malar J (2017) 16:469*

Page 3 of 14

Fig. 1 Maps of Oromia Region, Ethiopia displaying insecticides used for IRS activities in PMI-supported districts from 2010 to 2016. Note there have been changes in district boundaries over this time period. Additionally, deltamethrin LLINs have been distributed en masse across all regions since 2010

DDT (4%), deltamethrin (0.05%), etofenprox (0.5%), fenitrothion (1%), lambda-cyhalothrin (0.05%), malathion (5%), permethrin (0.75%), pirimiphos-methyl (0.25%) and propoxur (0.1%)) were conducted according to WHO guidelines [17]. The diagnostic dose of insecticides used on papers is generally twice the LC_{99} values systematically determined from baseline studies in multiple locations [18]. In 2012, four replicates of 20–25 non-blood fed, 2-3 days old adult female mosquitoes were exposed to different insecticide-impregnated papers in WHO tubes for 1 h (except in the case of fenitrothion where mosquitoes were exposed for 2 h) and in parallel, one replicate of control mosquitoes (20–25 mosquitoes per tube) was exposed to oil-impregnated papers; from 2013 onwards, two control replicates using 25 mosquitoes were performed. For all assays, mosquito mortality was recorded after a 24-h holding period. Cotton wool soaked in 10% sugar solution was provided to mosquitoes on top of the holding tube and optimum temperature and relative humidity was maintained using a damp towel placed on top of boxes where holding tubes were kept.

CDC bottle bioassays
CDC bottle bioassays for seven insecticides (alpha-cypermethrin, bendiocarb, DDT, deltamethrin, lambda-cyhalothrin, permethrin and propoxur) were conducted according to published guidelines [19]. Stock solutions were prepared by diluting technical grade insecticide in 50 mL of acetone. Each Wheaton 250 mL bottle

along with its cap was coated with 1 mL of stock solution (12.5 μg/bottle for alpha-cypermethrin, bendiocarb, deltamethrin, lambda-cyhalothrin and propoxur, 100 μg/bottle for DDT and 21.5 μg/bottle for permethrin) by rolling and inverting the bottles. In each test, a control bottle was coated with 1 mL of acetone. Following coating, bottles were covered with mats and left to dry. Approximately, 10–25 non-blood fed, 2–3 days old adult female mosquitoes were introduced into each bottle using a mouth aspirator and mortality was recorded at 15 min intervals up to 30 min for all insecticides except DDT; for this assay mosquitoes were exposed for 45 min. From 2014 onwards, CDC resistance intensity assays were performed by testing 1, 2, 5 and 10 times the concentration required to kill all mosquitoes (LC_{100}), as determined in a series of baseline experiments [19], and in selected sites, synergist assays were also conducted by pre-exposing mosquitoes to PBO for 1 h (100 μg/bottle).

Molecular identification of *Anopheles gambiae* species complex
Mosquitoes used in bioassay tests were identified morphologically using standard keys [20]. In 2015 and 2016, sub-samples of both surviving and dead mosquitoes from WHO tests were randomly selected by insecticide (DDT and deltamethrin), site and region for molecular species identification and *kdr* allele detection at the Molecular Biology Laboratory, Tropical and Infectious Diseases Research Centre (TIDRC) of Jimma University. Genomic

Messenger *et al. Malar J* (2017) 16:469

Page 4 of 14

DNA was extracted following the procedure described by Collins et al. [21]. DNA was re-suspended in 25 ml sterile TE-buffer (10 mM Tris–HCl pH 8, 1 mM EDTA). Molecular identification of *An. gambiae* s.l. was carried out by species-specific PCR using primers for *An. gambiae* s.s., *An. arabiensis* and *Anopheles quadriannulatus* species B (*Anopheles amharicus*) according to Scott et al. [22], with modifications [9, 23]. Briefly, genomic DNA was mixed with primers AR (5′-AAGTGTCCTTCTCCATCCRA-3′; specific for *An. arabiensis*), AG (5′-CTGGTTTGGTCG-GCACGTTT-3; specific for *An. gambiae* s.s.), QD-b (5′-AGTGTCCAATGTCTGTGAAG-3′; specific for *An. quadriannulatus* species B) and UN (5′-GTGTGCC-CCTTCCTCGATGT-3′; common for all species) in a 25 μL reaction. Amplification reactions contained 1 μL of DNA, 1.5 mM MgCl$_2$, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100, 200 μM of dNTPs (Amersham, Buckinghamshire, United Kingdom), 25 pmol of primers AR, AG, QD-b and UN and 0.25 U of SilverStar DNA polymerase (Eurogentec, Seraing, Belgium) [24]. PCR reaction conditions are described in Scott et al. [22]. Amplified PCR products were visualized on 2% agarose gels, stained with ethidium bromide. *An. arabiensis* strain from the Sekoru colony, maintained at the Vector Biology and Control Research Unit, TIDRC, Jimma University, was used as a positive control.

Detection of resistance mutations

West African *kdr* (L1014S) and East African *kdr* (L1014F) alleles were detected using adapted protocols [24] for allele-specific PCR (AS-PCR), developed by Martinez-Torres et al. [25] and Ranson et al. [26]. Primers Agd1 (5′-ATAGATTCCCCGACCATG-3′), Agd2 (5′-AGACAAGGATGATGAACC-3′), Agd3 (5′-AATTT-GCATTACTTACGACA-3′) and Agd4 (5′-CTGTAGT-GATAGGAAATTTA-3′) were used to detect the L1014F allele (AS-PCR Agd3), whereas primers Agd1, Agd2, Agd4 and Agd5 (5′-TTTGCATTACTTACGACTG-3′) were used to detect the L1014S allele (AS-PCR Agd5). Amplifications were performed in 50 μL reactions containing 2 μL DNA, 1× Qiagen PCR buffer, 0.5 mM MgCl$_2$, 100 nM of each primer, 200 μM dNTPs, and 1U *Taq* DNA polymerase (*Taq* PCR core kit, Qiagen, Hilden, Germany). The cycling conditions were: initial 94 °C denaturation for 5 min, 10 cycles of 1 min denaturation at 94 °C, 30 s annealing at 54 °C and 30 s extension at 72 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 30 s annealing at 47 °C and 30 s extension at 72 °C, and a final extension at 72 °C for 10 min. Amplification products were visualized on 2% agarose gels, stained with ethidium bromide. *An. arabiensis* from the Sekoru colony (a *kdr* negative mosquito strain) was used as a negative control.

Data analysis

Data were interpreted according to the WHO guidelines [17]; mortality of 98% or higher in susceptibility tests indicates susceptibility, mortality of 90–97% is suggestive of resistance and mortality of less than 90% indicates resistance. Mortality was corrected using Abbott's formula, when mortality in control assays was between 5 and 20% [17, 27]. A bioassay was repeated if control mortality exceeded 20%. Per site, mean percent mosquito mortality was determined across all replicates for a given insecticide. Pearson's Chi squared tests were used to evaluate the association of *kdr* frequency with WHO assay results and to test for deviations from Hardy–Weinberg equilibrium. Cohen's Kappa (κ) was calculated to quantify the magnitude of agreement between WHO susceptibility tests and CDC bottle bioassays [28]; values were interpreted as poor (κ ≤ 0), slight (0 < κ ≤ 0.2), fair (0.2 < κ ≤0.4), moderate (0.4 < κ ≤0.6), substantial (0.6 < κ ≤0.8) and almost perfect agreement (0.8 < κ ≤1.0) [29]. All statistical analyses were performed in Stata/IC 14.2 (Stata Corp., College Station, USA) with the level of significance set at α = 0.05.
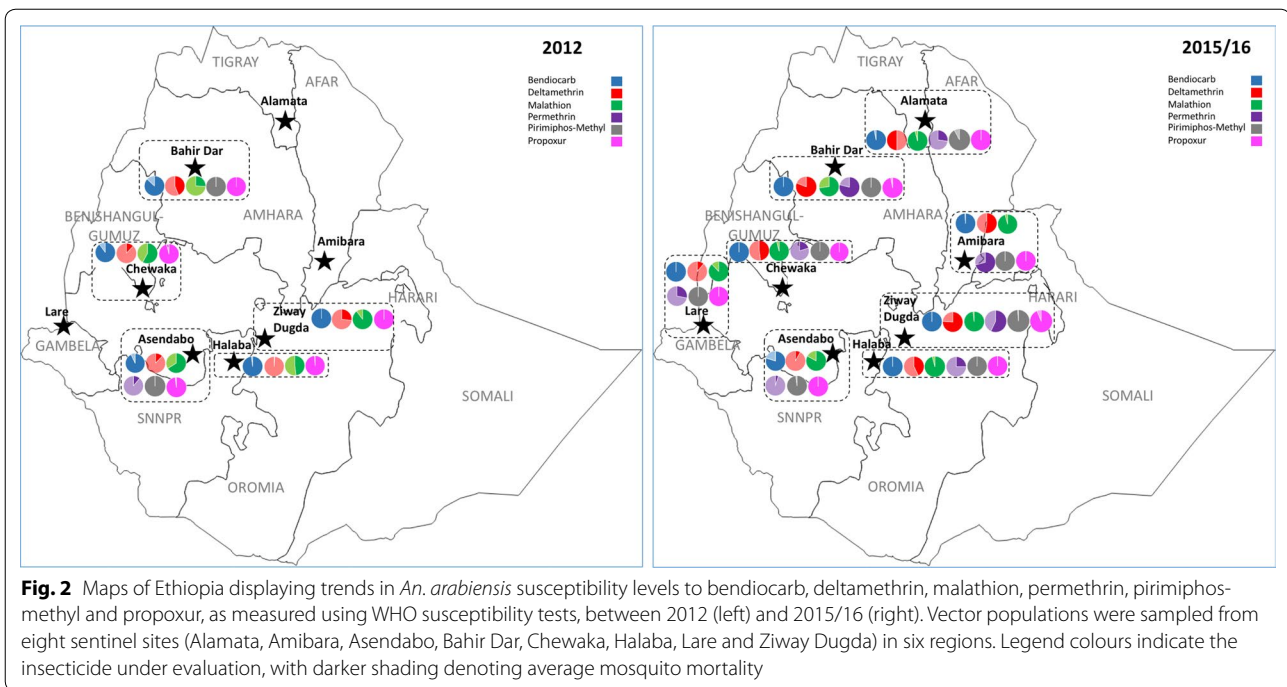
Results

WHO susceptibility tests

Results from WHO susceptibility tests conducted in eight sentinel sites (Alamata, Amibara, Asendabo, Bahir Dar, Chewaka, Halaba, Lare and Ziway-Dugda) between 2012 and 2016 are summarized in Table 1 and Fig. 2. WHO tests conducted in additional areas from 2013 onwards are detailed in Additional file 1: Tables S3–S6 by year.

In 2012, high levels of pyrethroid resistance were evident across Ethiopia, with *An. arabiensis* mortality levels of 50% or less for alpha-cypermethrin, deltamethrin, etofenprox, lambda-cyhalothrin and permethrin. Similarly, all mosquito populations were highly resistant to DDT (mortality ranging from 0 to 13%) and demonstrated variable levels of susceptibility to the organophosphate malathion (26–90% mortality) (Table 1). In contrast, *An. arabiensis* was fully susceptible to organophosphates fenitrothion and pirimiphos-methyl and the carbamate propoxur, with the exception of Chewaka, where average mortality for the latter was 96%. Low levels of developing bendiocarb (carbamate) resistance were detected in three study sites, Asendabo, Bahir Dar and Chewaka (mortality of 93, 87 and 90%, respectively).

In 2015–2016, most *An. arabiensis* populations presented consistent susceptibility profiles, with minor variations in some resistance levels; notably in four sites (Asendabo, Chewaka, Halaba and Ziway-Dugda) susceptibility to malathion increased over time (from 66 to 83%, 58 to 96%, 48 to 96% and 90 to 98% average mosquito mortality, respectively), while mosquito mortality to

Messenger *et al. Malar J* (2017) 16:469

Page 5 of 14

Table 1 Percentage corrected mortality (and numbers tested) of *Anopheles arabiensis* in WHO susceptibility tests conducted in eight national sentinel sites in Ethiopia 2012–2016

Insecticide Class	Insecticide
Carbamate	Bendiocarb
	Propoxur
Organochlorine	DDT
	Fenitrothion
	Malathion
Organophosphate	Pirimiphos-methyl
	Alpha-cypermethrin
	Deltamethrin
	Etofenprox
	Lambda-cyhalothrin
Pyrethroid	Permethrin

Messenger *et al. Malar J* (2017) 16:469

Page 6 of 14

Fig. 2 Maps of Ethiopia displaying trends in *An. arabiensis* susceptibility levels to bendiocarb, deltamethrin, malathion, permethrin, pirimiphos-methyl and propoxur, as measured using WHO susceptibility tests, between 2012 (left) and 2015/16 (right). Vector populations were sampled from eight sentinel sites (Alamata, Amibara, Asendabo, Bahir Dar, Chewaka, Halaba, Lare and Ziway Dugda) in six regions. Legend colours indicate the insecticide under evaluation, with darker shading denoting average mosquito mortality

pirimiphos-methyl began to decline slightly in Asendabo and Ziway-Dugda (from 100 to 98% and 100 to 99% mortality, respectively) and possible resistance to propoxur emerged in Ziway-Dugda (94% mortality) and Bahir Dar (97% mortality). Resistance to pirimiphos-methyl and propoxur was also detected in additional study sites from Oromia region; *An. arabiensis* mortality was 85% in Babile in 2016 to pirimiphos-methyl, and 75% and 95% to propoxur in Abaya in 2013 and in Nono in 2016, respectively (Additional file 1: Tables S3, S6). Interestingly, vectors from an area of sesame cultivation sampled in 2016 (Metema; Amhara Region) demonstrated almost complete susceptibility to deltamethrin (average mortality of 99%; Additional file 1: Table S6).
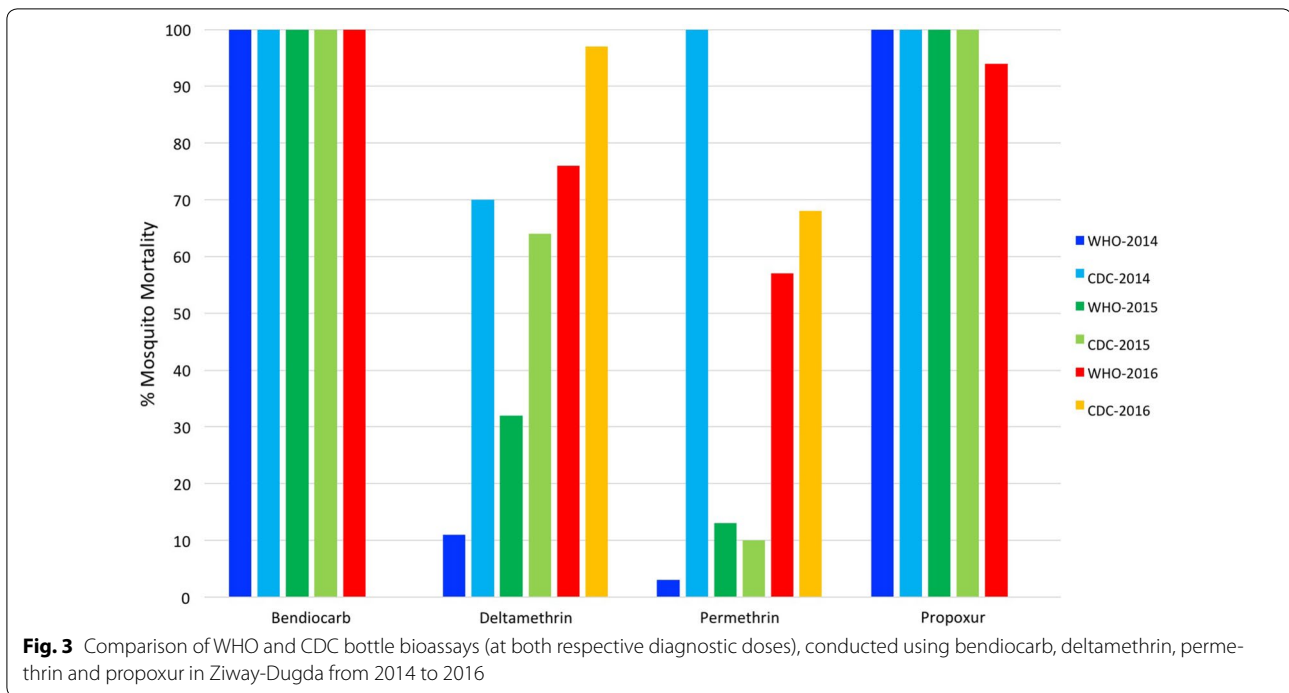
Similar longitudinal trends were observed in Alamata, Lare and Amibara, where resistance monitoring began in 2014 (Table 1). In these areas, at baseline, vector populations were also resistant to pyrethroids and DDT, fully susceptible to fenitrothion, pirimiphos-methyl and propoxur and potentially resistant to bendiocarb in Alamata and Lare (mortality of 96 and 92%, respectively). In these latter two sites, resistance to malathion was also detected (mortality of 89 and 95%, respectively). By 2016, putative resistance to pirimiphos-methyl had developed in Alamata (mortality of 92%) and to malathion in Amibara (mortality of 96%). On a yearly basis, levels of resistance fluctuated within sites, in some cases modestly (e.g. in Asendabo, mortality to bendiocarb oscillated from 93% in 2012, to 86% in 2014, 95% in 2015 and 80% in 2016)

and in others more dramatically, beyond what might be expected of stochastic variation (e.g. in Bahir Dar, mortality to malathion ranged from 26% in 2012, to 89% in 2014 and 43% in 2015).

CDC bottle bioassays
From 2013 onwards, CDC bottle bioassays, resistance intensity and synergist assays were conducted in additional study sites, which differed between years (Additional file 1: Tables S7–S10). Data from Ziway-Dugda, where these tests were performed routinely throughout the monitoring period are presented in Fig. 3 and Additional file 1: Table S11, in comparison with concurrent WHO bioassays. Results from both WHO tests (diagnostic dose) and CDC bottle bioassays (2X) were concordant for bendiocarb and propoxur; vector populations were fully susceptible to both insecticides until 2016. However, levels of pyrethroid resistance were not comparable between techniques, e.g. *An. arabiensis* mortality in 2014 to deltamethrin was 11% in WHO tests, compared to 70% in CDC bottle bioassays at the same discriminatory dose ($\kappa \leq 0$, for all comparisons between pyrethroid assays in 2014, 2015 and 2016).

While consistently high levels of pyrethroid resistance were observed using WHO tests, examination of CDC bioassays from additional study areas identified some completely susceptible *An. arabiensis* populations, e.g. mortality was 100% to deltamethrin in Abedogoro at the equivalent WHO diagnostic dose (2X) in 2015. Focal

Messenger *et al. Malar J* (2017) 16:469

Page 7 of 14

Fig. 3 Comparison of WHO and CDC bottle bioassays (at both respective diagnostic doses), conducted using bendiocarb, deltamethrin, permethrin and propoxur in Ziway-Dugda from 2014 to 2016

patterns of pyrethroid resistance were also apparent in 2013, where vector populations, susceptible to one or more pyrethroids, were identified in eleven areas across three regions (Additional file 1: Table S7). In these sites resistance to a particular pyrethroid was not necessarily associated with increased tolerance to another, e.g. in Gobu-Seyo (Oromia Region) mosquito mortality was 76, 49, 98 and 8% to alpha-cypermethrin, deltamethrin, lambda-cyhalothrin and permethrin at the CDC diagnostic doses, respectively. In other areas with high levels of pyrethroid resistance, CDC resistance intensity assays detected some mosquitoes capable of surviving ten times the diagnostic dose of deltamethrin or permethrin (e.g. average mortality of 75 and 65% in Asendabo in 2015, respectively; Additional file 1: Table S9). High intensities of DDT resistance were also observed in these areas, e.g. 95% of mosquitoes survived ten times the diagnostic dose in Wondogenet in 2014 (Additional file 1: Table S8).

In all sites where mosquitoes were pre-exposed to PBO, a synergist that interferes with oxidase activity, resistance to both deltamethrin and permethrin was reduced, increasing mortality to 87–100% when mosquitoes were exposed to the 1X dose (Additional file 1: Tables S8–S11).

Molecular detection of species and resistance mutations

Following WHO susceptibility tests in 2015, three hundred and sixty-four surviving and dead *An. gambiae* s.l. specimens were randomly selected from eight study sites

in six regions [Asendabo, Chewaka, Ziway-Dugda (Oromia region), Bahir Dar (Amhara region), Amibara (Afar region), Alamata (Tigray region), Halaba (SNNPR) and Abobo (Gambela region)] and were assayed to determine species. Of these, 94.8% (345/364) of samples amplified and were identified as *An. arabiensis*. Non-amplifiers were evenly distributed across all study sites, except Amibara and Ziway-Dugda where all specimens were successfully classified. In 2016, six hundred and sixty-two *An. gambiae* s.l. were collected from ten study sites in six regions: Asendabo, Babile, Nono and Ziway-Dugda (Oromia region), Metema (Amhara region), Amibara (Afar region), Abobo (Gambela region), Alamata and Humera (Tigray region) and Arba Minch (SNNPR), and 86.7% (574/662) of specimens were confirmed as *An. arabiensis*. Higher proportions of non-amplifiers were collected from Metema (17.6% of total mosquitoes sampled), Nono (30%), Arba Minch and Abobo (both 17.5%), than other neighboring areas (range of remaining sites: 5–12.5%).

In 2015, the presence of *kdr* was also assessed in matched mosquito samples (Table 2). The West African (L1014F) *kdr* allele was identified in 75.5% (n = 275/364) of specimens (215 alive and 60 dead), with allele frequencies ranging from 50 to 100% and 13 to 88% in surviving and dead *An. arabiensis* from DDT bioassays, respectively, and from 36 to 100% and 13 to 100% in surviving and dead *An. arabiensis* from deltamethrin bioassays, respectively. The majority of vectors surviving bioassays were homozygous for *kdr* (54.0%; 116/215) compared to those

Messenger *et al. Malar J* (2017) 16:469

Page 8 of 14

Table 2 Genotypic and *kdr* allele frequencies in *Anopheles arabiensis* from eight national sentinel sites in Ethiopia, 2015

Region	Village/site	Insecticide	Survival status after exposure	# mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	Kdr allele frequency R	S	χ^2 test	p value	% mortality in WHO tests	Resistance status
Oromia	Chewaka	DDT	Alive	15	1	6	6	0.31	0.69	0.345	0.557	14	R
			Dead	5	1	1	3	0.3	0.7	1.372	0.242		
		Deltamethrin	Alive	15	2	4	5	0.36	0.64	1.441	0.23	48	R
			Dead	5	1	1	0	0.75	0.25	1.889	0.169		
	Asendabo	DDT	Alive	30	14	6	0	0.85	0.15	3.749	0.0528	4	R
			Dead	4	0	1	3	0.13	0.88	0.0892	0.765		
		Deltamethrin	Alive	30	16	4	4	0.75	0.25	7.126	0.0076	32	R
			Dead	6	2	2	1	0.6	0.4	0.282	0.595		
	Ziway-Dugda	DDT	Alive	15	3	4	3	0.5	0.5	1.988	0.164	–	–
			Dead	–	–	–	–	–	–	–	–		
		Deltamethrin	Alive	15	3	4	3	0.5	0.5	1.988	0.164	32	R
			Dead	10	0	3	5	0.19	0.81	0.734	0.391		
Amhara	Bahir Dar	DDT	Alive	15	3	4	1	0.63	0.37	3.287	0.0698	11	R
			Dead	5	0	1	3	0.13	0.87	0.262	0.609		
		Deltamethrin	Alive	15	3	6	3	0.5	0.5	0.6	0.439	25	R
			Dead	5	0	1	3	0.13	0.87	0.262	0.609		
SNNPR	Halaba	DDT	Alive	15	8	1	0	0.94	0.06	2.42	0.12	25	R
			Dead	5	1	0	2	0.3	0.7	2.855	0.0911		
		Deltamethrin	Alive	15	5	2	1	0.75	0.25	3.741	0.0531	43	R
			Dead	5	2	1	1	0.63	0.37	0.906	0.341		
Tigray	Alamata	DDT	Alive	9	9	0	0	1	0	–	–	40	R
			Dead	5	3	1	0	0.88	0.12	0.271	0.602		
		Deltamethrin	Alive	8	6	0	0	1	0	–	–	57	R
			Dead	3	1	0	0	1	0	–	–		
Afar	Amibara	DDT	Alive	14	6	2	1	0.78	0.22	2.535	0.111	48	R
			Dead	5	1	2	2	0.4	0.6	0.139	0.709		
		Deltamethrin	Alive	15	2	7	3	0.46	0.54	0.893	0.345	49	R
			Dead	5	1	3	1	0.5	0.5	0.2	0.655		
Gambela	Lare	DDT	Alive	30	17	10	3	0.74	0.26	0.733	0.392	24	R
			Dead	5	0	4	0	0.5	0.5	3.4	0.0652		
		Deltamethrin	Alive	30	18	3	3	0.81	0.19	7.746	0.00538	11	R
			Dead	5	1	1	0	0.75	0.25	1.889	0.169		

Messenger *et al. Malar J* (2017) 16:469

Page 9 of 14

that died (23.3%; 14/60); 29.3% (63/215) and 36.7% (22/60) were heterozygous for *kdr*, respectively. The *kdr*-West allele was present in 100% of surviving vectors from one study site (Alamata; Tigray region) and 94% in another (Halaba; the SNNPR). Deviations from Hardy–Weinberg equilibrium were only observed in *An. arabiensis* survivors of deltamethrin bioassays in Asendabo and Lare ($\chi^2 = 7.13$; $p = 0.0076$ and $\chi^2 = 7.75$; $p = 0.0054$ respectively) (Table 2). There was no significant association between *kdr* allele frequency and *An. arabiensis* mortality following exposure to DDT or deltamethrin ($p = 0.227$ and $p = 0.208$, respectively). The East African (L1014S) *kdr* allele was not detected in any samples assayed.

In 2016, all mosquito specimens confirmed as *An. arabiensis* (n = 562; with the exception of 8 and 4 samples from Metema and Alamata, respectively) were assayed for *kdr* (Table 3). L1014F *kdr* was detected in 73.5% (n = 413/562) of *An. arabiensis* (322 alive and 91 dead), with allele frequencies ranging from 31 to 89% and 10 to 100% in surviving and dead mosquitoes from DDT bioassays, respectively, and from 33 to 100% and 6 to 94% in surviving and dead mosquitoes from deltamethrin bioassays, respectively. There was no significant differences in *kdr* allele frequency between years among DDT or deltamethrin bioassay survivors ($p = 0.229$ and $p = 0.158$, respectively). As previously, the majority of *An. arabiensis* surviving bioassays were homozygous for *kdr* (42.2%; 136/322) compared to those that died (28.6%; 26/91); 33.2% (107/322) and 28.6% (26/91) were heterozygous for *kdr*, respectively. The *kdr*-West allele was fixed in surviving vectors from two study sites (Babile and Nono; Oromia region); in Alamata, *kdr* frequencies had declined from 100% in 2015 to 62% and 57% among survivors of DDT and deltamethrin bioassays, respectively. Consistent with results from 2015, deviations from Hardy–Weinberg equilibrium were identified among *An. arabiensis* in Asendabo ($\chi^2 = 9.71$; $p = 0.0018$ for deltamethrin survivors) (Table 3) and in additional vector populations in Abobo ($\chi^2 = 13.60$; $p = 0.00023$, $\chi^2 = 3.83$; $p = 0.05$ for survivors of DDT and deltamethrin bioassays, respectively), Arba Minch ($\chi^2 = 4.87$; $p = 0.027$ for DDT survivors), Alamata ($\chi^2 = 4.83$; $p = 0.028$ for deltamethrin survivors) and Amibara ($\chi^2 = 5.62$; $p = 0.018$ and $\chi^2 = 6.53$; $p = 0.011$ for DDT and deltamethrin survivors, respectively). There was no significant association between *kdr* allele frequency and *An. arabiensis* mortality following exposure to DDT or deltamethrin ($p = 0.231$ and $p = 0.267$, respectively).

Discussion

The development of pervasive insecticide resistance across sub-Saharan Africa threatens to jeopardize the long-term effectiveness of both IRS and LLINs for malaria control [30, 31]. This study presents data from the largest nationwide, longitudinal monitoring of insecticide resistance among *An. arabiensis* populations to four classes of insecticides in Ethiopia. Intense resistance to pyrethroids (alpha-cypermethrin, deltamethrin, etofenprox, lambda-cyhalothrin and permethrin) and DDT were commonplace, and in many sites, vectors were able to survive exposure to five to ten times the diagnostic dose. These high levels of resistance are likely a direct consequence of historic DDT use for IRS, as well as its considerable application between 2000 and 2005, where 255,000–298,000 kg/year were used [32], alongside mass distributions of LLINs [2].

Patterns of resistance to bendiocarb, malathion, propoxur and pirimiphos-methyl also corresponded to shifts in the national insecticide policy [2]. Since 2012, *An. arabiensis* susceptibility to malathion increased in some areas, potentially attributable to the discontinuation of this insecticide for IRS; malathion was last used extensively for malaria control from 2003 to 2005 by the NMCP in areas with reported DDT resistance [5, 9]. Between 2011 and 2015, bendiocarb (with deltamethrin in 2011–2012) was the insecticide of choice for PMI-supported IRS activities in Oromia Region, where low levels of mosquito resistance were initially detected in 2012. However, because bendiocarb was largely abandoned due to its short residual efficacy, relative to other organophosphates and carbamates [33], only moderate levels of resistance developed in a few areas. Concurrent propoxur spraying in 2012 was accompanied by the emergence of potential resistance in some *An. arabiensis* populations by 2014 and likewise, decreased *An. arabiensis* susceptibility to pirimiphos-methyl was observed in 2016, concomitant with the switch to this insecticide in selected districts in 2015; however it should be noted that these sites fell outside the districts where pirimiphos-methyl was used for IRS. In general, the reactive, and in some cases, heterogeneous use of different insecticides has resulted in highly focal, volatile resistance profiles across sentinel sites [34], complicating the prospective deployment of interventions for vector control. Study observations are consistent with earlier cross-sectional evaluations from Ethiopia [35], which also describe widespread resistance to DDT and pyrethroids, as well as more restricted decreases in *An. arabiensis* susceptibility to malathion, bendiocarb and propoxur [5–15]. Furthermore, results from Sudan, Kenya and Eritrea corroborate large-scale resistance trends in *An. arabiensis* documented across this region [35–38].

Study results raised concerns pertaining to the comparability of WHO and CDC insecticide resistance tests. While in some areas, outcome measurements from both assays are reported to be equivalent in terms of resistance

Messenger et al. Malar J (2017) 16:469

Page 10 of 14

Table 3 Genotypic and kdr allele frequencies in Anopheles arabiensis from ten national sentinel sites in Ethiopia, 2016

Region	Site	Insecticide	Survival status after exposure	# mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	kdr allele frequency R	kdr allele frequency S	χ² test	P value	% mortality in WHO tests	Resistance status
Oromia	Babile	DDT	Alive	13	7	2	0	0.89	0.11	1.33	0.249	11	R
			Dead	9	9	0	0	1	0	–	–		
		Deltamethrin	Alive	15	14	0	0	1	0	–	–	22	R
			Dead	9	8	1	0	0.94	0.167	1.431	0.232		
	Nono	DDT	Alive	12	2	3	3	0.44	0.56	1.635	0.201	29	R
			Dead	9	0	3	3	0.25	0.75	1.444	0.229		
		Deltamethrin	Alive	8	7	0	0	1	0	–	–	52	R
			Dead	6	2	2	2	0.5	0.5	0.667	0.414		
	Asendabo	DDT	Alive	28	9	11	6	0.56	0.44	0.635	0.426	10	R
			Dead	0	0	0	0	0	0	–	–		
		Deltamethrin	Alive	25	3	12	6	0.43	0.57	9.706	0.00184	8	R
			Dead	7	1	1	3	0.3	0.7	1.551	0.213		
	Ziway-Dugda	DDT	Alive	30	9	10	11	0.47	0.53	3.272	0.0705	27	R
			Dead	10	0	2	8	0.1	0.9	0.123	0.725		
		Deltamethrin	Alive	9	2	4	3	0.44	0.56	0.092	0.762	76	R
			Dead	8	0	1	7	0.06	0.94	0.04	0.841		
Amhara	Metema	DDT	Alive	11	1	3	4	0.31	0.69	0.914	0.339	74	R
			Dead	8	0	1	4	0.2	0.8	1.516	0.218		
		Deltamethrin	Alive	1	1	0	0	1	0	–	–	99	S
			Dead	0	0	0	0	0	0	–	–		
SNNPR	Arba Minch	DDT	Alive	28	12	7	7	0.6	0.4	4.869	0.0273	51	R
			Dead	10	1	3	4	0.31	0.69	0.505	0.477		
		Deltamethrin	Alive	22	12	8	2	0.73	0.27	0.157	0.692	41	R
			Dead	6	2	1	3	0.41	0.59	2.619	0.106		
Tigray	Alamata	DDT	Alive	30	10	12	4	0.62	0.38	0.552	0.458	34	R
			Dead	9	0	4	3	0.29	0.71	1.301	0.254		
		Deltamethrin	Alive	20	6	5	4	0.57	0.43	4.834	0.0279	50	R
			Dead	10	0	1	3	0.13	0.87	3.631	0.0567		
	Humera	DDT	Alive	27	14	6	4	0.71	0.29	3.685	0.0549	33	R
			Dead	10	0	2	5	0.14	0.86	1.041	0.308		
		Deltamethrin	Alive	24	7	6	5	0.56	0.44	2.935	0.0867	39	R
			Dead	9	2	2	4	0.38	0.62	1.646	0.2		

Messenger *et al. Malar J (2017) 16:469*

Page 11 of 14

Table 3 continued

Region	Site	Insecticide	Survival status after exposure	# mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	*kdr* allele frequency		χ^2 test	*P* value	% mortality in WHO tests	Resistance status
								R	S				
Afar	Amibara	DDT	Alive	30	6	6	13	0.36	0.64	5.617	0.0178	67	R
			Dead	10	0	1	4	0.1	0.9	2.531	0.112		
		Deltamethrin	Alive	24	3	3	8	0.33	0.68	6.529	0.0106	53	R
			Dead	9	0	0	5	0	1	–	–		
Gambela	Abobo	DDT	Alive	19	0	2	1	0.33	0.67	13.593	0.000227	37	R
			Dead	9	1	0	2	0.33	0.67	5.01	0.0252		
		Deltamethrin	Alive	30	11	7	3	0.73	0.27	3.827	0.05	33	R
			Dead	8	0	1	5	0.08	0.92	0.541	0.462		

Messenger *et al. Malar J* (2017) 16:469

Page 12 of 14

monitoring [39, 40], our data align with others reporting considerable discrepancies [28, 41, 42], which were problematic to interpret, particularly when susceptibility profiles annually fluctuated above or below the thresholds of resistance set by the WHO [18, 28]. Previous studies have suggested that the extent of inter-assay agreement may reflect levels of susceptibility heterogeneity, whereby tests conducted on vector populations with highly variable resistance profiles are more prone to inconsistent results [28]. Indeed, direct comparisons between our WHO and CDC bottle bioassays performed using bendiocarb and propoxur (where *An. arabiensis* were completely susceptible), and against pyrethroids (where resistance was more capricious) supports this supposition. Others have proposed that CDC bioassays may over-estimate pyrethroid resistance, as insecticide repellency can reduce the already relatively short contact time (30 min) of mosquitoes in coated bottles [40]. In this regard, our results demonstrated the opposite; higher and more uniform levels of pyrethroid resistance were obtained for WHO tests, when results were pooled across study sites.

There are a number of other technical and biological factors which could contribute to discordance between assays. WHO papers are distributed from a centralized source, which may render them prone to inter-batch variation and depending upon procurement schedules, can result in filter papers of different ages being used for the same monitoring activities in a given year. Ideally, to ensure consistency between study sites, all batches of papers would have been tested initially using a susceptible laboratory strain. CDC bottle bioassays are coated in-house which also introduces issues of standardization based on the proficiency of individual laboratory technicians, conditions of insecticide storage and numbers of consecutive times bottles are re-used [43–45]. *Anopheles* larvae were sampled from a range of the most productive breeding sites with different effective population sizes, genetic compositions, temperatures, nutritional access and chemical exposures depending upon local ecology. One important factor that was not investigated in this study was the influence that intensive agriculture pesticide use has had on resistance levels in Ethiopia (reviewed by [46]). Once collected, bioassays were conducted on emergent adult mosquitoes who were presumed to be *An. arabiensis*, based on PCR validation of a sub-set in 2015 and 2016. It should be noted that 10.4% of all PCR reactions did not amplify either because of technical errors or the specimens did not belong to the species under investigation (*An. gambiae* s.s., *An. arabiensis* or *An. quadriannulatus* species B/*An. amharicus*). Finally, WHO tests assess mortality after a 24-h holding period, while CDC bioassays measure knock-down and acute toxicity, which depending upon the degree of

vector tolerance, may not necessarily be interchangeable [28].

Regarding the underlying mechanisms of resistance, the restoration of pyrethroid susceptibility following pre-exposure to PBO and the non-association of L1014F *kdr* allele frequency with levels of *An. arabiensis* mortality in WHO bioassays, suggests that both over-expression of detoxification enzymes and target-site mutations are driving insecticide resistance. Moderate to high *kdr* frequencies were detected in a number of sites and were fixed or approaching fixation in a minority. However, by comparison with earlier reports from the same areas, *kdr* allele frequencies were lower overall [9, 14, 47] and between our study years we also observed a slight, albeit not statistically significant, decline. In 2015, the lack of deviations from Hardy–Weinberg equilibrium in most areas indicated that selection for *kdr* heterozygotes was not ongoing, allowing this allele to be lost to genetic drift in some populations, but by 2016, study sites from five regions demonstrated evidence for *kdr* selection, potentially resulting from a mass distribution of LLINs by the NMCP beginning in August 2015. Given the lack of association between mosquito bioassay mortality and presence/absence of *kdr* mutation, there are likely to be other mechanisms at play in the development of resistance. Future local surveillance programmes may wish to consider screening for additional, recently identified genetic markers of metabolic resistance in *An. arabiensis*, e.g. *CYP6P4* [48].

Conclusions

To date, the presence of insensitive acetylcholinesterase mutations (*ace-1*^{R} or G119S), known to mediate resistance to both organophosphates and carbamates in *An. gambiae* s.s. and *An. arabiensis* [49, 50], has not been reported in Ethiopia among vectors resistant to malathion or propoxur [9]. The bioassay results in this study were not indicative of any cross-resistance both between organophosphates and carbamates and among chemicals belonging to the same insecticide class. This observation suggests the existence of additional metabolic resistance mechanisms that can confer insecticide-specific resistance and also has implications for the development of an insecticide resistance management strategy. If inter- and intra-class rotation of different insecticides could be exploited to reduce selection pressures, this may have the potential to safeguard continued efficacy of IRS and other vector control strategies in Ethiopia and proactively mitigate the development of future insecticide resistance. Furthermore, additional epidemiological studies are warranted, in parallel with future resistance monitoring activities, to determine the operational impact of insecticide resistance on malaria vector control in Ethiopia.

Messenger *et al. Malar J* (2017) 16:469

Page 13 of 14

Additional file

Author details

[1] Entomology Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30329-4027, USA. [2] President's Malaria Initiative Africa Indoor Residual Spraying Project, Abt Associates, Gerji Road, Sami Building, 1st Floor, Addis Ababa, Ethiopia. [3] U.S. Agency for International Development (USAID), Entoto Street, Addis Ababa, Ethiopia. [4] President's Malaria Initiative Africa Indoor Residual Spraying Project, Abt Associates, 4550 Montgomery Ave., Suite 800 North, Bethesda, MD 20814, USA. [5] Tropical and Infectious Diseases Research Center, Jimma University, Jimma, Ethiopia. [6] Medical and Entomology Unit, Institute of Bio-Medical Sciences, College of Health Sciences, Mekelle University, Mek'ele, Ethiopia. [7] National Malaria Control Programme, Federal Ministry of Health, Addis Ababa, Ethiopia. [8] President's Malaria Initiative, United States Agency for International Development, Bureau for Global Health, Office of Infectious Disease, 2100 Crystal Drive, Arlington, VA 22202, USA. [9] Department of Medical Laboratory Sciences and Pathology, College of Health Sciences, Jimma University, Jimma, Ethiopia.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the institutional review board (IRB) of the College of Health Sciences at Jimma University.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. WHO. World Malaria Report 2016. Geneva: World Health Organization; 2016.
2. President's Malaria Initiative. Malaria Operational Plan FY 2016—Ethiopia. USAID; 2016.
3. Africa Indoor Residual Spraying Project, A.A.I. Ethiopia: Entomological Monitoring of 2015 IRS Activities. Final Report. Addis Ababa, Ethiopia; 2015.
4. MOH. National malaria strategic plan (2014–2020). Addis Ababa: Federal Democratic Republic of Ethiopia, Ministry of Health; 2014.
5. Abose T, Yeebiyo Y, Olana D, Alamirew D, Beyene Y, Regassa L, et al. Reorientation and definition of the role of malaria vector control in Ethiopia. The epidemiology and control of malaria with special emphasis on the distribution, behaviour and susceptibility of insecticides of Anopheline vectors and chloroquine resistance in Zwai, central Ethiopia and other areas. WHO/MAL/98.1085; 1998.
6. Nigatu W, Petros B, Lulu M, Adunga N, Wirtz R, Tilahun D. Some aspects of malaria prevalence, vector infectivity and DDT resistance studies in Gambella Region, South Western Ethiopia. Ethiop J Health Dev. 1994;8:1–10.
7. Balkew M, Gebre-Michael T, Hailu A. Insecticide susceptibility level of *Anopheles arabiensis* in two agro-development localities in eastern Ethiopia. Parassitologia. 2003;45:1–3.
8. Balkew M, Ibrahim M, Koekemoer LL, Brooke BD, Engers H, Aseffa A, et al. Insecticide resistance in *Anopheles arabiensis* (Diptera: Culicidae) from villages in central, northern and south west Ethiopia and detection of *kdr* mutation. Parasites Vectors. 2010;3:40.
9. Yewhalaw D, Wassie F, Steurbaut W, Spanoghe P, Van Bortel W, Denis L, et al. Multiple insecticide resistance: an impediment to insecticide-based malaria vector control program. PLoS ONE. 2011;6:e16066.
10. Asale A, Getachew Y, Hailesilassie W, Speybroeck N, Duchateau L, Yewhalaw D. Evaluation of the efficacy of DDT indoor residual spraying and long-lasting insecticidal nets against insecticide resistant populations of *Anopheles arabiensis* Patton (Diptera: Culicidae) from Ethiopia using experimental huts. Parasites Vectors. 2014;7:131.
11. Abate A, Hadis M. Susceptibility of *Anopheles gambiae* s.l. to DDT, malathion, permethrin and deltamethrin in Ethiopia. Trop Med Int Health. 2011;16:486–91.
12. Yewhalaw D, Asale A, Tushune K, Getachew Y, Duchateau L, Speybroeck N. Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant *Anopheles arabiensis* from South-Western Ethiopia. Parasites Vectors. 2012;5:159.
13. Massebo F, Balkew M, Gebre-Michael T, Lindtørn B. Blood meal origins and insecticide susceptibility of *Anopheles arabiensis* from Chano in South-West Ethiopia. Parasites Vectors. 2013;6:44.
14. Fettene M, Olana D, Christian RN, Koekemoer LL, Coetzee M. Insecticide resistance in *Anopheles arabiensis* from Ethiopia. Afr Entomol. 2013;21:89–94.

Messenger *et al. Malar J* (2017) 16:469

Page 14 of 14

15. Balkew M, Getachew A, Chibsa S, Olana D, Reithinger R, Brogdon W. Insecticide resistance: a challenge to malaria vector control in Ethiopia. Malar J. 2012;11:139.
16. FMoH. Insecticide resistance monitoring and management strategy for malaria vector control in Ethiopia. Disease prevention and control directorate. National malaria control and elimination programme. Ethiopia; 2016.
17. WHO. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization; 2013.
18. WHO. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. Report of the WHO informal consultation 28–30 September 1998. WHO/CDS/MAL/98.12; 1998.
19. Brogdon W, Chan A. Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay. https://www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf. 2013.
20. Gillies T, Coetzee M. Supplement of the Anopheles of Africa south of Sahara (Afrotropical region) Johannesburg: Republic of South Africa. Publication of the South African Institute of Medical Research; 1987.
21. Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. Am J Trop Med Hyg. 1987;37:37–41.
22. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg. 1993;49:520–9.
23. Fettene M, Temu EA. Species-specific primer for identification of *Anopheles quadriannulatus* sp. B (Diptera: Culicidae) from Ethiopia using a multiplex polymerase chain reaction assay. J Med Entomol. 2003;40:112–5.
24. Verhaeghen K, Van Bortel W, Roelants P, Backeljau T, Coosemans M. (Detection of the East and West African *kdr* mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt curve analysis. Malar J. 2006;5:16.
25. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, et al. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vectors *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7:179–84.
26. Ranson H, Jensen B, Vulule J, Wang X, Hemingway J, Collins FH. Identification of point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000;9:491–7.
27. Abbott WS. A method of computing the effectiveness of insecticide. J Econ Entomol. 1925;18:265–7.
28. Owusu HF, Jancaryova D, Malone D, Müller P. Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology? Parasites Vectors. 2015;8:357.
29. Viera AJ, Garrett JM. Understanding interobserver agreement: the kappa statistic. Fam Med. 2005;37:360–3.
30. Strode C, Donegan S, Garner P, Enayati AA, Hemingway J. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African Anopheline mosquitoes: systematic review and meta-analysis. PLoS Med. 2014;11:e1001619.
31. Riveron JM, Chiumia M, Menze BD, Barnes KG, Irving H, Ibrahim SS, et al. Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: a major concern for malaria vector control. Malar J. 2015;14:344.
32. Sadasivaiah S, Tozan Y, Breman JG. Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control? Am J Trop Med Hyg. 2007;77:249–63.
33. Yeebiyo Y, Dengela D, Tesfaye AG, Anshebo GY, Kolyada L, Wirtz R, et al. Short persistence of bendiocarb sprayed on previous walls and its implications for the indoor residual spray program in Ethiopia. Parasites Vectors. 2016;9:266.
34. Ranson H, Abdallah H, Badolo A, Guelbeogo WM, Kerah-Hinzoumbé C, Yangalbé-Kalnoné E, Sagnon N, et al. Insecticide resistance in *Anopheles*

gambiae: data from the first year of a multi-country study highlight the extent of the problem. Malar J. 2009;8:299.
35. Deressa W, Loha E, Balkew M, Hailu A, Gari T, Kenea O, et al. Combining long-lasting insecticidal nets and indoor residual spraying for malaria prevention in Ethiopia: study protocol for a cluster randomized controlled trial. Trials. 2016;17:20.
36. Abdalla H, Wilding CS, Nardini L, Pignatelli P, Koekemoer LL, Ranson H, et al. Insecticide resistance in *Anopheles arabiensis* in Sudan: temporal trends and underlying mechanisms. Parasites Vectors. 2014;7:213.
37. Abuelmaali SA, Elaagip AH, Basheer MA, Frah EA, Ahmed FTA, Elhaj HFA, et al. Impacts of agricultural practices on insecticide resistance in the malaria vector *Anopheles arabiensis* in Khartoum State, Sudan. PLoS ONE. 2013;8:e80549.
38. Wanjala CL, Mbugi JP, Ototo E, Gesuge M, Afrane YA, Atieli HE, et al. Pyrethroid and DDT resistance and organophosphate susceptibility among *Anopheles* spp. mosquitoes, Western Kenya. Emerg Infect Dis. 2014;21:2178–81.
39. MoH. Malaria Vector Control Situation Analysis. National Malaria Control Programme. Ministry of Health. The State of Eritrea; 2014.
40. Aïzoun N, Osse R, Azondekon R, Alia R, Oussou O, Gnanguenon V, et al. Comparison of the standard WHO susceptibility tests and the CDC bottle bioassay for the determination of insecticide susceptibility in malaria vector and their correlation with biochemical and molecular biology assays in Benin, West Africa. Parasites Vectors. 2013;6:147.
41. Ochomo E, Bayoh MN, Brogdon WG, Gimnig JE, Ouma C, Vulule JM, et al. Pyrethroid resistance in *Anopheles gambiae* s.s. and *Anopheles arabiensis* in western Kenya: phenotypic, metabolic and target site characterizations of three populations. Med Vet Entomol. 2013;27:156–64.
42. Fonseca-Gonzalez I, Cardenas R, Quiñones ML, McAllister J, Brogdon WG. Pyrethroid and organophosphates resistance in *Anopheles* (N.) *nuneztovari* Gabaldón populations from malaria endemic areas in Colombia. Parasitol Res. 2009;105:1399–409.
43. Perea EZ, León RB, Salcedo MP, Brogdon WG, Devine GJ. Adaptation and evaluation of the bottle bioassay for monitoring insecticide resistance in disease vector mosquitoes in the Peruvian Amazon. Malar J. 2009;8:208.
44. Aïzoun N, Azondekon R, Aïkpon R, Gnanguenon V, Osse R, Asidi A, et al. Study of the efficacy of a Wheaton coated bottle with permethrin and deltamethrin in laboratory conditions and a WHO impregnated paper with bendiocarb in field conditions. Asian Pac J Trop Biomed. 2014;4:492–7.
45. Aïzoun N, Azondekon R, Akogbéto M. Determination of the days storage and re-use of a Wheaton coated bottle with DDT, fenitrothion, and bendiocarb in laboratory conditions. Int J Curr Microbiol App Sci. 2014;3:203–10.
46. Reid MC, McKenzie FE. The contribution of agricultural insecticide use to increasing insecticide resistance in African malaria vectors. Malar J. 2016;15:107.
47. Yewhalaw D, Bortel WV, Denis L, Coosemans M, Duchateau L, Speybroeck N. First evidence of high knockdown resistance frequency in *Anopheles arabiensis* (Diptera: Culicidae) in Ethiopia. Am J Trop Med Hyg. 2010;83:122–5.
48. Ibrahim SS, Riverson JM, Stott R, Irving H, Wondji CS. The cytochrome P450 *CYP6P4* is responsible for the high pyrethroid resistance in knockdown resistance-free *Anopheles arabiensis*. Insect Biochem Mol Biol. 2016;68:23–32.
49. Djogbénou L, Dabiré R, Diabaté A, Kengne P, Akogbéto JM, Chandre F. Identification and geographic distribution of the *ACE-1R* mutation in the malaria vector *Anopheles gambiae* in South-Western Burkina Faso, West Africa. Am J Trop Med Hyg. 2008;78:298–302.
50. Dabiré RK, Namountougou M, Diabaté A, Soma DD, Bado J, Toé HK, et al. Distribution and frequency of kdr mutations within Anopheles gambiae s.l. populations and first report of the Ace. 1G119S mutation in Anopheles arabiensis from Burkina Faso (West Africa). PLoS ONE. 2014;9:e101484.