**Efficacy of *Wolbachia*-infected mosquito deployments for the control of dengue in Indonesia**

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**Background**:

*Aedes aegypti* mosquitoes infected with the *Wolbachia* *pipientis* (*w*Mel strain) have reduced potential to transmit dengue viruses.

**Methods**:

We conducted a randomised trial to assess the efficacy of deployments of *Wolbachia* (*w*Mel strain)-infected *Ae. aegypti* to reduce the incidence of virologically-confirmed dengue (VCD) cases in Yogyakarta City, Indonesia. Twenty-four geographic clusters were randomly allocated to one of two study arms; one arm received *w*Mel deployments as an adjunct to local dengue control measures, the untreated arm continued with local dengue control measures only. A test-negative design was used to measure efficacy. Study participants were aged 3-45 years old presenting with acute undifferentiated fever at any of 18 primary care clinics between January 2018 and March 2020. Laboratory testing identified VCD cases and test-negative controls. The intention-to-treat (ITT) analysis compared the odds of residence in a *w*Mel-treated cluster between VCD cases and test-negative controls.

**Results:**

Following successful introgression of *w*Mel in intervention clusters, a total of 8144 participants were enrolled; 3721 from *w*Mel-treated clusters and 4423 from untreated clusters. There were 385 VCD cases and 5921 test-negative controls amongst 6306 participants in the analysis set. *w*Mel deployments were associated with a 77.1% (95% CI, 65.3, 84.9) reduction in VCD case incidence in the ITT analysis. Protective efficacy was similar for the four serotypes. Efficacy against hospitalisation for VCD was 86.2% (66.2, 94.3).

**Conclusions**:

*w*Mel introgression into *Ae. aegypti* populations reduced the incidence of symptomatic dengue and led to fewer dengue hospitalisations.

**Trial registration number:** ClinicalTrials.gov Identifier: NCT03055585 and INA-A7OB6TW

**Introduction**

Dengue is a mosquito-borne, acute viral syndrome caused by any of the four serotypes of dengue virus (DENV).1 In 2019 the World Health Organisation nominated dengue as one of the top ten global health threats.2 Estimates suggest 50-100 million symptomatic cases occur globally each year.3,4 Annual (seasonal) and multi-annual epidemic surges in case numbers place considerable pressure on health services in endemic countries.5

*Aedes aegypti* mosquitoes are the primary vectors of dengue. For decades, efforts to control this vector have used combinations of methods directed at killing or suppressing the immature aquatic stages and/or the adult mosquitoes.6 The extent to which any of these methods reduce dengue incidence is unknown because so few clinical trials have been conducted. Those trials that have been reported have not demonstrated benefit.7

*Wolbachia pipientis* are maternally-inherited, obligate intracellular bacteria that infect insects and nematodes but don’t naturally occur in *Ae. aegypti* mosquitoes.8 Stable transinfection of *A*. *aegypti* with some strains of *Wolbachia* confers on the mosquito resistance to disseminated infection by DENV and other arboviruses.9-12 Thus, the introgression of “virus blocking” strains of *Wolbachia* into field-populations of *Ae. aegypti* is an emerging dengue control method.13-16 The approach works by delivering regular pulses of *Wolbachia*-infected mosquitoes into the wild mosquito population over a period of several months. Helpfully, *Wolbachia* self-propels its own population introgression by manipulating reproductive outcomes between wild-type and *Wolbachia-*infected mosquitoes – the only viable mating outcomes are those where the progeny are *Wolbachia*-infected.12

Here we report results of a city-wide cluster randomised trial to measure the efficacy of *w*Mel-infected mosquito deployments in reducing the incidence of virologically-confirmed dengue in Yogyakarta City, Indonesia. The trial builds on earlier entomological and epidemiological pilot studies in this setting.13,17,18

**Methods**

***Trial*** ***design and oversight***

The “Applying Wolbachia to Eliminate Dengue” (AWED) trial was financially supported by the Tahija Foundation and the trial sponsor was the Universitas Gadjah Mada (UGM), Indonesia. The protocol was published19,20 and is provided in the Supplementary Appendix.

Community support for randomised *w*Mel releases was obtained from leaders of 37 urban villages following a community engagement and mass communications campaign. For enrolment into the clinical cohort in primary health care facilities, written informed consent was obtained from all participants or their guardian where the participant was a minor. In addition, participants aged between 13 and 17 years gave written informed assent to participate. The trial was conducted in accordance with the principles of the Good Clinical Practice guidelines of the International Conference on Harmonisation and was approved by the UGM and the Monash University Human Research Ethics Committees. The trial data was analysed by the independent trial statisticians (NJ and SD). The funders had no role in the analysis of the data, in the preparation or approval of the manuscript, or in the decision to submit the manuscript for publication. 

***Randomisation***

The baseline characteristics of the study site are described in Table S1. Briefly, the study site was a continuous urban area of 26 km2 and with a population of approximately 311,700. The study site was subdivided into twenty-four contiguous clusters, each approximately 1km2 in size and where possible having borders that would slow the dispersal of mosquitoes between clusters. Of the 24 clusters, 12 were randomly allocated to receive open label *Wolbachia* deployments and 12 left untreated. No placebo was used. Constrained randomisation was used to prevent a chance imbalance in the baseline characteristics or spatial distribution of treated and untreated clusters (described in Supplementary Appendix).

***Wolbachia deployment and entomological monitoring***

*w*Mel-infected *Ae. aegypti* were sourced from an outcrossed colony described previously.13 Mosquitoes were released as eggs into intervention clusters between March and December 2017. Each cluster received between 9-14 rounds of releases. Mosquito releases, and the monitoring of *w*Mel frequencies via a network of 348 BG-Sentinel adult mosquito traps (BioGents), are described in the Supplementary Appendix.

***Participant enrolment***

Participant enrolment to measure the efficacy endpoint was performed at a network of 18 government-run primary care clinics in Yogyakarta City and adjacent Bantul District. Patients presenting to the clinics were eligible for the study if they met the inclusion criteria, a) fever (either self-reported or objectively measured, defined as forehead or axillary temperature >37.5oC) with a date of onset between 1-4 days prior to the day of presentation, b) aged between 3-45 years old and c) resided in the study area every night for the 10 days preceding illness onset. Participants were not eligible if, a) they had localising features suggestive of a specific diagnosis other than an arboviral infection, e.g. severe diarrhea, otitis, or pneumonia, or b) were enrolled in the study within the previous 4 weeks.

***Procedures***

Enrolled participants provided demographic information, geolocated residential address and a detailed travel history (durations and geolocations) for the past 10 days. A 3 ml venous blood sample was collected for arbovirus diagnostic tests. Participants were followed up 14-21 days later to determine whether they were, a) alive, and b) had been hospitalised since their enrolment in the study. No further information on disease severity or clinical diagnoses was acquired.

***Diagnostic investigations and classifications***

Study participants were classified as VCD cases if their enrolment plasma sample was DENV test-positive in a multiplex (DENV, CHIKV and ZIKV) reverse-transcriptase polymerase chain reaction (RT-PCR) and/or in an enzyme-linked immunosorbent assay (ELISA) for dengue NS1 (BioRad Platelia). Study participants were classified as test-negative controls if their enrolment plasma sample was test-negative by RT-PCR for DENV, chikungunya and Zika viruses, and also test-negative for DENV NS1 and negative in dengue IgM and IgG capture ELISAs. The diagnostic algorithm is shown in Figure S1. Details of the diagnostic methods are provided in the Supplementary Appendix

***Primary and secondary endpoints***

The primary endpoint was the efficacy of community-based deployments of *w*Mel*-*infected *Ae. aegypti* mosquitoes in reducing the incidence of symptomatic, virologically-confirmed dengue cases of any severity in Yogyakarta residents aged 3-45 years in release (intervention) areas, relative to non-release (untreated) areas. Secondary endpoints reported here include the efficacy against each of the four DENV serotypes.

***Sample Size***

Reflective of the novel design, the sample size requirements to demonstrate a 50% reduction in dengue incidence, which was considered the minimum effect size for public health value, evolved over time. The full sample size narrative is provided in the Supplementary Appendix. Briefly, 400 VCD cases and four times as many controls was determined to be sufficient to detect a 50% reduction in VCD case incidence with 80% power. The emergence of SARS-Cov-2 in Yogyakarta in March 2020 prevented the continued enrolment of participants in clinics, with enrolment stopping on 18th March 2020. On 5th May 2020, the trial steering committee endorsed the recommendation from the trial investigators to terminate the trial having recruited 385 VCD cases.

***Statistical Analysis***

The statistical analysis plan was published21 and is available in the Supplementary Appendix. The primary intention-to-treat (ITT) analysis considered *Wolbachia* exposure as a binary classification based on residence in a cluster allocated to *Wolbachia* deployment or not. Residence was defined as the primary place of residence during the 10 days prior to illness onset. The intervention effect was estimated from an aggregate odds ratio comparing the exposure odds (residence in a *Wolbachia*-treated cluster) among VCD cases versus test-negative controls, using the constrained permutation distribution as the foundation for inference. The null hypothesis was that the odds of residence in a *Wolbachia*-treated cluster was the same among VCD cases as test-negative controls. Efficacy of the intervention was calculated as 100\*(1-aggregate odds ratio). A predefined exploratory analysis evaluated the efficacy of the intervention in preventing hospitalised virologically-confirmed dengue cases.

An additional predefined cluster-level ITT analysis was performed by calculating the VCD case proportion in each cluster. The difference in the average proportion of VCD cases between the intervention clusters and untreated clusters was used to test the null hypothesis of no intervention effect (a t-test statistic) but basing inference on the constrained permutation distribution.22,23

The same intention-to-treat analyses described above were applied for the secondary endpoint of serotype-specific efficacy, with case populations restricted to each of the DENV serotypes in turn, and with the same test-negative control population as for the primary analysis.

Per protocol analyses considered exposure contamination by assigning a *Wolbachia* exposure index to each participant based on the *w*Mel frequency in their cluster of residence only, or by combining this frequency with the participant’s recent travel history. A mixed effects logistic regression model was fitted, incorporating cluster membership as a random effect, to estimate the relative risk of VCD and associated confidence interval. Detailed methods are provided in the Supplementary Appendix.

**Results**

***Establishment of wMel in Ae. aegypti populations***

This city-wide cluster randomised trial was performed in Yogyakarta, Indonesia (Figure 1). *w*Mel was durably established in the *Ae. aegypti* populations in each of the 12 intervention clusters (Figure S2). The monthly median (interquartile range) cluster level *w*Mel prevalence was 95.8% (91.5-97.8%) during the 27 months of clinical surveillance.

***Study participants***

53,924 patients were screened for study eligibility at 18 primary care clinics between January 8th 2018 and March 18th 2020 and 8144 persons were enrolled. Of these, 6306 participants met the requirements for the primary analysis dataset; 2905 participants were resident in the *w*Mel intervention arm and 3401 in the untreated arm (Figure 2). Four virologically-confirmed chikungunya cases (1 in the *w*Mel-treated arm and 3 in the untreated arm) were excluded from the primary analysis dataset. No Zika cases were detected. The median age (interquartile range) of participants was 12.0 years (7.0, 21.1) and 48.7% of participants were female (Table S2). 295 (4.7%) of the 6306 participants in the analysis dataset were hospitalised in the time between their enrolment and follow-up 14-21 days later. Of these 295 hospitalisations, participants from the untreated arm (n=214, 6.3%) were over-represented compared to the *w*Mel-treated arm (n=81, 2.8%) and this was independent of the clinic of enrolment (Table S3 and Figure S3). 385 (6.1%) of 6306 participants in the analysis dataset had VCD and 5921 (93.8%) were test-negative controls. No study participants died.

***Intention to treat analyses***

The incidence of VCD cases was significantly lower in the *w*Mel-treated arm (67 VCDs amongst 2905 participants (2.3%)) than in the untreated arm (318/3401 (9.4%)) (odds ratio 0.23, 95% confidence interval [CI], 0.15 to 0.35; P=0.004). This represented a protective efficacy of 77.1% (95% CI, 65.3 to 84.9) (Figure 3). The intervention effect was evident by 12 months after *w*Mel-establishment (Figure S4). Protective efficacy was similar across serotypes, being highest for DENV-2 (83.8%; 95% CI, 72.2 to 90.6) and lowest for DENV-1 (71.0%; 95% CI, 18.2 to 89.7) (Figure 3). For all four serotypes the lower bound of the 95% CI for protective efficacy was greater than 0. There were 13 hospitalisations for VCD amongst 2905 participants (0.4%) from the *w*Mel treated arm compared to 102 hospitalisations for VCD amongst 3401 participants (3%) from the untreated arm, for a protective efficacy of 86.2% (95% CI, 66.2 to 94.3) (Figure 3 and Table S4).

An additional prespecified ITT analysis compared VCD cases as a proportion of total participants in each cluster, between study arms. In all but one of the *w*Mel-treated clusters the VCD proportion was lower than untreated clusters, yielding a relative risk of 0.23 (95% CI, 0.06 to 0.47; P=0.004) (Figure 4A). When stratified by serotype, the relative risk of VCD caused by the two most prevalent serotypes, DENV-2 and DENV-4, was significantly lower in the *w*Mel-treated arm (Figure 4B).

***Per protocol analyses***

Per protocol analyses assigned a *Wolbachia* exposure index to each participant based on the *w*Mel frequency in their cluster of residence only, or by accounting also for *w*Mel frequencies and time spent in other locations. Protective efficacy against VCD increased with incremental increases in participants’ *Wolbachia* exposure index when cluster of residence and recent travel history were considered (Figure 5A). When only the *w*Mel frequency in the cluster of residence was considered, the results aligned tightly with the ITT analysis in that high *w*Mel frequencies in the cluster of residence were protective (Figure 5B).

**Discussion**

Our results show establishment of *w*Mel in *Ae. aegypti* mosquitoes in Yogyakarta reduced the relative risk of symptomatic VCD amongst 3-45 year olds by 77%. Even if the lower boundary of the 95% confidence interval (65%) represented the truth, the effect size would still represent a major public health benefit. Similar protective efficacy was observed against all four DENV serotypes. Efficacy against VCD requiring hospitalisation, a major clinical burden in dengue endemic countries, was 86%. Protective efficacy against VCD was nearly homogeneous across clusters, with 11 of the 12 *w*Mel treated clusters having a lower proportion of VCD cases than untreated clusters.

The conceptual underpinnings of the test negative design used in this trial, and the statistical framework for population inference, have been described.22 Trial operational procedures, particularly blinding of research staff, were designed to prevent issues of selection bias at the point of participant enrolment, follow-up, laboratory diagnosis and outcome classification. Since the intervention (mosquito releases) was delivered openly, and not placebo controlled, we cannot exclude performance bias affecting the measured outcomes. One scenario is that VCD cases who resided in the treated clusters chose not to seek health care, or attended other health care providers, both of which are highly unlikely given the natural history of the illness and the limited availability of other health care services in Yogyakarta.

*w*Mel-infected mosquito populations are not static and increasing contamination of untreated clusters was observed in year 2 of the trial. The per protocol analyses accounted for exposure contamination that occurred through changes in cluster-level *w*Mel frequencies and the movement of participants. The strongest signal of protective efficacy was derived from the cluster-level *Wolbachia* frequency only, rather than the travel history as well. These data point to the participant’s residence as the most prominent location for acquisition of VCD and thus the expression of the intervention’s protective effect in this trial.

The efficacy results reported, which conceivably underestimate the epidemiological effect size that might be achieved by city-wide *w*Mel establishment, are consistent with a body of laboratory and field observations. Predictions from an ensemble of mathematical models have suggested that the reduced infectiousness observed in *w*Mel-infected *Ae. aegypti* could be sufficient to reduce R0 (the basic reproductive number) to below 1 in many dengue endemic settings, resulting in local elimination of disease.3,24,25 Previous non-randomised field studies in Australia15,16 and Indonesia13 provided evidence of epidemiological impact after *w*Mel was introgressed. Together with the trial reported here, this body of work suggests that when *w*Mel is established at high prevalence in local *Ae. aegypti* populations then reductions in dengue incidence follow. Another *Wolbachia* strain, wAlbB, also has pathogen-blocking properties and can be introgressed into *Ae. aegypti* field populations.14 This suggests the possibility of a portfolio of *Wolbachia* strains, each with different strengths and weaknesses, for application as public health interventions in *Ae. aegypti* populations.

Stable *w*Mel transinfection imparts an antiviral state in *Ae. aegypti* mosquitoes that attenuates superinfection by several medically-important *Flaviviruses* and *Alphaviruses*. Multiple mechanisms have been proposed to explain this phenotype, including *Wolbachia*-induced triggering of innate immune effectors26,27 and changes in intracellular cholesterol transport.28 DENV could plausibly evolve resistance to *w*Mel however the requirement for alternating infection of human and mosquito hosts could be a constraint on the adaptive emergence of resistant virus populations. Future research should survey arbovirus populations for signals of *Wolbachia*-associated selective pressure.

The *w*Mel introgression approach represents a novel product class for the control of dengue.

It is predicted to be highly cost-effective when deployed at scale in urbanised settings.29 Future trials should explore *w*Mel’s multivalency in the field, since laboratory studies11,30-33 suggest it should also attenuate transmission of Zika, chikungunya, Yellow Fever and Mayaro viruses by *Ae. aegypti.*

**Figure legends**

**Figure 1: Map of study location.**  In panel A, the location of the City of Yogyakarta is shown in Indonesia. In panel B, the distribution of wMel-treated and untreated clusters, and the locations of primary care clinics where enrolment occurred, are shown on a Yogyakarta City map.

**Figure 2:** **Cluster randomisation, participant enrolment, inclusion in analysis dataset, and follow-up of safety endpoints.**  The commonest reasons for exclusion from the analysis dataset were enrolment before the predefined time point of *Wolbachia* establishment (January 8th 2018), enrolment in a calendar month without any VCD cases (September 2018) or having positive or equivocal dengue IgM or IgG serology that precluded classification as a test-negative control .

**Figure 3:** **Intention-to-treat efficacy.** Shownis the protective efficacy of *w*Mel-infected *Aedes aegypti* deployments against virologically-confirmed dengue of any serotype (All VCDs), by infecting DENV serotype, and against hospitalised VCD as a proxy for severe dengue disease. VCDs with ‘Unknown serotype’ were test-negative by DENV RT-PCR and test-positive for DENV NS1 antigen. Seven participants had two DENV serotypes detected during the same febrile episode: 4 with serotypes 1 and 2, 2 with serotypes 1 and 4, and 1 with serotypes 2 and 4.

**Figure 4**: **Cluster-level proportions of virologically-confirmed dengue cases.** A) VCD cases and B) serotype-specific VCD cases as a proportion of all participants in *Wolbachia*-treated (closed circles) and untreated (open circles) clusters. Circle size in Panel A is proportionate to the total number of participants in the cluster. Horizontal bars show the mean VCD proportion in intervention and untreated clusters; the relative risk and P-value are derived from a comparison of these mean proportions (see Methods). \*When the number of serotype-specific VCD cases is small while the number of test-negative controls remains large, the test-positive fraction method is not sufficiently precise to distinguish between very low values of the relative risk that are close to zero; in this case the method yields a lower confidence interval of 0 as reported here.

**Figure 5: Efficacy of the *Wolbachia* intervention against virologically-confirmed dengue according to *Wolbachia* exposure index (per-protocol analysis).** Markers show stratum-specific efficacy (and 95% confidence intervals) against VCD by quintile of *Wolbachia* exposure index, with WEI based on A) duration-weighted *w*Mel frequencies in the cluster of residence and other visited locations, or B) *w*Mel frequency in cluster of residence only. Lines show the increase in efficacy (with 95%CI) for each increase in WEI quintile. The number of VCD cases and test-negative controls with WEI values in each quintile is shown beneath the plots.

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