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► **To cite this version:**

Louis Lambrechts, Neil M Ferguson, Eva Harris, Edward C Holmes, Elizabeth A McGraw, et al.. Assessing the epidemiological effect of wolbachia for dengue control.. The Lancet Infectious Diseases, 2015, 15 (7), pp.862-6. 10.1016/S1473-3099(15)00091-2 . pasteur-01176423

**HAL Id: pasteur-01176423**

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Submitted on 16 Jul 2015

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1 **Assessing the epidemiological impact of *Wolbachia* deployment for**  
2 **dengue control**

3

4

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36

37 **Summary**

38

39 Dengue viruses cause more human morbidity and mortality than any other arthropod-borne  
40 virus. Dengue prevention relies primarily on vector control but the failure of traditional  
41 methods has promoted the development of novel entomological approaches. Although use of  
42 the intracellular bacterium *Wolbachia* to control mosquito populations was proposed half a  
43 century ago, it has only gained significant interest as a potential agent of dengue control in  
44 the last decade. Here, we review the evidence that supports a practical approach for dengue  
45 reduction through field release of *Wolbachia*-infected mosquitoes and discuss the additional  
46 studies that must be conducted before the strategy can be validated and operationally  
47 implemented. A critical next step is to assess the efficacy of *Wolbachia* deployment in  
48 reducing dengue virus transmission. We argue that a cluster-randomized trial is currently  
49 premature because *Wolbachia* strain choice for release as well as deployment strategies are  
50 still being optimized. We therefore present a pragmatic approach to acquiring preliminary  
51 evidence of efficacy via a suite of complementary methodologies: prospective cohort study,  
52 geographical cluster investigation, virus phylogenetic analysis, virus surveillance in  
53 mosquitoes, and vector competence assays. This multi-pronged approach could provide  
54 valuable intermediate evidence of efficacy to justify a future cluster-randomized trial.

55

56 Dengue is a major public health problem in tropical and sub-tropical regions, where almost  
57 400 million infections are estimated to occur each year.<sup>1</sup> The etiological agents are four  
58 dengue virus serotypes (DENV-1 to -4) in the genus *Flavivirus* that are transmitted among  
59 humans by mosquitoes. These viruses cause a systemic, debilitating though mostly self-  
60 limiting illness, which without careful management can lead to hypovolemic shock and  
61 death.<sup>2</sup> In the absence of a licensed vaccine or therapeutic drug, dengue prevention efforts  
62 are currently limited to the control of its main mosquito vector, *Aedes aegypti*. With a few  
63 exceptions, the implementation of vector control methods has been largely unsuccessful due  
64 to the lack of sustained commitment of resources<sup>3</sup> and inability to effectively scale-up and  
65 successfully apply interventions over large geographic areas and modern mega-cities. Novel  
66 entomological approaches to dengue control have been developed<sup>4</sup> and some are now  
67 advancing to field testing.<sup>5</sup>

68

69

#### 70 ***Wolbachia*-based strategies for dengue control**

71

72 One of the most promising entomological strategies being developed for dengue control  
73 relies on introduction of the intracellular bacterium *Wolbachia* into *Ae. aegypti*.<sup>6</sup> *Wolbachia*  
74 *pipientis* is a bacterial endosymbiont that was originally identified in ovaries of *Culex*  
75 mosquitoes in the 1920s<sup>7</sup> and is thought to infect two-thirds of all living insect species.<sup>8</sup> The  
76 extraordinary evolutionary success of *Wolbachia* is attributed to their ability to manipulate the  
77 biology of their hosts in diverse ways.<sup>9</sup> For example, *Wolbachia* can induce reproductive  
78 abnormalities such as feminization and sperm-egg cytoplasmic incompatibility (CI). Because  
79 *Wolbachia* is transmitted vertically via the egg, these female-biased reproductive  
80 manipulations can drive *Wolbachia* infections to high frequencies in wild populations. CI, the  
81 most common manipulation in insects, occurs when *Wolbachia*-infected males mating with  
82 *Wolbachia*-free females lead to the production of non-viable offspring. *Wolbachia*-infected

83 females, in contrast, produce successful offspring regardless of the *Wolbachia* infection  
84 status of their mate.

85

86 The potential of *Wolbachia* to control pest insect populations was realized as early as half a  
87 century ago (Figure 1). *Wolbachia*-induced CI was then proposed to eliminate *Culex*  
88 mosquitoes<sup>10</sup> or to introduce desirable genes into wild vector populations.<sup>11</sup> To date,  
89 however, *Wolbachia* have never been operationally implemented as a vector control  
90 measure. A significant hurdle was the fact that several major vectors of human pathogens  
91 are not naturally infected by *Wolbachia*, including the main DENV vector *Ae. aegypti*. The  
92 mosquito vectors (*Anopheles* spp.) of human malaria parasites were also thought to be  
93 *Wolbachia*-free until a recent study reported evidence for *Wolbachia* in field populations of  
94 *An. gambiae*.<sup>12</sup>

95

96 A resurgence of interest for *Wolbachia*-based strategies to control vector-borne diseases  
97 occurred about a decade ago with the advent of transinfection techniques (Figure 1). Stable  
98 *Wolbachia* infections in naïve hosts can now be established by embryonic microinjections  
99 into the developing embryo germline. In general, *Wolbachia* transinfection is more likely to be  
100 successful between closely related donor and recipient hosts, and the expression of  
101 *Wolbachia*-induced phenotypes is conserved across hosts. In 2005, a stable infection by a  
102 *Wolbachia* strain from the mosquito *Aedes albopictus* was established in *Ae. aegypti*, which  
103 caused high rates of CI and rapidly spread to high frequencies in experimental populations.<sup>13</sup>  
104 This was quickly followed by double transinfections of *Ae. aegypti* with two different  
105 *Wolbachia* strains from *Ae. albopictus*.<sup>14</sup>

106

107 A second wave of breakthroughs occurred a few years later with the discovery of *Wolbachia*-  
108 induced phenotypes in mosquitoes that had a direct effect on pathogen transmission (Figure  
109 1). Until then *Wolbachia* was primarily considered a gene drive system, but the possibility to  
110 transinfect *Wolbachia* strains from more distant hosts by cell culture adaptation prior to

111 microinjection,<sup>15</sup> combined with the wide diversity of available *Wolbachia* strains and  
112 properties, resulted in new mosquito-*Wolbachia* associations. Stable introduction of a life-  
113 shortening strain of *Wolbachia* from *Drosophila* into *Ae. aegypti* halved the adult mosquito  
114 life-span under laboratory conditions, making mosquitoes unlikely to live long enough to  
115 transmit DENV.<sup>16</sup> More importantly, this life-shortening *Wolbachia* strain directly inhibited the  
116 ability of a range of pathogens, including DENV, to infect and replicate in *Ae. aegypti*.<sup>17</sup>  
117 Finally, semi-field and field trials in Australia demonstrated that *Wolbachia* can be  
118 persistently established in wild *Ae. aegypti* populations.<sup>18,19</sup> Together, these properties form  
119 the basis of a practical approach for suppression of DENV transmission through field release  
120 of *Wolbachia*-infected mosquitoes.

121

122

### 123 **Current status of *Wolbachia* deployment for dengue control**

124

125 The critical next step is to assess the efficacy of medium-scale *Wolbachia* deployment in  
126 reducing human DENV infection. The gold standard, a cluster-randomized trial (CRT) of  
127 *Wolbachia*, has been considered in detail previously.<sup>20</sup> CRT is a type of randomized  
128 controlled trial in which groups of subjects, instead of individual subjects, are randomly  
129 assigned to the alternative treatments under study. The CRT design is particularly useful  
130 when the intervention cannot be directed toward selected individual subjects, such as the  
131 release of *Wolbachia*-infected mosquitoes. In the classical two-armed CRT, clusters without  
132 intervention provide contemporaneous controls. In a stepped wedge CRT, the intervention is  
133 rolled-out sequentially to all the clusters so that the clusters are their own controls over time.

134

135 We believe that at this time a CRT is premature for the *Wolbachia*-based approach for  
136 several reasons. First, there are multiple strains of *Wolbachia* available for deployment, each  
137 with its own characteristic effects on DENV blocking and mosquito fitness. A process of  
138 selection through field-testing is still required before one or more final strain(s) can be chosen

139 for a particular release area. In addition, while deployment in North Queensland has provided  
140 a basic template for release, this environment differs substantially from the large urban  
141 centers in Southeast Asia and Latin America where a CRT would likely be carried out. It is  
142 crucial to retain the capacity to learn during deployment about the effectiveness of release  
143 strategies and community engagement and to adjust practice accordingly. Past examples of  
144 adaptive changes made during deployment include releasing larger numbers of mosquitoes,  
145 changing the intensity of trap grids to monitor *Wolbachia* spread, supplementing releases  
146 with different mosquito developmental stages, and altering locations of deployment based on  
147 community concerns.<sup>18,21</sup> In contrast, the standard CRT approach would lock-in all aspects of  
148 the release, preventing ‘on the fly’ improvements in design. Finally, a classical two-armed  
149 CRT would have to be large, with >40 clusters that each include approximately 100 study  
150 subjects who are monitored for infection to detect a 50% reduction in dengue with 90%  
151 power.<sup>20</sup> Rough estimates of cost for such a design suggest it would exceed 5-10 million  
152 USD.

153

154

### 155 **A pragmatic approach to optimize *Wolbachia* deployment**

156

157 Here, we argue that well-designed observational studies could provide a suite of valuable  
158 indirect evidence that supports *Wolbachia* as a dengue intervention and, hence, justifies  
159 continued development, ultimately leading to a definitive efficacy trial. Ideally, several  
160 observational studies would be conducted in different settings and their outcomes combined  
161 in a meta-analytic framework to assess the impact on disease and infection incidence. Below  
162 we describe five possible approaches that could be used separately or in combination for  
163 acquiring such evidence.

164

165 **Pediatric cohort study.** A prospective longitudinal cohort study that tracks seroconversion  
166 rates in children could measure both the true incidence of DENV infections and the relative



167 risk of infection between *Wolbachia*-treated and untreated areas.<sup>20</sup> Because the overall  
168 DENV seroconversion rate is generally 5-10% per annum in endemic countries,<sup>22</sup> a cohort  
169 would need to include at least several thousand individuals to be compatible with the  
170 statistical requirements of a CRT with sufficient power to detect a moderate intervention  
171 effect.<sup>20</sup> A smaller cohort of 1,000-1,500 children, although underpowered in the context of a  
172 CRT, could be significantly enhanced by the concurrent approaches described below. Fine-  
173 scale entomological surveillance (e.g., a grid of traps) would allow monitoring the spatio-  
174 temporal dynamics of *Wolbachia* prevalence to distinguish, in real time, *Wolbachia*-free areas  
175 from areas where *Wolbachia* had established. The raw entomological data could be  
176 interpolated over time and space using standard methodology and serve as a covariate for  
177 DENV seroconversion. As in other epidemiological investigations, participants residing in the  
178 study area, but acquiring infections outside of the intervention area, represent a complication  
179 to this approach.<sup>23,24</sup> However, geographical cluster studies of dengue cases and fine-scale  
180 spatiotemporal phylogenetic analyses of genomic DENV strain sequences (see below) would  
181 help to address this concern.

182

183 **Geographical cluster investigation.** DENV infections are acute, often mild, inapparent or  
184 with non-specific signs and symptoms, and thus are difficult to detect across populations in  
185 real time. Active surveillance of human infections can be efficiently achieved using  
186 geographical cluster sampling around dengue index cases.<sup>25,26</sup> Here, 'index case' refers to  
187 the laboratory-diagnosed clinical dengue case that initiates a cluster investigation within a  
188 geographically restricted area around the home of a person with a documented DENV  
189 infection. Geographical cluster investigations could be used to compare the fine-scale spatial  
190 signature of DENV transmission in areas with and without *Wolbachia* (Figure 2). This  
191 methodology would test the hypothesis that concurrent and/or subsequent infections around  
192 an index case are reduced in areas where *Wolbachia*-infected mosquitoes are established.  
193 Inward migration of dengue infections acquired outside the treatment area would also be a  
194 confounding factor,<sup>23,24</sup> although again potentially resolvable through detailed phylogenetic

195 analysis of virus sequences and/or monitoring movement patterns of study participants.  
196 Nonetheless, if a *Wolbachia* intervention reduces local transmission at a micro-scale, it  
197 should be detectable by a cluster investigation methodology. An efficacious intervention  
198 would result in a lower overall number of index cases in the *Wolbachia*-treated areas and/or  
199 reduction in concurrent infections measured by a lower frequency of cases that are  
200 spatiotemporally linked to the index case.

201

202 **Virus sequence analysis.** Increasing access to viral genome sequence data has promoted  
203 the development of new methodologies to infer dengue epidemiological dynamics based on  
204 analyses of changing patterns in viral genetic diversity in time and space.<sup>27,28</sup> Assuming that  
205 multiple lineages of various DENV serotypes co-circulate prior to an intervention, a reduction  
206 in local DENV transmission is expected to result in a decrease in viral genetic diversity  
207 across serotypes in the intervention area due to a major viral demographic bottleneck, and in  
208 an increase in the average dispersion distances travelled by DENV into the intervention area  
209 (Figure 3). Phylogenetic analysis provides a simple means to identify importation of 'foreign'  
210 viral lineages into the study area, provided that genetic diversity accumulates at a sufficiently  
211 high rate. Previous studies on DENV microevolution in Southeast Asia suggested that spatial  
212 patterns of genetic diversity are shaped by frequent virus immigration and highly focal  
213 transmission.<sup>28-30</sup> Although the level of phylogenetic resolution to be obtained is uncertain,  
214 deep sequencing methods have recently undergone dramatic improvement, increasing the  
215 power of this approach. We expect that if local DENV transmission is reduced in *Wolbachia*-  
216 treated areas, some viruses will continue to be imported by human-mediated dispersal but  
217 will not persist locally, reducing the strong spatial clustering that is typically observed in  
218 DENV phylogenies.

219

220 **Virus detection in mosquitoes.** The release of *Wolbachia*-infected mosquitoes will require  
221 monitoring of the local *Ae. aegypti* population for changes in *Wolbachia* frequency and  
222 possibly in mosquito density. Recently, several sampling methods that effectively capture

223 female *Ae. aegypti* have been developed.<sup>31-34</sup> Virus detection could be combined with routine  
224 molecular tests for *Wolbachia* presence. Detecting DENV-infected *Ae. aegypti* mosquitoes is  
225 challenging because of the low infection rates (typically ~0.1%) in the adult females across  
226 the population, although infection rates can be higher in locations of geographical cluster  
227 investigations.<sup>25</sup> Because mosquitoes that test positive for virus are not necessarily  
228 infectious, the proportion of DENV-infected mosquitoes does not directly translate into an  
229 estimate of virus transmission unless virus disseminated from the mosquito midgut or in  
230 saliva is also assayed, and even this approach is limited by the sensitivity of assays and  
231 variation of *in vitro* saliva collections. Nonetheless, a successful intervention is expected to  
232 reduce the incidence of viremic and infectious humans and, therefore, similarly reduce the  
233 incidence of DENV infection in mosquitoes in areas where *Wolbachia* infection predominates.

234

235 **Vector competence assays.** Following the release of *Wolbachia*-infected mosquitoes, it will  
236 be necessary to verify that the phenotype of reduced vector competence is maintained over  
237 time in field-collected mosquitoes.<sup>35</sup> Vector competence assays consist of experimentally  
238 exposing laboratory-reared mosquitoes to either an artificial infectious blood meal or the  
239 blood of a viremic person.<sup>36</sup> The proportion of infectious mosquitoes (i.e., with virus detected  
240 in saliva) is then measured over time. *Wolbachia*-infected mosquitoes have a strongly  
241 reduced ability to deliver DENV in their saliva compared to *Wolbachia*-free mosquitoes.<sup>19</sup>  
242 Ideally, vector competence experiments would be extended to human-to-mosquito-to-human  
243 transmission experiments in a human challenge model.<sup>37</sup> Vector competence assays will  
244 provide additional indirect evidence on the impact of the intervention, especially if the virus  
245 interference effect is strong.

246

247

248 **Conclusions and perspectives**

249

250 The current challenge is to convert a promising strategy into a validated public health  
251 intervention through rigorous assessment of its epidemiological impact. The suite of  
252 approaches described above is not a substitute for a CRT. Nonetheless, this strategy has at  
253 least two major strengths that can lay the foundations for a future CRT. First, the proposed  
254 investigations are not dependent on the uniform application of the intervention, which by  
255 nature will vary through time and space. Instead, an association between *Wolbachia*  
256 presence and proxies of DENV transmission (e.g., DENV seroconversion or occurrence of  
257 secondary cases around index cases) can be inferred dynamically from the spatiotemporal  
258 correlation between these factors. Second, comprehensive observation and detection of  
259 correlations between multiple environmental and biological factors will likely improve  
260 fundamental understanding of dengue epidemiology that will inform and underpin future trial  
261 designs. A multi-pronged approach would also help to evaluate potential impacts on other  
262 *Ae. aegypti*-borne arboviruses (e.g., chikungunya virus), and the likelihood of unexpected  
263 outcomes such as viral evolution to escape the inhibitory effects of *Wolbachia*, or other  
264 unanticipated, adverse events.

265

266 Measuring the epidemiological impact of a *Wolbachia* deployment to reduce DENV  
267 transmission is challenging. The intervention is not based on individuals, as a vaccine trial  
268 would be, but on populations defined by spatial areas. The fundamental test of the impact of  
269 the intervention is a comparison between areas where *Wolbachia*-infected mosquitoes are  
270 present versus areas where they are not (Figures 2, 3). Although limited dispersal of *Ae.*  
271 *aegypti*<sup>38</sup> and, therefore, spread of *Wolbachia*, is expected to maintain spatial delineation of  
272 the intervention, a buffer zone will be necessary to avoid unanticipated overlap between  
273 treatment and control areas. The intervention needs to be deployed over a large enough  
274 geographic area to ensure that a sufficient number of dengue cases (or absence of cases if  
275 the intervention is effective) is captured. Prior knowledge of the study area will help to assign  
276 intervention and control areas with similar baseline transmission trends. Virus importation  
277 into the intervention area (through human-mediated dispersal<sup>23,24</sup>), which is likely to occur

278 and may reduce the signal-to-noise ratio, can be explored with geographic cluster studies  
279 and by accounting for movement of study subjects.

280

281 One advantage of our proposed approach is that interpretation of seroconversion data from a  
282 small-scale pediatric cohort can be enhanced by data from geographical cluster  
283 investigations, viral sequencing and virus detection in mosquitoes, collectively resulting in a  
284 body of evidence that could support continued development of *Wolbachia* as public health  
285 tool. In any case, virus importation by study participants exposed to infected mosquitoes  
286 outside of the treatment area would result in false positive cases in the *Wolbachia*-treated  
287 area and conservatively lead to an underestimation of efficacy. A true placebo treatment (i.e.,  
288 release of *Wolbachia*-free mosquitoes) is not ethically possible. The human and mosquito  
289 samples can, however, be blinded prior to laboratory testing.

290

291 We have described a pragmatic approach for evaluation of novel entomological interventions  
292 for dengue control through a coordinated, cross-disciplinary, ecological study that combines  
293 several proxies of efficacy at the epidemiological, entomological and virological levels. It  
294 relies on a combination of methodologies that have been successfully used to monitor  
295 dengue epidemiological dynamics, as well as novel methodologies. Although this approach  
296 has no precedent for dengue, it has the potential to provide valuable intermediate evidence  
297 of efficacy that supports the *Wolbachia* methodology and justifies funding for a CRT or  
298 deployment.

299

300 **Acknowledgements**

301

302 We thank Stephanie James and Fil M. Randazzo for their insights, and two anonymous  
303 reviewers for helpful comments. This work was supported by a grant from the Foundation for  
304 the National Institutes of Health through the Grand Challenges in Global Health Initiative of  
305 the Bill and Melinda Gates Foundation.

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307

308 **Contributors**

309

310 LL wrote the first draft of the manuscript. All other authors contributed equally to edit the  
311 manuscript.

312

313

314 **Conflicts of interest**

315

316 We declare that we have no conflict of interest

317

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419 **Figure Legends**

420

421 **Figure 1. Key dates in the development of *Wolbachia*-based dengue control strategies.**

422 The timeline shows major achievements over the last century that have supported the  
423 development of *Wolbachia* as a dengue control tool.

424

425

426 **Figure 2. Geographical cluster methodology.** The central dot represents the home of a  
427 confirmed dengue case (orange: area with *Wolbachia*; green: area without *Wolbachia*).

428 People living within a 100-m radius (black dots) are screened for concomitant and/or  
429 secondary DENV infection (crosses denote homes of additional DENV-infected individuals).

430

431

432 **Figure 3. Schematic representation of how *Wolbachia* intervention might change  
433 patterns of virus genetic diversity.** Assuming that multiple lineages of various DENV

434 serotypes (colored dots) co-circulate prior to the intervention, a reduction in local DENV  
435 transmission is expected to result in a decrease in viral genetic diversity in the intervention

436 area and a relative increase in the average dispersion distances.

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