UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON D.C., 20460

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

April 6, 2018

MEMORANDUM

SUBJECT: Science Review of the Published Research Article: Popovici et al. (2010), which assesses the potential for human exposure to *Wolbachia* and *Wolbachia* antigens through exposure to *Wolbachia*-infected *Ae. aegypti* mosquitoes.

FROM: Milutin S. Djurickovic, M.S., Biologist
Biopesticides and Pollution Prevention Division
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THROUGH: Eric W. Bohnenblust, Ph.D., Acting Senior Biologist
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs

John Kough, Ph.D., Senior Scientist
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs

TO: Mike Mendelsohn, Chief, Emerging Technologies Branch
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs

REF: Popovici, J., L.A. Moreira, A. Poinsignon, I. Iturbe-Ormaetxe, D. McNaughton and S.L. O'Neill. 2010. Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes. Memorias do Instituto Oswaldo Cruz 105: 957-964.

O'Neill, S.L, I. Iturbe-Ormaetxe. 2011-2015. Institutional Approval Form for Experiments on Humans Including Behavioral Research. Rearing of Mosquitoes Using Blood from Human Volunteers 21/03/2011 – AMENDMENT. The University of Queensland.

ACTION REQUESTED

Conduct a science review of Popovici et al. (2010). This study is proposed for use as part of a weight-of-evidence approach to assess the potential human exposure to *Wolbachia* through bites from *Wolbachia*-infected mosquitoes. For this review, the assays assessing if *Wolbachia* can be transferred into the environment are not considered because they are not relevant to the Human Health Risk Assessment.

CONCLUSIONS

The results of Western blots and ELISA conducted as part of Popovici et al. (2010) suggest humans who regularly blood-feed *Aedes aegypti* mosquitoes are not exposed to *Wolbachia* microbial pesticide because they do not develop an immune response to *Wolbachia,* and that *Wolbachia* and *Wolbachia* antigens are unlikely to be transferred to people when they are bitten by an *Ae. aegypti* mosquito harboring *Wolbachia.* When evaluated according to OPP's guidance document "Guidance for Considering and Using Open Literature Toxicity Studies to Support Human Health Risk Assessment (USEPA, 2012)," Popovici et al. (2010) is classified as *supplemental/qualitative*. Thus, when the data from Popovici et al. 2010 are considered along with the additional scientific evidence below as part of a weight of evidence approach, they support the hypothesis that humans are not exposed to the *Wolbachia* microbial pesticide through the release of *Wolbachia*-infected *Aedes* spp. mosquitoes as part of a sterile male insect release program. The Human Studies Review Board is being asked to comment on this study.

BACKGROUND AND PURPOSE

Wolbachia-infected *Aedes albopictus* mosquitoes (EPA Reg. No. 89668-4) are currently registered for use as a pesticide with EPA. EPA also approved an experimental use permit (EPA Reg. No. 89668-EUP-3) to produce efficacy data to support a registration for *Wolbachia*-infected *Aedes aegypti* mosquitoes. As part of the human health risk assessments to support regulatory decisions for the two products, EPA concluded exposure to *Wolbachia* microbial pesticide through the release of *Wolbachia*-infected male mosquitoes was negligible based the low contamination rate of females in male batches (1 female per 250,000 male mosquitoes) (EPA 2017). EPA relied on the conclusion of negligible risk for previous regulatory decisions due to the low contamination rate and because *Wolbachia* is naturally present in several mosquito species. EPA is now refining its characterization of the potential risk associated with human exposure to *Wolbachia* microbial pesticide through release of *Wolbachia*-infected mosquitoes. As part of refining its risk assessment, EPA believes the Popovici et al. 2010 article provides useful data. The Popovici et al. 2010 data are the only known data directly assessing exposure of humans to *Wolbachia* microbial pesticide through *Wolbachia*-infected biting female mosquitoes. When considered as a part of a weight of evidence, this study provides useful information suggesting that even if humans are exposed to biting *Wolbachia*-infected mosquitoes, humans will not be exposed to *Wolbachia* or *Wolbachia* antigens.

Additional Weight of Evidence

1. *Wolbachia pipientis* is a common obligate intracellular bacterium that is found in an estimated 65% of insect species (Hilgenboecker et al. 2008). *Wolbachia* infection was identified in wild Culex spp. mosquitoes in 1971 (Yen and Barr 1971), and cytoplasmic incompatibility resulting from *Wolbachia* infection was discovered in the early to mid-20th century (Hertig and Wolbach 1924; Laven 1951). There are no reports of transmission of *Wolbachia* to humans bitten by naturally infected mosquitoes or other arthropods.
2. Only infected males are released in the sterile insect technique program. The estimated female contamination rate for releases is 1 female per 250,000 males and therefore exposure to biting females is considered negligible (U.S. EPA 2017).
3. The presence of different *Wolbachia* strains in a mating causes cytoplasmic incompatibility and karyogamy failure in the zygote. No offspring are produced when *Wolbachia*-infected males are introduced into a population of mosquitoes that do not have *Wolbachia* present or carry different strains of *Wolbachia* resulting in greatly reduced reproduction. Cytoplasmic incompatibility arises because of asynchrony between the maternal and paternal pronucleus during mitosis. Therefore, when males carrying *W. pipientis* are introduced into a population with no *Wolbachia* or a different strain of *Wolbachia,* cytoplasmic incompatibility is complete and offspring are not produced (Dobson et al. 2001, Dobson et al. 2004), as is the case for *Ae. aegypti* and *Ae. Albopictus* (Dobson et al. 2001). Therefore, there is no exposure to an offspring generation.
4. *Wolbachia pipientis* does not typically survive outside of the intracellular environment of its host (Werren et al. 2008).

BPPD has used OPP's guidance document "Guidance for Considering and Using Open Literature Toxicity Studies to Support Human Health Risk Assessment (USEPA, 2012)[1]," for evaluating the scientific quality of Popovici et al. (2010). EPA concludes the study does not meet the criteria for quantitative use, because a dose cannot be determined, the data are not reported in units comparable to other studies, and methods, information, and raw data are not provided to definitively substantiate the limit of dectection. However, because the data show immune response to proteins in mosquito saliva, clearly identify the band for *Wolbachia* surface protein in the control (Braig et al. 1998), and exposure to *Wolbachia*-infected mosquitoes is high, the study is appropriate for qualitative use and for use in a weight of evidence evaluation as proposed here.

[1] https://www.epa.gov/sites/production/files/2015-07/documents/lit-studies.pdf

SCIENCE REVIEW

Study objective: The objective of the study was to determine if humans bitten by *Wolbachia*-infected *Ae. aegypti* develop an immune response specific to *Wolbachia.*

Methods:

Identification of the test system and experimental design: To provide a blood meal for mosquito colonies, over a six-week period 17 human volunteers (blood-feeder group (BF)) placed their arm for 15 minutes into a cage containing 150 *Ae. aegypti* mosquitoes infected with *Wolbachia pipientis*. Each volunteer fed between 2 and 4 cages of mosquitoes twice per week totaling 600 - 1200 mosquitoes per week for six weeks. Over the six-week period, subjects received an estimated 3600 – 7200 mosquito bites per person in the BF group. The non-blood-feeder (NBF) control group consisted of 5 human volunteers who never blood-fed any mosquito lab colony. After sustained feeding by the BF group, blood serum from human volunteers in the BF and NBF groups was collected to test for immunoreactivity to *Wolbachia* or mosquito proteins. Under standard aseptic procedures, a maximum of 10 ml of blood was drawn from BF and NBF groups by a trained phlebotomist at the University of Queensland Health Service. Blood was stored in the laboratory.

Western Blots: To detect the immunological antibody response to *Wolbachia* and *Ae. aegypti* antigens, Western blots were conducted using blood serum from NBFs (n = 2) and BFs (n = 7). First, *Wolbachia* extracts from *Ae. aegypti* cells were loaded and run through SDS-PAGE gels (12%). Once blotted, the membranes were incubated with either a rabbit anti-*Wolbachia* surface protein (WSP) antibody or with serum from individuals in the NBF and BF groups. As a control, *Ae. aegypti* thorax extracts were incubated with a mouse anti-mosquito saliva antibody or blood serum from NBF and BF groups to determine if the human volunteers were immunologically responding to mosquito bites.

Enzyme-linked Immunosorbent Assays: ELISAs were conducted to detect and measure immunoglobin G (IgG) antibodies specific to *Wolbachia* antigens in the NBF (n = 5) and BF (n = 17) groups in response to *Ae. aegypti* salivary glands not infected with *Wolbachia, Wolbachia* extracts purified from *Ae. aegypti* cells, and *Wolbachia* extracts purified from *Drosophila* adult flies, the original source of the *Wolbachia pipientis* introduced into *Ae.aegypti*. *Wolbachia* antigens were purified from infected insects as follows. Cells from confluent monolayers of *Ae. aegypti* harboring *Wolbachia* were removed and centrifuged (1000 x g) to remove culture medium. The cellular pellet was washed in SPG buffer, resuspended, and the supernatant removed two more times. The pellet was resuspended and subjected to sonication to disrupt the insect cells. This suspension was centrifuged and filtered at 5 µm. The filtrate was centrifuged (12,000 x g), the supernatant was removed, and the pellets were buffered with SPG and centrifuged (300 x g) to remove any remaining cellular debris. The supernatant containing cells with *Wolbachia* was removed, transferred to a clean tube, and stored on ice until use for assays (<3 hrs; McMeniman et al., 2008). Each well of the ELISA plate was coated with antigens (either *Wolbachia* extract purified from 3×10^4 *Ae. aegypti* cells, or *Wolbachia* extract from one *Drosophila* fly harboring *Wolbachia*). Serum from a single BF or NBF individual was added and incubated overnight.

Bound human IgG was detected using a goat anti-human IgG antibody. IgG levels specific to *Wolbachia* were expressed as the difference in optical density between the background optical density of the salivary gland without *Wolbachia* and the optical density for each purified *Wolbachia* sample. Controls consisted of ELISA plates coated with 1/10th of the extract from a single salivary gland from *Ae. aegypti* which were not infected with *Wolbachia* and incubated with human serum to detect a response to mosquitoes.

The non-parametric Mann Whitney test was used to determine differences in optical density of ELISA plates containing blood sera from the BF and NBF groups exposed to *Ae. aegypti* salivary glands, *Wolbachia* from *Ae. aegypti* cells, and *Wolbachia* from *Drosophila* adults.

Results and analyses:

Western Blots: The Western blot with *Wolbachia* extracts from *Ae. aegypti* cells incubated with serum from NBF and BF groups did not produce any bands for either group (Fig. 1A). The rabbit anti-WSP lane did have one band at 26 kDa (Fig. 1A; correct size for Wolbachia WSP, Braig et al. 1998)) suggesting NBF and BF groups did not react with WSP antigen and *Wolbachia* or WSP is not transferred through mosquito bites. In the control group where thorax extracts of *Ae. aegypti* not infected with *Wolbachia* were incubated with sera from the BF and NBF groups, one NBF sample showed no bands, and most BF lanes had bands at both 46 kDa and 37 kDa although some lanes only had one of those bands, which correspond to *Ae. aegypti* saliva proteins which are known to produce an immune response in humans (Fig 1B).

Figure 1. Western blot results from Popovici et al., 2010.

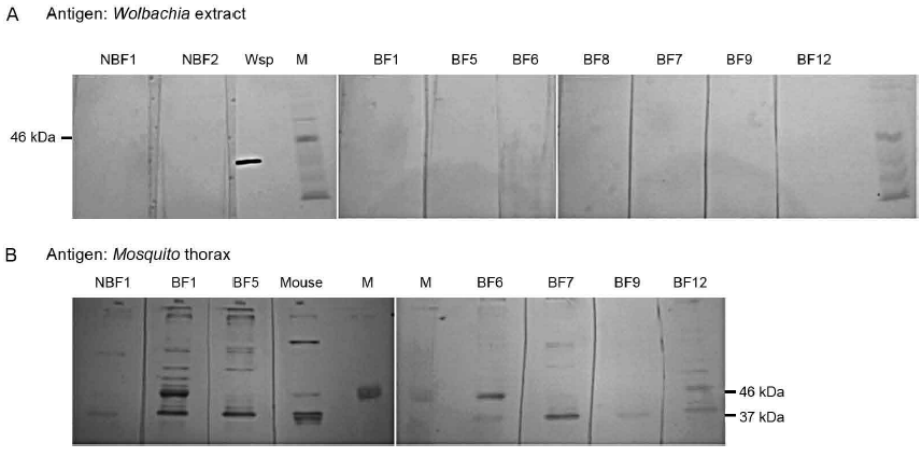

Fig. 1: Western blot analysis to detect any potential anti-*Wolbachia* antibody in human sera. *Wolbachia* (A) or mosquito thorax extracts (B) were run in a 12% SDS-PAGE and incubated with either human sera or anti-WSP (*Wolbachia* surface protein) antibody (A) or with human sera or mouse anti-mosquito saliva serum (B). Non blood-feeder (NBF)1 and NBF2 are sera from NBF volunteers. Blood-feeder (BF)1, BF5, BF6, BF7, BF8, BF9, BF12 are sera from BF volunteers. M: Kaleidoscope prestained standards (Bio-Rad).

Enzyme-linked Immunosorbent Assays: The ELISA analysis showed a difference (p=0.0129) in optical density in the BF group compared to the NBF group for the *Ae. aegypti* salivary glands not

infected with *Wolbachia* (Fig 2). The change in optical density indicates the different level of IgG antibodies in the sera from the NBF and BF groups. As expected, plates with blood sera from the BF group reacted to *Ae. aegypti* salivary glands had higher IgG levels than plates with blood sera from the NBF group because the BF group continually blood-fed mosquitoes and the NBF group did not blood-feed mosquitoes during the study. Low level reaction in the NBF group as seen in Fig. 2 is expected, because the NBF subjects were likely bitten by *Ae. Aegypti* mosquitoes during their lifetime. These results suggest humans develop antibodies against mosquito antigens after being bitten by mosquitoes. Optical density was similar for sera from BFs and NBFs incubated with *Wolbachia* extracts from *Ae. aegypti* cells or *Drosophila* suggesting IgG antibodies are not being produced against *Wolbachia* antigens. The similarity in optical density between NBF and BF groups on plates where blood sera were exposed to extracts of insects harboring *Wolbachia* suggest *Wolbachia* and *Wolbachia* antigens are not being transferred to the humans who are repeatedly bitten by *Ae. aegypti*.

Figure 2. ELISA analysis from Popovici et al., 2010.

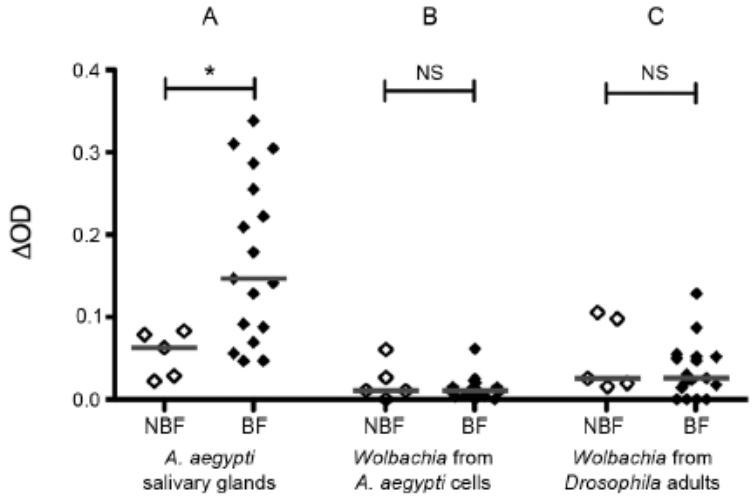

Fig. 2: ELISA analysis to detect IgG antibody levels specific to *Aedes aegypti* salivary glands or to *Wolbachia* antigens in human sera. Individuals difference of optical density (ΔOD) values are shown for blood-feeders (BF) and for non-blood-feeders (NBF) and bars indicate the median value. Results are presented according to antigens: *Wolbachia*-uninfected *Ae. aegypti* salivary gland extracts (A), *Wolbachia* extracts purified from *Ae. aegypti* cell culture (B) and *Wolbachia* extracts purified from *Drosophila* flies (C). NS: non significant. Asterisk means statistical differences between groups (non parametric Mann Whitney test).

Uncertainties: Numerous methods were omitted that are necessary for evaluating the Western blots and ELISAs. Information was not provided regarding the amount of sample run in both analyses, the limits of detection, WSP antibody reactions, or the staining methods. Furthermore, in the Western blot (Fig. 1), three molecular ladders were pre-stained and are visible, however the fourth molecular ladder does not show any bands. This may have occurred because there was not

enough of the molecular ladder available for the final gel. Also, for the Western blots we cannot determine whether a single specific antibody was reacted with the blotted gels multiple times or if multiple gel membranes were each reacted with a specific antibody. Moreover, the second molecular ladder was not labeled. The *Wolbachia* surface protein WSP band in the Western blot (Fig. 1A Antigen *Wolbachia* extract), is much darker than any of the other bands and has a white halo around the band. The reasons for this band being so much darker than other bands are unclear, but may be due to a punctured gel or too much protein. Also, ELISA plates were not provided, only the summarized data with statistics in Fig. 2. are available. In addition, raw data and methods addressing the noted deficiencies are unavailable, EPA requested the raw data and additional details relating to methods but the corresponding author was unable to provide any raw data or methods (Appendix 1).

Conclusions: Uncertainties were noted in the Western Blots and ELISA. However, despite these uncertainties, the results provide evidence that humans who regularly blood-feed *Ae. aegypti* mosquitoes do not develop an immune response to antigens present in *Wolbachia* extracts. The lack of reactivity also suggests *Wolbachia* and *Wolbachia* antigens are unlikely to be transferred to humans when they are bitten by an *Ae. aegypti* mosquito infected with *Wolbachia.* This study can be used in a weight of evidence when considered with the additional evidence presented above to indicate that humans are not exposed to the *Wolbachia* microbial pesticide through the release of *Wolbachia*-infected *Aedes* spp. mosquitoes as part of a sterile male insect release program.

References:

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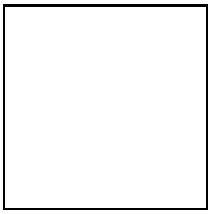
Appendix 1. Email correspondence between EPA and corresponding author with regard to obtaining raw data and additional methods.

Request 1: Raw Data

From: Scott O'Neill [mailto:scott.oneill@worldmosquito.org]
Sent: Sunday, February 04, 2018 5:33 PM
To: Striegel, Wiebke <Striegel.Wiebke@epa.gov>
Subject: Re: U.S. EPA Inquiry about "Assessing key safety concerns of a Wolbachia-based strategy"

Hi Wiebke,
I have made some enquiries about the raw data used in this paper. This work was done nearly a decade ago and the people involved have long since left my lab. I have enquired with them if they have the original raw data and the answer was that it was lost with a stolen computer. So we are not able to assist further with your enquiry.
Sorry I cant be of more help.
Scott

Prof. Scott O'Neill
Director, World Mosquito Program
Director, Institute of Vector-Borne Disease



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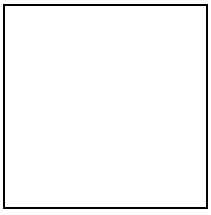
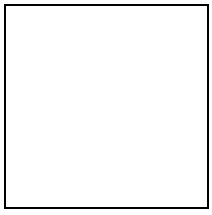
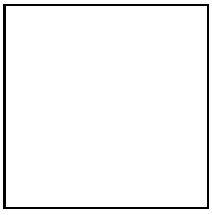
Join the conversation:   
www.worldmosquitoprogram.org

On 2 February 2018 at 00:25, Striegel, Wiebke <Striegel.Wiebke@epa.gov> wrote:

Dear Dr. O'Neill,

I would like to follow up on my November 16, 2017 email to Scott.O'Neill@monash.edu requesting the permission to use the raw data in the Popovici *et al*., 2010 publication "Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes" as published in Memorias Instituto Oswaldo Cruz Vol. 105(8), 957-964.

As described in the email correspondence below, we are requesting the raw data from the article listed above. The initial assessment of the article, and the raw data (if you agree to provide it) would be conducted by EPA scientists. We would then present the results of our internal review to an advisory committee, the Human Studies Review Board (HSRB), which consists of subject matter experts in bioethics, human exposure assessment, and statistics, from the public, universities, and other U.S. Federal Government agencies. The HSRB meets a few times a year and all meetings are public. While not all of your data may be initially presented to the public as part of the review by EPA and HSRB, if your data are requested by a third party under the Freedom of Information Act (FOIA), at the time of the request, EPA will determine whether or not the information must be released publicly. The link below sends you to EPA's HSRB website, which provides current schedules, final reports, and background information. We hope that this will convey a more tangible sense of the process and the types of reviews the Board conducts.

https://www.epa.gov/osa/meetings-human-studies-review-board

Your article came to EPA's attention as part of an application to use *Wolbachia*-infected male mosquitoes for population suppression. Last year, EPA registered the ZAP Males®, which are male *Aedes albopictus* mosquitoes infected with the *Wolbachia* wPip (ZAP) strain.

You can find more information on the ZAP Males and EPA's risk assessment by following this link to www.regulations.gov:

https://www.regulations.gov/docket?D=EPA-HQ-OPP-2016-0205

If possible, please respond by February 15, regardless of whether you can provide the raw data. Thank you in advance for any assistance you can provide. I am available for any questions you may have regarding this request and look forward to hearing back from you soon.

With kind regards,

Wiebke

Request 2. Scientific Methods.

From: Scott O"Neill

To: Arling, Michelle
Cc: Bohnenblust, Eric; Djurickovic, Milutin; Scott.O"Neill@monash.edu; Striegel, Wiebke; scott.oneill@eliminatedengue.com
Subject: Re: Request for information about "Assessing key safety concerns of a Wolbachia-based strategy"
Date: Tuesday, March 13, 2018 5:45:09 PM

I'm currently on leave returning to work next week - I will attend to your request when I am back.

Scott

On Wed, 14 Mar 2018 at 6:04 am, Arling, Michelle <Arling.Michelle@epa.gov> wrote:
Hi Dr. O'Neill,

We corresponded last summer about your research summarized in the article "Assessing key safety concerns of a *Wolbachia*-based strategy." I wrote with a request for some additional information last month. I'm hoping that you will be able to provide responses to the questions in my email below. Even if you cannot respond to all of the questions, we would appreciate any information you can provide.

If you are unable to respond to the questions, do you mind sending a quick email to that effect? Again, thank you so much for your time and attention to EPA's requests about your work on this research.

Regards,

Michelle

Michelle Arling
Human Research Ethics Review Officer
Office of Pesticide Programs (S-4248)
1200 Pennsylvania Avenue NW MC 7501P
Washington DC 20460
703-308-5891
arling.michelle@epa.gov

From: Arling, Michelle
Sent: Tuesday, February 27, 2018 7:08 AM
To: 'scott.oneill@eliminatedengue.com' <scott.oneill@eliminatedengue.com>; 'Scott.O'Neill@monash.edu' <Scott.O'Neill@monash.edu>
Cc: Bohnenblust, Eric <Bohnenblust.Eric@epa.gov>; Striegel, Wiebke <Striegel.Wiebke@epa.gov>;
Djurickovic, Milutin <Djurickovic.Milutin@epa.gov>
Subject: RE: U.S. EPA Inquiry about "Assessing key safety concerns of a Wolbachia-based strategy"

Hello Dr. O'Neill,

Thanks for your assistance in getting the protocol and amendment approval from the University of Queensland HREC last year. I have a few follow-up ethics-related questions, and am forwarding an inquiry from my colleagues about methods (see below). As discussed in an earlier email, EPA is obligated to seek out ethics-related information and to present to an independent advisory group (Human Studies Review Board) our reviews of research with human subjects submitted to EPA. The meeting of the Human Studies Review Board is scheduled for April 24-26, 2018, and our reviews are due to the committee by March 16. I would be happy to share more information about the meeting (virtual meeting, open to the public) as well as EPA's reviews of the article if you are interested.

After reviewing the article and the materials forwarded by the University of Queensland ethics committee (attached for your convenience), I have a few more questions and would appreciate your assistance in answering them as much as possible so I can complete my review. I understand that records of the study may not be available – even your recollection of the study conduct would be helpful! Please let me know if it would be easier to have a conversation rather than reply by email – I appreciate that you are busy and travel a lot.

Ethics questions

1. In the amendment approved on 24-3-2011, the request was to transfer oversight of the research to the HREC at Monash University.
a. How long did the research proceed at the 2nd site?
b. Was blood collected from subjects at the 2nd site tested for the presence of Wolbachia antibodies?
c. If human subject blood samples were collected at the 2nd site and tested as part of the research reported in the published article, would it be possible for me to request the materials reviewed and approved by the Monash University HREC?
(https://www.monash.edu/researchoffice/contact; Executive Officer Human Ethics - Dr. Souheir Houssami; muhrec@monash.edu)
2. The Application Form (received date: 27 Sept 2007) notes that subjects were recruited from personnel working in Dr. O'Neill's lab. "personnel working at the O'Neill laboratory will be asked for voluntary unpaid participation in blood feeding *Aedes aegypti* mosquitoes." p. 3.
a. How were subjects recruited? (E.g., Flyers, email, personal contact)
b. If possible, can you share the materials used in the recruitment process?
c. What steps were taken to ensure that no potential subject felt pressured to participate? The Application Form (received date: 27 Sept 2007) notes that "right to refuse will not be considered detrimental to their research or work status in the group. Any volunteer will be able to refuse at any time once the experiment is underway and no explanation will be required for their decision." p. 4
d. Did any subjects refuse to participate after going through the consent process or withdraw during their participation?
3. What were the circumstances and methods by which informed consent was obtained from the test subjects? E.g., in person, one on one meeting?

a. The consent form included at the end of the Amendment Form (received date: 10 June 2009) notes that "Further information can be read in our Human Ethics documents if necessary." What are the Human Ethics documents, and is it possible to get a copy?
4. What were the inclusion and exclusion criteria for participants?
5. The article notes that sera from 17 bloodfeeders was tested. (p. 960) Were these all of the people enrolled as feeders from the initiation of the research, or only those who consented after the protocol was amended to draw blood?
6. Can you please confirm that no person under 20 years old was enrolled? The Application Form (received date: 27 Sept 2007) notes that "only people >20 years old will be able to volunteer for the bloodfeeding." p. 5
7. Were any female subjects enrolled in the study?
a. If so, how many?
b. Were female subjects tested for pregnancy before participation? If so, please explain.
c. Were any women known to be pregnant or nursing/lactating included as subjects?
8. Were there stopping rules for the study? If so, please explain.
9. Did any of the subjects require medical treatment as a result of their participation in the study?
a. If so, please explain.
10. Can you explain the steps taken during the study to reduce risks to participating subjects? E.g., did you provide over the counter topical ointments to relieve itchiness/irritation associated with mosquito bites?
11. What steps did you take to protect the identity of the subjects in the raw data and study?
12. What was the approximate length of a subject's participation in the study? The materials provided by UQ note that subjects were requested to feed the mosquitoes twice per week, for about 15 minutes per session. In addition, it noted that the approximate lifespan of mosquitoes being fed was about 6 weeks (30 minutes/week * 6 weeks = 3 hours per cage of mosquitoes fed). Did subjects participate for only one lifecycle of mosquitoes?
13. How were the control subjects recruited/enrolled into the study?
14. Did subjects receive any non-monetary compensation for their participation in the study?

Science questions
The article discusses generally the methods used to test the sera samples. Would it be possible for you to share the detailed methods or protocol for Western blot, ELISA (i.e., amount of sample tested , staining methods, limits of detection, and dose response), and the ELISA plates to supplement Fig. 2. in the article?

I appreciate any additional information you can provide. Please let me know if you need any other information related to this request.

Michelle

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