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Regarding: NBB REF NO: NRE(S)609-2/1/3

APPLICATION FOR APPROVAL FOR LIMITED MARK-RELEASE-RECAPTURE OF *Aedes aegypti* (L.) WILD TYPE AND OX513A STRAINS

Dear Madam / Sir

Concerning the planned release of genetically modified mosquitoes strain OX513A, I would like to offer my comments for your consideration with the intent and wish to be of help. I have made these comments in my capacity as a geneticist and biologist with a particular expertise in gene regulation and biosafety issues, including genetic use restriction technologies. I have been involved in the Cartagena Biosafety Protocol processes for 15 years (since 1995) and am currently serving on its AHTEG (ad hoc technical expert group) on risk assessment and risk management, where we produced amongst others the 'Draft Guidance Document for the Risk Assessment of LMO Mosquitoes', to be presented to MOP5 this October. I participated in the AHTEG as a civil society expert and representative of the Federation of German Scientists, the organisation I also represent at the CBD and Cartagena Protocol negotiations.

Please note, that my analysis and commentary regarding the application for approval of OX513A, can only be based on the information available to me, in particular the fact sheet provided on your website and the publication on RIDL (Release of Insects carrying a Dominant Lethal) technology and mosquito control covering strain OX513A by Phuc et al. (2007).

It is my interpretation from the information provided, that the intended release is an open release and that there have been no previous field cage trials studying the LMO mosquito under open air conditions, but contained by netting. I consider that such field cage trials can provide crucial and invaluable data not obtainable in laboratory experiments and are widely recommended for use prior to open field releases. They are thus another option available to further gather data and knowledge that is still missing. I would like to refer here to Marshal (2008) and to the AHTEG Draft Guidance Documents for LMO Mosquitoes and its bibliography.

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The underlying question is, does the open field release as designed hold risks and if so, what are these risks and how can they be clarified and minimised or eliminated? Further, is sufficient data available to identify possible hazards, to determine the consequences and calculate the risks?

In the following I offer my analysis where I see – given the information available to me – shortcomings in the design of the field release trials as well as the information available or known to date.

(1) Changes present in *Aedes aegypti* strain OX513A – identification and consequences

Please let me briefly recall and summarise the different changes that have been or might have been introduced into *Aedes aegypti*, due to the insertion of the transgenes and the use of genetic engineering techniques.

(a) ***Intended and predicted changes***: trait of red fluorescence (DsRed2 coding sequence under control of the Act5C promoter) and conditional lethality in the absence of tetracycline during development (tTAV coding sequence under the heat shock promoter hsp70 regulated by the tTAV binding site tetO7).

* tTAV is a transcriptional activator and was found to be toxic at high levels of expression. Yet its mode of action and the underlying mechanism(s) leading to cell death are not fully understood.

* The tTAV/tetO7 system is tetracycline-repressible - in brief: in the absence of tetracycline tTAV will continuously be produced. To this effect it binds to the tetO7 site of its own promoter sequence and thus activates its own expression. In the presence of tetracycline, tTAV will bind to tetracycline and will in this form no longer be able to bind to the tetO7 site of its own promoter. In this state, only a very reduced rate of tTAV is being produced.

(b) ***Intended but unpredictable***: the declared aim, i.e. intention was to have a late onset of the conditional lethality. *However, there is insufficient knowledge to design a tTAV system for this precise and predictable effect. With its mode of action not fully understood, it is also not known which levels of expression are required to for lethality and how this is or can be controlled.

In strain OX513A affected individuals died around the larval-pupal boundary whilst in another strain (513B) death occurred in early larval stages, despite using the same construct (Phuc et al., 2007). The authors stipulate that this variation (of phenotype) is due to a "positional effect", i.e. where on the chromosomes the transgenes have inserted in the random process. It remains unclear though, whether or to which extent the timing and level of expression of tTAV are influenced by the activity of other genes and whether for example environmental conditions, including stresses, alter the expression of tTAV to sub-lethal levels.

(c) ***Unpredicted and unintended changes / side effects****:* It is widely recognised that the insertion of transgenes can lead to changes that have neither been intended nor predicted and are seemingly unrelated to the nature of the gene inserted. Documented cases include higher lignin content in transgenic herbicide tolerant soybean plants and Bt corn plants, lowered vitamin content in transgenic squash, increased rate of out-crossing and altered root formation in herbicide tolerant Thale Cress. The reasons why and the mechanisms behind these changes are not always understood and would require further investigation. The following underlying causes have been observed frequently: *positional* effects (due to the particular chromosomal location where the transgene inserted; this can affect the expression either of the transgene or the genes nearby the insertion site); *mutational* effects (due to mutations within the gene adjacent to the insertion site, or in the wider genome due to insertion and transformation processes common to genetic engineering). *Pleiotropic* effects (due to the transgene, its activity, its gene product and its involvement in seemingly unrelated metabolic pathways – resulting in qualitative or quantitative changes of molecular compounds). Other classifications refer to *synergistic*, *antagonistic* or *cumulative* effects.

Obviously the identification of such changes and effects is laborious and not always possible. Whilst such changes do not necessarily result in negative effects or consequences, they might do so.

> An Australian group for example transferred a gene from the common bean to peas. The gene coded for a seed storage protein (alpha-AI or alpha-amylase inhibitor gene). Unexpectedly, the protein product from the bean gene changed its characteristics and became immunogenic, i.e., causing immune reactions, when expressed in the pea (Prescott *et al.* 2005). Although the original gene and the modified transgene both coded for exactly the same protein, the pea produced a structurally different protein from the same genetic information. Furthermore, the transgenic protein also gave rise to "immunological cross priming", also known as "adjuvant effect", thus leading to allergic reactions to many other pea proteins. The reasons for these changes are still not understood.

As I do not have access to the full dossier I do not know whether molecular data was provided with regards to genomic changes and compositional changes, and whether the LMO mosquitoes have been tested for unexpected or unpredicted changes other than testing for some fitness parameters.

Questions of interest could for example be but are not limited to:

Has the bite of **female LMO mosquitoes** changed? Do they bite more or less frequently? Has the reaction of humans (or animals) to the mosquito bite changed, e.g. is there a different immune reaction due to compositional changes in the saliva of the mosquito?

Has the pathogen-vector interaction changed in female mosquitoes? Has the level of the dengue virus present in the saliva changed? Has the affinity to the dengue virus changed? Or are there new interactions with other disease viruses? Are the mosquitoes less or more aggressive?

Do different conditions (including biotic and abiotic stresses encountered in the wild) result in different or altered phenotypic and behavioural characteristics?

(2) Risk of survival, spread and establishment

Phuc et al. (2007) state, that in the absence of tetracycline 3-4% of the first larval instar of OX513A survive to adulthood. This means that due to yet not known reasons, the RIDL system can and is breaking down at a measurable frequency. A possible reason for this is the onset of gene silencing, which has been repeatedly observed with transgenes (mostly studied in plants but first discovered in animals). There are a number of mechanisms available to cells and organisms to achieve gene silencing, some of which can be 'passed on' to offspring for generations to come, but have also been observed to be reversible. 'Passed on' here does not mean in form of genetic code, but can be for example in form of imprinting and epigenetics, such as chemical alterations of the DNA in question.

Another possibility is any one or more of the weak links of genetic use restriction technologies, as outlined in a CBD Information paper on GURTs (Steinbrecher, 2006, pp.6-7). There are other possible reasons for the RIDL system not functioning, such as point mutations within the tetO7 sequence, the hsp70 promoter or the tTAV coding sequence.

Determining the reasons and mechanisms behind the observed 3-4% of surviving is crucial, as it will bear directly on the risk assessment and the risk determination.

This is for two main reasons:

(a) The male *A. aegypti* LMO mosquitoes released into the open will – according to design – mate with female *A. aegypti* wild type mosquitoes. These females will lay such fertilised eggs and if the same ratio of survival applies, 3-4% of the F1 generation will survive and mate again. This, over time, would potentially select for those mosquitoes that have the capacity to overcome the RIDL system.

(b) If the mechanism is a form of gene silencing that can be passed on to the offspring (e.g. epigenetic changes), or the mechanism is mutation, then the likelihood and speed of survival and spread of LMO mosquitoes increases.

In this context other questions also arise, such as:

The figure for 3-4% is given for laboratory experiments.

- What is the figure for field cage trials (i.e. under natural out door conditions but contained by netted structures)?
- Do different conditions (including biotic and abiotic stresses that can be encountered in the wild) result in different survival rates?
- If the individuals from the 3-4% surviving mosquitoes are used for further breeding, does the percentage number of surviving mosquitoes (i.e. failed RIDL mechanism) increase? This means, does progeny (F2) from the surviving 3-4% (F1 parents) have an increased survival rate, thus possibly indicating a mechanism that can be passed on?
- What is the survival rate for offspring from LMO males and wild type females and vice versa, and are they any different from offspring from an all LMO parental line setting?

*Whilst Phuc et al. state that the observed leakiness at the 3-4% (or 3-5%) level does not compromise the RIDL strategy and effectiveness, their model does not take at least two major points into due consideration:

- *Non-sterility occuring in the RIDL system is fundamentally different from the one occurring in the traditional radiation-induced sterile insect technique (SIT) and should not be treated in the same way in population modelling. The 3-4% leakiness of the RIDL system represent offspring from the transgenic mosquitoes where the system failed to kick in or was overcome in other ways. This group could – in theory at least - become the basis for a sub-population capable of surviving and flourishing despite any further RIDL releases.
- *The effectiveness of the system also depends on the late onset of the lethality. If the time of onset is altered due to environmental conditions (incl. biotic and abiotic stresses) then a 3-4% represents a much bigger problem then currently allowed for in the Phuc et al modelling.

Another source for spread would be the accidental release of female LMO mosquitoes. It is not clear from the documentation to which extent the sorting of pupae according to size is reliably segregating male and female individuals and what the error rate might be. Is there an additional segregation step that ensures and guarantees that no female LMO mosquitoes are released amongst the male? (for some potential adverse effects of female LMO mosquitoes see Questions under point (1) above).

In the production process, are there monitoring and control mechanisms in place that continuously assesses the survival rate of the progeny in the absence of tetracycline as well as assess the percentage of females being mistakenly assorted to the male faction/cohort?

(3) Gene transfer

The fact sheet provided states: "Furthermore, the repressible lethal gene conferred to OX513A *Aedes aegypti* confers a selective disadvantage to the organism, therefore highly unlikely to be maintained in an organism in the unlikely event that genetic material is transfered by horizontal gene transfer."

I am not sure this argument indeed is relevant to 'horizontal' gene transfer. If the transfer is envisaged to be to completely unrelated species such as microorganisms, birds or frogs, tTAV might not result in a lethal trait at all, as this will depend on the receiving organism as well as possibly on the position of insertion into the genome. If the transfer is envisaged to be to more closely related species, such as other mosquitoes or other insects, RIDL can be silenced or somehow circumvented and positional effects may also come into play. In short, the fact that RIDL is to a large extent lethal in OX513A mosquitoes should not be relied upon as a hindrance for horizontal gene transfer.

Furthermore, both in this section as within the whole document provided there has been no mention or discussion of the red fluorescence marker gene or assessment of its potential adverse effects on the environment or to human health.

(4) Risks to biodiversity, the environment and ecosystems

It is not clear whether this aspect has been covered by the applicant, as it is not covered in the fact sheet provided. The Draft Guidelines for the Risk Assessment of LMO Mosquitoes produced by AHTEG pays particular attention to this aspect, recognising that when mosquitoes are an integral part of ecosystems / the environment, there are potential knock-on effects from the release of LMO mosquitoes to many other organisms.

Questions include: What will the knock-on effects be if there is an increased or reduced presence of mosquitoes or what if the mosquitoes are eliminated altogether (e.g. what would happen to those feeding on the larvae, the adult, like some fish, frogs, other insects and arthropods)? What if their interactions with other organisms in the environment change? It has been emphasised in the Draft Guidelines, that thorough knowledge of the biology, behaviour and role within the ecosystem of the particular mosquito in question is required as to be able to undertake a reliable risk assessment.

I would like to suggest that the investigation of one non-target species cannot replace an environmental and biodiversity risk assessment, though it can be part of it.

There is also the question of what will fill the gap or occupy the niche should the target mosquitoes have been eliminated? *Will other pests increase in number? Will targeted diseases be able to switch vectors? Will these vectors be easier or more difficult to control? Other than being a question relevant to biological biodiversity, it is a question relevant to **human and animal health**.

Concerning human health: Could the native *A. albopictus* fill the gap? I have been informed that it is not only a vector for dengue, but also for chikungunya. If *A. albopictus* were to fill the gap, could that result in both diseases being present? And would that be a negative result rather than the envisaged positive idea of eliminating Dengue? These points will probably already have been brought up by medical and epidemiological experts, and I am looking forward to the outcome of the debate.

(5) Conclusion

In my assessment – given the information available to me - there are numerous open questions that appear not to have been investigated. Given the importance of such missing data, experiments, investigation and knowledge for ensuring the "protection of human, plant and animal health, the environment and biological diversity", I would suggest that it is too early for any open field releases. This is particularly the case since RIDL is not 100% reliable and thus LMO mosquitoes will escape via surviving progeny.

But there are valuable avenues of investigation open to the applicant and the wider scientific community.

If field cage trials should be considered, these would not only offer more time for investigation, production of data and opportunities to ask open questions, but also enable the testing of assumptions for better accuracy.

I kindly thank the Director General and the NBB for the opportunity to submit my comments.

I wish you success in your upcoming deliberations.

Ricarda A. Steinbrecher, Ph.D.

References

Marshall JM (2008). Biosafety training course for Africa related to genetically modified disease vectors: Containment issues during planned field cage trials. At: http://johnmm.bol.ucla.edu/containment.htm

Phuc HK et al. (2007). Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology*. 5: 11

Prescott VE, Campbell PM, Moore A, Mattes J, Rothenberg ME, Foster PS, Higgins TJV, and Hogan SP (2005). Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *Journal of Agricultural and Food Chemistry* 53, 9023-9030

Steinbrecher RA (2006). V-GURTs (Terminator Technology): Design, Reality and Inherent Risks. CBD Information Document: UNEP/CBD/WG8J/4/INF/17, available on the CBD website (http://www.biodiv.org/doc/meetings/tk/wg8j-04/information/wg8j-04-inf-17-en.pdf)

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