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Title: Performance of wireball bioassay tests as a method to measure insecticidal activity in used Long-lasting Insecticidal Nets; findings from a study in Afghanistan.

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Introduction: Mass-scale distribution of long-lasting Insecticidal Nets (LLINs) is an effective strategy to reduce malaria incidence in endemic countries. LLINs have a dual protective effect, creating both a physical and a chemical barrier against malaria-transmitting *Anopheles* mosquitoes. In order to ascertain the functional life (and thus the replenishment intervals) of LLINs in field circumstances, field studies are crucial to measure durability and insecticidal activity of LLINs after a specific usage period. Insecticidal activity (i.e. the capacity of a LLIN to cause knock-down and/or death of an *Anopheles* mosquito coming into contact with the fabric) is traditionally measured with the cone bioassay method. Although WHO guidelines recommend the wireball assay as a good alternative method, little is known about its performance, also in relation with cone bioassay and insecticidal content measurements on the same LLIN positions. In this study we compared results of the three methods on the same LLIN positions.

Methods: This sub-analysis was performed as part of a larger study among 500 randomly selected households in five Afghan provinces. From the LLINs that they received three years earlier one LLIN was randomly selected and further examined. Insecticidal activity testing was done on a sub-selection of LLINs, both by cone and wireball bioassay testing on four predefined positions. Insecticidal content testing was done on a smaller sample of these LLINs. All tests were done with susceptible lab-reared 3-day old *Anopheles stephensi* mosquitoes and followed WHO guidelines.

Outcome measures were mosquito knock-down rate (kdr), mosquito mortality and Deltamethrin concentration.

Results: Combined cone and wireball measurements were available for 800 LLIN pieces, originating from 200 individual bed nets. Wireball mortality showed a weak correlation with cone mortality (Spearman rank correlation coefficient (ρ)=0.34, $p<0.0001$), while the correlation between wireball kdr and cone kdr was even weaker (ρ =0.15, $p<0.0001$). In the scatter plots a wide deviation was seen between measured bioassay results for the same LLIN piece.

Combined bioassay and Deltamethrin concentration measurements were available for 132 LLIN pieces, originating from 33 individual bed nets. Deltamethrin concentration showed the strongest correlation with cone mortality (ρ =0.56, $p<0.0001$), followed by wireball mortality (ρ =0.51, $p<0.0001$), cone kdr (ρ =0.49, $p<0.0001$) and wireball kdr (ρ =0.25, $p=0.0041$).

Discussion: Our analysis shows that the cone and wireball assays measure differently and thus cannot be used interchangeably. While the wireball assay is more difficult to perform, the cone bioassay seems to be the preferred method. Our analysis also shows a clear relation between Deltamethrin concentration and (cone and wireball) bioassay mortality in used LLINs. This finding is of high interest, as LLIN experts increasingly recognize the complexity and variability present in the bioassays.