Assessment of Mosquito Collection Methods for Dengue Surveillance

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Abstract

Background

Several methods exist to collect and assess the abundance of dengue vector mosquitoes, i.e. morning adult collection using an aspirator, pupal collection, various ovitraps, whole night collection using human landing methods, and larval collection. This diversity of methods might be a source of variability and lack of statistical significance when trying to correlate mosquito density and risk of dengue outbreak. There is also a lack of published data regarding the effectiveness of these methods

Methods

A mosquito survey was conducted in 39 locations corresponding to 15 dengue endemic provinces in Indonesia. The larval surveys were performed by collecting at least a single *Aedes* larva from each container, and then reared up until hatching. Three adult mosquito sampling methods were also used, including morning resting collection, human landing collection, animal baited trap. All field samples were tested for dengue. Factor Analysis of Mixed Data (FAMD) was conducted to analyze the effectiveness of the collection methods against mosquito species and dengue incidence.

Results

A total of 44,675 mosquitoes were collected. The single larva method was the most efficient method. Out of a total of 89 dengue-positive pools, the most frequently encountered virus was DENV2, which made up half of the positive samples, followed by DENV3 and DENV1, respectively. FAMD showed that no correlation could be found between any methods and the presence of dengue virus in mosquitoes. Moreover, no correlation could be found between either any methods or the incidence.

Conclusions

There was no concistency in the efficacy of a given method and the incidence of dengue in the human population. There was no correlation between any of the parameters considered, i.e. method, incidence of dengue, location and presence of dengue virus in mosquitoes. This indicates that entomological factors are not reliable indicators.

Background

Dengue is the most rapidly spreading arboviral disease worldwide [1]. Recent studies estimate that 55 to 100 million dengue cases are reported annually with 3.9 billion people at risk [2,3]. Indonesia is an hyperendemic dengue country, i.e. all four serotypes are circulating, with the highest number of dengue cases in South-East Asia [4,5]. Dengue incidence in Indonesia has increased significantly over the last four decades from 0.05 per 100,000 in 1968 to 78.8 per 100,000 in 2016 [6]. The Dengue virus is transmitted to humans by the bite of infected *Aedes aegypti* mosquitoes, the main vector, and *Ae. albopictus,* the secondary vector. These species are anthropophilic, i.e. they live in human environments

and breed in various sites, such as water containers, flowerpots, birdbaths, disposed water-holding vessels, waste disposal areas, small containers, discarded tyres, natural holes in vegetation, etc. [7–10]. Both are present in urban and sub-urban areas. With no treatment and while an effective vaccine is still under study, vector control remains the only effective way to prevent and control dengue.

Vector surveillance methods have remained mostly unchanged for more than three decades [11]. Larval survey is the most widely adopted dengue vector surveillance method to locate larval habitats and to measure the abundance of *Ae. aegypti* and *Ae. albopictus* [12,13]. The *Stegomyia* indices, i.e. House index (HI), Container Index (CI) and Breteau index (BI) to which a specific Free Larval Index (FLI) is added in Indonesia, are used for calculating mosquito abundance and for predicting the risk of dengue transmission [11]. However, previous studies have demonstrated the lack of correlation between *Stegomyia* indices and the risk of dengue outbreak [12,14-18], while a correlation was found between human population density and incidence of dengue [18]. Several methods exist to collect mosquitoes, i.e. morning adult collection using an aspirator, pupal collection, various ovitraps, whole night collection using human landing methods, and larval collection. This diversity of methods might be a source of variability and lack of statistical significance when trying to correlate mosquito density and risk of dengue outbreak. Furthermore, there is a lack of published data regarding the effectiveness of these methods [12,19-22]. Therefore, we conducted a comparative analysis to assess their relative effectiveness.

Methods

Study Sites

The study was conducted in 39 locations corresponding to 39 districts/municipalities in 15 dengue endemics provinces in Indonesia (Figure 1). These provinces include Aceh, West Sumatra, Lampung, Bangka-Belitung, West Kalimantan, South Kalimantan, North Sulawesi, East Java, South-East Sulawesi, Maluku, West Nusa Tenggara, North Maluku. This study is part of the Indonesia national project, Rikhus Vektora that started in 2016

Study design

A mosquito survey was performed in all study sites from July to August 2016, during the rainy season. Single larval methods were performed randomly in at least 100 households in each study site during the study. *Aedes* larvae were collected and then reared in a field laboratory by using plastic trays with tap water and fish food for 3-4 days. Adult collection of *Aedes* mosquitoes were performed in the morning (morning resting) on mosquitoes resting inside houses using manual aspirators. Adult mosquitoes were also collected outside using standard procedures for all night human-landing collection methods from 6.00 pm to 6.00 am. Each adult collection method was performed in every study site. All methodologies used in this study have been previously described [22]. Field data collections for larva and adult *Aedes* mosquitoes were performed by trained collectors in collaboration with local volunteers, local authorities and staff from district/municipality dengue control programs.

Single larva and rearing methods

The larval surveys were performed by collecting at least a single *Aedes* larva from each container. All larvae then were taken to the field laboratory located in the study sites and reared up to the fourth days until adult hatching. Emerged adult mosquitoes were then killed in a freezer (-20°C) or by using ethyl acetate for 5 to 10 minutes and immediately stored in 1.5 ml vial tubes with RNAlater (Qiagen, Hilden, Germany) by pools of 25 mosquitoes and kept refrigerated at 4°C prior to further analysis. Reared larvae and pupae that did not hatch to adult stage up to the fourth days were preserved under the same conditions for further analysis.

Adult mosquito collection methods

Three adult mosquito sampling methods were conducted simultaneously in all study sites, including: (1) morning resting collection, (2) human landing collection, (3) animal baited trap.

(1). Morning resting collections were made by eight collectors using hand nets and aspirators. Collections were conducted from 7.00 am to 9.00 am and included any resting locations within the house. All adults mosquitoes were placed into labelled paper caps and taken to the field laboratory for further analysis.

(2). Human landing collection were performed by eight local volunteers as collectors in three selected houses of each study sites for sampling adult mosquitoes using mouth aspirators. They were all trained before collecting mosquitoes. Three teams of two people sampled outdoors (up to 5 meters from the house) and indoors (inside the house). Each collector sat on chairs with exposing their legs for 50 of 60 minutes per hour. Sampling was conducted all night from 6.00 pm to 6.00 am. The team changed roles regularly every 2 hours with a 2 hour-break. Although, the targeted *Aedes* mosquitoes are diurnal, the Indonesia law does not allow human landing collections during day time. Therefore, collections had to be conducted at night. This introduces a strong bias in the sampling but since it is what surveillance teams do in accordance with the law, this method was nevertheless performed. Mosquitoes that have been collected per hour were then taken to the field laboratory for species identification and further analysis.

(3). Animal baited trap was conducted by using tame animal placed inside a net all night. Mosquito collections were carried out for 15 minutes per hour inside the nets by 3 collectors. Collected mosquitoes were then similarly preserved as for the human landing collection method.

All mosquitoes from these three collecting methods identified as *Ae. aegypti* and *Ae. albopictus* were then killed with ethyl acetate, pooled up to 25 mosquitoes in labelled 1.5 ml vial tubes with RNAlater (Qiagen, Hilden, Germany) and preserved based on the same cold chain management than the one above used for larvae.

Detection of Dengue virus from mosquitoes

The *Ae. aegypti* and *Ae. albopictus* mosquito pools were homogenized in 1.5ml tubes containing 200 µl PBS 1x by using pellet pestles. RNA was extracted using QIAamp®Viral RNA Mini Kit (Qiagen®,

Courtaboeuf, France). RNAs were extracted from 200-µl homogenized samples following the manufacturer's instructions.

All RNA extracted samples were analyzed for Dengue detection using Lanciotti's protocol [23]. The nested RT-PCR for Dengue was performed using SimpliAmp Thermal Cycler Applied Biosystems™ (ThermoFisher Scientific®, United States). Amplification of Dengue RNA was carried out with following specific primers : D1 (5'-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3'), D2 (5'-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3'), TS1 (5'-CGT CTC AGT GAT CCG GGG G-3'), TS2 (5'-CGC CAC AAG GGC CAT GAA CAG-3'), TS3 (5'-TAA CAT CAT CAT GAG ACA GAG C- 3'), and TS4 (5'-CTC TGT TGT CTT AAA CAA GAG A-3').

The first amplification of Dengue virus was performed using Superscript III one-step RT-PCR kit (Invitrogen, Carlsbad, CA). The cycling conditions consisted of initial 95°C denaturation step for 2 minutes, followed by 40 cycles of 95°C denaturation for 30 seconds, 60°C annealing for 1 minute, and 72°C extension for 1 minute 30 seconds, and a final extension step 72°C for 10 minutes. Samples were then stored at 4°C. First step PCR products were run on 2% agarose gel under 120 V current for 1 hour and followed by visualization using SYBR® safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) under UV condition in GelDoc system and check for presence of the 511 bp control band corresponding to dengue virus (DENV) positive. Subsequent serotyping was conducted by using 1st step PCR product with thermal cycle setting as follow : initial denaturation step at 95°C for 2 minutes, followed by 10 cycles of denaturation step at 95°C for 30 seconds, 60°C annealing for 1 minute, and an extension step at 72°C for 1 minute and 30 seconds. The final extension step was conducted at 72°C for 10 minutes. Subsequently, samples were stored at 4°C. Amplification product on 2% agarose gel were then carried out under 80V current for 1 hour and check under UV condition. Multiplex serotyping reaction is expected to produce single-specific band with the size of 482bp for DEN-1 , 119bp for DENV-2, 290bp for DENV-3, and 389bp for DENV-4. All of field samples were tested for the presence of dengue virus after being pooled by 25 individuals of the same species.

Statistical analyses

A first Factor Analysis of Mixed Data (FAMD) [24] was conducted using the incidence data, the number of mosquitoes and the number of positive pools for each dengue serotype as quantitative parameters, and mosquito species, methods of collection and provinces as qualitative parameters. The effectiveness of the collection methods (qualitative data) against mosquito species (quantitative data) was assessed using a second FAMD. These analyses were performed using the R software with FactoMineR [25].

Results

Mosquito sampling

A total of 44,675 mosquitoes were collected from 39 locations (Figure 1, Supplementary Table 1). Out of these 44,675 mosquitoes collected, 32,525 (72.8%) were *Ae. aegypti* and 10,300 (23.1%) were *Ae. albopictus*, while 1,850 (4.1%) were undetermined. When considering the method of capture, the highest

number of captured individuals was, as expected, obtained when targeting larvae. The single larva method was the most efficient in terms of number of individuals collected. A total of 36,500 larvae were collected with this method out which 27,475 were *Ae. aegypti*, 7,775 were *Ae. albopictus* and 1,250 were not identified. The rearing method, although less efficient also yielded large numbers of individuals. Out of 6,450 larvae collected and reared, 4,325 were *Ae. aegypti,* 1,575 were *Ae. albopictus* and 550 were not identified. With both larval methods a bias was observed in favor of *Ae. aegypti* which represented 75.27% and 37.05% of all samples for the single lava and rearing methods, respectively. Very different results were obtained with the adult capture method. From the three methods used, human landing was the most efficient even though a bias is introduced by the legal obligation to perform this approach by night. Out of 1,325 adult mosquitoes captured 325 were *Ae. aegypti*, 975 were *Ae. albopictus* and 25 were not identified. The higher proportion of *Ae. albopictus* might be related to the fact this species is more crepuscular than *Ae. aegypti*. The animal baited trap method yielded only 25 mosquitoes, all being *Ae. albopictus*. The ratio between *Ae. aegypti* and *Ae. albopictus* was reversed with a bias this time in favor of *Ae. albopictus*. It represented 73.58% and 53.33% for the human landing and morning resting methods, respectively. The animal baited trap method yielded only *Ae. albopictus*, but considering the very low number of mosquitoes captured, i.e. 25, this is not significant.

Distribution of dengue virus

A total of 89 pools were positive for dengue virus. The most frequently encountered virus was DENV2 (n=44), which made up half of the positive samples. DENV3 and DENV1 followed with 20 and 17 positive pools, respectively. DENV4 was detected in only one pool. Combinations were also detected. 8 pools contained a combination of DENV1 and DENV2, whereas the combination of DENV1 and DENV3 was found in only one pool. Another single pool contained the triple combination DENV1-DENV2-DENV3. With respect to the geographic distribution, a strong imbalance was observed. A large part of the detected dengue viruses, i.e. 56 (63%), were found in mosquitoes collected in the province of Aceh. All four dengue virus serotypes and all positive combinations were found in this province. The other provinces where positive pools were detected are: West Sumatra (n=5), Lampung (n=6), Bangka-Belitung (n=4), West Kalimantan (n=2), South Kalimantan (n=1), North Sulawesi (n=2), East Java (n=7) and Maluku (n=6). A strong imbalance was also observed when considering the nature of the positive samples. Mosquito larvae were the almost exclusive source of virus, i.e. 93.3% (n=83), with 70.8% (n=63) found with the single larva method and 22.5% (n=20) for the rearing method. Only 6 pools (6.7%) of adult mosquitoes were found positive with the human landing method totalizing 2 pools (2.3%), while 4 pools (4.4%) were found positive in mosquitoes collected with the morning resting method. An imbalanced result was also found regarding the mosquito species with 76.4% (n=68) of the positive pools corresponding to *Ae. aegypti* and 23.6% (n=21) corresponding to *Ae. albopictus*.

Correlation assessment

A Factor Analysis of Mixed Data (FAMD) was performed to determine the potential correlation between the various parameters considered: mosquito species, province, number of mosquitoes, collection

method, dengue virus and dengue incidence (Figure 2a). The only correlation which could be found was between the province and the incidence (Figure 2a). However, the global level of explanation was low (20%) indicating a lack of correlation between any of the parameters with the exception of province and incidence of dengue. A similar result was found when comparing the different collection methods with the mosquito species. The only, but rather weak, correlation which could be found was the preferred association of the larval methods with *Ae. aegypti* and the adult methods with *Ae. albopictus* (Figure 2b, Supplementary Table 1).

Discussion

In the absence of commercialized vaccines and of any medical treatment, the management of dengue relies only on mosquito control and on prevention. Finding efficient and reliable descriptors for assessing the risk of dengue outbreaks is thus a priority in all dengue-endemic countries. The main tools currently in use for assessing this risk of dengue outbreak is the *Stegomiya* indices [15,26] which rely on the calculation of the relative density of mosquito larvae present in containers and in households through the Container Index (CI), the House Index (HI) and the Breteau Index (BI) [26,27]. However, these indices were shown to have no correlation with dengue infection rates and are thus not reliable descriptors [18,28-30].

The development of entomological indices other than the *Stegomiya* indices could then be envisioned. However, as entomological indices, they will still be based on the capture of mosquitoes regardless of the calculation model applied. This work is to our knowledge the most extensive one with 44,675 mosquitoes collected in 39 different sampling locations over Indonesia in a short period of two months, allowing thus a robust statistical analysis. The main conclusion of this work is that it is not the *Stegomiya* indices only, but any kind of entomological indices that might be at best of very limited use. Not only no correlation could be found between any methods and the presence of dengue virus in mosquitoes but no correlation could be found between either any methods or the incidence. Finally, there was no consistency in the efficiency of a given method for detecting dengue. The single-larva and rearing methods yielded 63% of all dengue-positive samples in the sole province of Aceh. However, the incidence of dengue in Aceh is not the highest among all provinces and is rather in the average. Provinces displaying the highest incidence such as Bangka-Belitung, South Kalimantan or North Sulawesi did not yield any dengue-positive larvae. The only single positive pool in these provinces was found in South Kalimantan among morning resting adults. This lack of correlation between incidence and dengue infection rate in mosquitoes is also a drawback for methods associating the capture of adults and the direct detection of dengue virus in the sampled mosquitoes [31-33].

The use of *Stegomiya* indices and the monitoring and collections of mosquitoes are today the main means of assessing the risk of dengue outbreaks and efficiency of mosquito control. Owing to the lack of correlation of the *Stegomiya* indices with the risk of dengue outbreaks and dengue incidence [18], the lack of consistency of the various collection methods, and the very low level of dengue detection in mosquitoes, the monitoring of mosquitoes to assess the risk of dengue outbreaks should be reconsidered. The risk with these methods is mostly that of misleading interpretation, and misguided

decisions and allocation of resources. The only factor found positively correlated with the incidence of dengue was the human population density [18].

Conclusions

The lack of correlation between dengue incidence and entomological factors demonstrated in this work and in a separate study on the *Stegomiya* indices [18] indicates that they are not reliable. Since the only correlation was found with a societal factor, i.e. the human population density [18], efforts should be devoted to the development of novel societal indices to achieve an efficient management of the risk of dengue outbreaks. It is even more important to communicate on this issue because dengue endemic countries worldwide, as well as WHO, still base their recommendations and dengue management procedures on entomological indices.

Abbreviations

HI: House Index, CI: Container Index, BI: Breteau Index, FAMD: Factor Analysis of Mixed Data

Declarations

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Ethics approval

This study involved the use of humans to collect adult mosquitoes in natural settings. Formal approval to conduct these activities was provided by the Ethical Commission Board of the NIHRD, Ministry of Health, Indonesia (No. LB.02.01/5.2/KE.003/2016 and No. LB.02.01/5.2/KE.020/2017).

Consent for publication

Not applicable

Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Competing interest

The authors declare that they have no competing interests.

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Authors Contributions

TAG, LS, TBT, WI, MTP conceived and designed the field studies. MO, SSN, DS prepared samples. TAG and MTP ran molecular analyses and laboratory experiments. TAG, LG and RF analyzed the data. MA prepared the map. TAG and RF wrote the manuscript. SM, LG and RF provided critiques and significant revisions to the manuscript.

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Figures
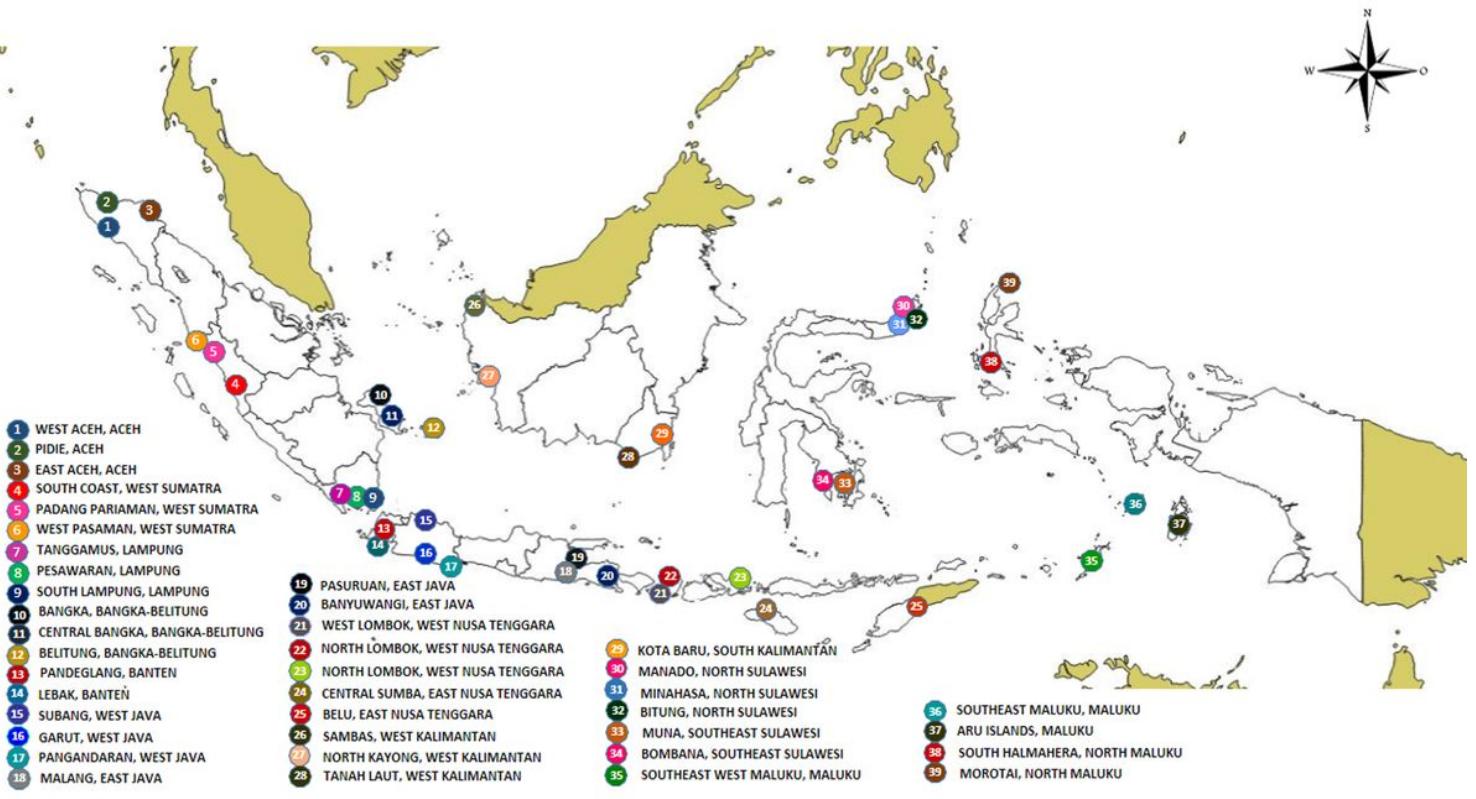
Figure 1

Map of the sampling sites throughout Indonesia. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
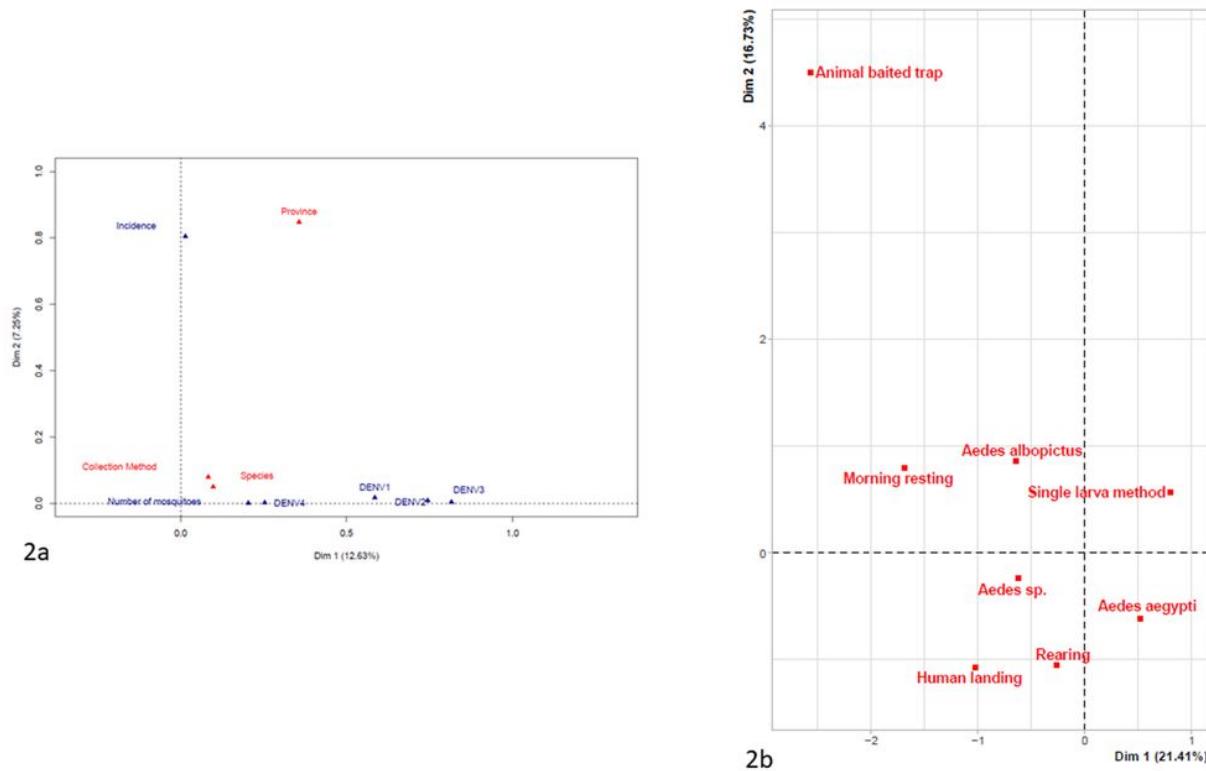
Figure 2

Multivariate analysis of parameters 2a. Global Factor Analysis of Mixed Data 2b. FAMD assessment of the effectiveness of the collection methods