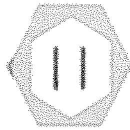

Centre de Recherche Public-Santé
and
Laboratoire National de Santé
Luxembourg

Prevalence and diversity of mosquitoes and mosquito-borne pathogens in Luxembourg

Principal investigators: Prof. Dr. Claude P. Muller,
Dr. Anna L. Reye
Institute of Immunology
Laboratoire National de Santé and
Centre de Recherche Public - Santé
20A rue Auguste Lumière
L-1950 Luxembourg

Tel : +352 490604-220
Fax: +352 490686
Email: claude.muller@crp-sante.lu

Abstract

Mosquitoes are important vectors of human diseases and recent climatic changes may facilitate the migration and establishment of invasive mosquito species in Central Europe and in Luxembourg.

In the present study, mosquitoes were collected at 90 collection sites from March to October in 2011 using Mosquito Magnet Executive traps. In total, 2553 mosquito-like insects were captured. The majority of mosquito-like insects was collected in July (42.0%; n=1073), lowest numbers were found in October (3.2%; n=82). Three individual collections made up 49.5% (n=1263) of all collected specimen, a phenomenon most likely caused by synchronized swarming of the same species.

Morphological identification is difficult when the mosquitoes are poorly preserved (e.g. partial or complete loss of legs, wings and/or mouthparts). Therefore, molecular identification was preferred to assess mosquito species diversity in Luxembourg. In collaboration with the Senckenberg Museum in Frankfurt am Main, Germany, a reference sequence library of endemic mosquito species is currently being established. So far, sequencing results for 204 specimen have been obtained which reveal that in Luxembourg *Culiseta annulata* is the predominant mosquito species (26.5%; n=54), followed by the *Culex pipiens* complex (17.2%; n=35), *Anopheles claviger* (12.7%; n=26) and *Anopheles plumbeus* (10.8%; n=22). Only occasionally *Aedes* (*Ochlerotatus*) *punctor* (3.4%; n=7) and *Anopheles maculipennis* (1.5%; n=3) were collected. In addition to the typing of the 204 samples by sequencing, all mosquitoes were provisionally identified by PCR fragment length analysis. Although this method is less sensitive and less specific similar prevalences were obtained. So far no invasive mosquito species was detected in Luxembourg.

In the coming weeks, we will finalise the sequence analysis of mosquitoes, complete the reference sequence library for European mosquito species and we will develop a new method for fast and reliable mosquito species identification.

Prevalence and diversity of mosquitoes and mosquito-borne pathogens in Luxembourg

Introduction

Despite the growing interest in vector-borne diseases, the actual risk for the population within Luxembourg is not clear for mosquito-borne pathogens. This is of particular concern in light of recent climatic changes facilitating the migration and geographic redistribution of mosquito species and (anecdotal) reports of accidental importation of mosquito species from foreign countries. In collaboration with the Inspection Sanitaire (Ministère de la Santé, Luxembourg) a surveillance study on the prevalence and distribution of mosquitoes was initiated by the Institute of Immunology (LNSI) in August 2010.

Selection of collection sites

In a first step, suitable collection sites were chosen. In each grid square of 10x10km raster, a collection site was placed either in a forested, agricultural or urbanised area. The number of sites per habitat represented the overall habitat distribution (50% agricultural plains, 33% forest and 17% urbanised or sealed area) of Luxembourg. Thus, in the 30 raster grid squares a total of 90 collection sites were chosen (Figure 1). One collection site was placed at the Luxembourg airport in order to catch potentially introduced mosquito species. In the complete study period ranging from March to October of 2011, each raster grid square was visited once per month at alternating collection sites. In 2010, a pilot study was carried out at 32 collection sites from August to October.

Collection of mosquitoes

The collection of mosquitoes was carried out using Mosquito Magnet Executive traps, which were placed at each collection site for 1 week. Parameters recorded for each collection were date, time and trap ID. A pluviometer was attached to each trap to record local rainfall during the collection period. Trapped mosquitoes were collected by the Inspection Sanitaire (Ministère de la Santé, Luxembourg) and nets were brought to the Institute of Immunology (LNSI). Mosquito-like insects were sorted from bycatch and individually stored at -80°C. Protocols for total nucleic acid extraction, mosquito

identification PCR and West Nile Virus (WNV) detection PCR were designed and optimised.

In the pilot study from 2010, 530 mosquito-like insects were collected. Of these, 64% (n=341) were collected at the 32 collection sites during 3 months, 7% (n=38) at the airport and 28% (n=151) for WNV detection at selected sites. 52% (n=277) of all mosquito-like insects were collected in October, 33% (n=173) in September and 15% (n=80) in August. Highest prevalence was observed in the East (33%, n=113) and South (31%, n=107), lowest in the West (13%, n=46) and North (22%, n=75). Rainfall seem to have an influence on the number of mosquitoes collected, as lowest rainfall and highest mosquito numbers were recorded in October. For WNV detection, total nucleic acids were extracted from 98 mosquitoes and detection PCR was carried out. All pools containing either 18 or 20 mosquitoes were negative for WNV.

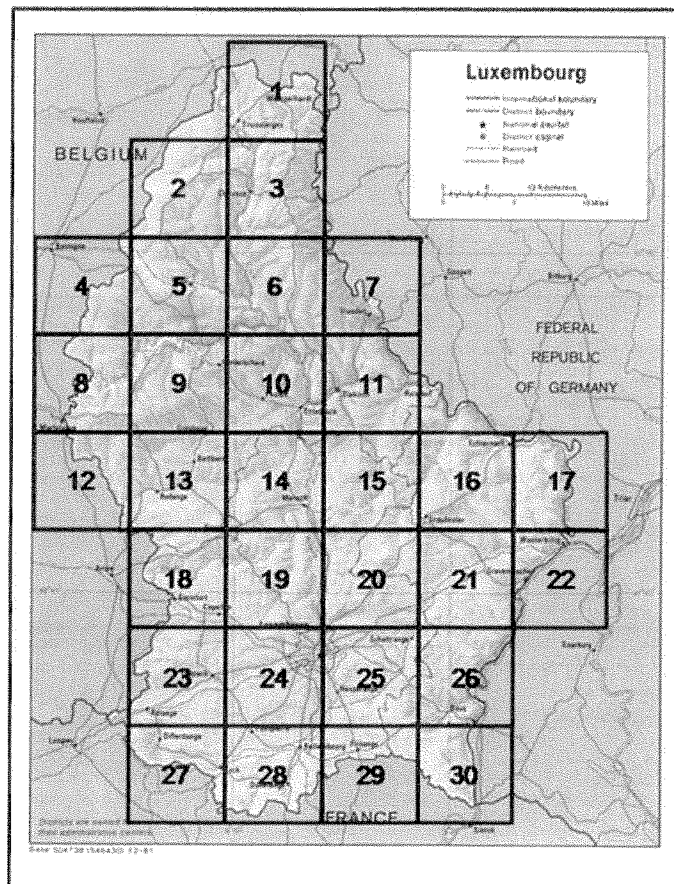

Figure 1. Raster grid used for placement of collection sites.

In 2011, 2553 mosquito-like insects were captured at the selected 90 collection sites (CS). Overall, the majority of mosquito-like insects was collected in July (42.0%; n=1073), lowest numbers were found in October (3.2%; n=82; Table 1).

Three individual collections made up 49.5% (n=1263) of all collected specimen, a phenomenon most likely caused by synchronized swarming of the same species. These extremely high abundances were observed in May and July at CS 28c (n=877 and n=203, respectively) and in June at CS 6a (n=183). These two collection sites are located in the North and South of Luxembourg (see Figure 1) and seem to provide excellent swarming habitat for mosquito-like insects. Of the three sites, only 100 specimen each were further used for total nucleic acid extraction.

Table 1: Seasonal variation of number of specimen collected in the raster grid squares.

Grid #	March	April	May	June	July	August	September	October
1		7		8	11		8	2
2	1	4	22	1	6		5	
3	14	7		4	52		16	6
4	2	2		4				
5	4	3	3				4	
6	2	4	3	183	3	12	20	
7	1	4	2	1	1	3	5	1
8	1		2		2			4
9				6	5	6	6	
10	4			18	3	59	6	2
11	4	1	5	18	1	16		14
12		1	4	9	1	6		
13	5	2	37	15	2	16		4
14	3		5	5	17	3		
15		4	5		4	35		
16	3	5	1	5		3		7
17			4	6	1	12		3
18	7	4			6			2
19			9	3	21		15	
20	3	4		3	7	26		2
21	14		5	2	10		9	
22	16	2	4	4	1		12	
23		3			2	4		
24	1	5	4		34	9	17	
25	3	8	10		7	4	8	24
26	6		30		3		15	9
27	3	3	9			7	10	
28	2	3	877		203	4	6	2
29	3	1	30	9	50	14		
30		12	2		18			
Total	102	89	1073	304	471	239	162	82

Identification of mosquito species

Currently, there are two possible approaches for mosquito species identification, based on either morphology or molecular biology. Even though the morphologic approach is widely accepted as gold standard for species identification it contains significant drawbacks, e.g. it is extremely time-consuming, requires excellent and rare entomological expertise and excellent specimen preservation. In contrast, molecular identification of species is a more efficient strategy of species identification which is more user-friendly e.g. for laboratories lacking a specialised entomology department, less dependent on sample quality, less error-prone and may even provide more specific information e.g. in terms of genetic population structure. However, this technique is quite expensive and requires a sufficient reference sequence library. Based on the second technique, we are currently developing an innovative third approach for mosquito species identification which combines the specificity and straight forwardness of sequencing approach but is less expensive and requires less data analysis. With this approach we will be able to identify mosquito species based on the unique length of their nuclear rDNA internal transcribed spacer region using highest resolution detector systems (available in-house at the Institute of Immunology). This species-typing will be done for the complete reference sequence library in combination with selected specimen from Luxembourg. In case two mosquito species share the exact same length of ITS PCR product, an additional genetic region like e.g. the Cytochrome C oxidase will be used to unambiguously identify mosquito species by PCR fragment length.
This technique is currently under development in our laboratory, the major challenge being the establishment of a complete reference library for all endemic mosquito species. To date, only few endemic mosquito species have been analysed molecularly, limiting the number accessible reference sequences. Nevertheless, sequences of the important invasive species e.g. the Asian mosquito species *Aedes albopictus* (syn. *Stegomyia albopicta*; Tiger mosquito), *Aedes japonicus* (syn. *Hulecoeteomyia japonica*; Asian bush mosquito) and *Aedes aegypti* (syn. *Stegomyia aegypti*; Yellow Fever mosquito) are already available on public databases. To overcome the current limitations we have initiated a collaboration with the Senckenberg Museum in Frankfurt am Main, Germany, which will provide us with reference mosquitoes morphologically identified by expert entomologists. So far, we have obtained reference specimen of seven endemic mosquito species. These were subjected to identification PCR to build up an own reference sequence library.

Identification of mosquito species based on sequences

Morphological identification could not be performed due to the bad preservation of specimen (e.g. partial or complete loss of legs, wings and/or mouthparts) probably caused by the prolonged collection duration of 1 week. Therefore, molecular identification was chosen to assess mosquito species diversity in Luxembourg. Primers amplifying a genetic region with sufficient interspecies variability were chosen from the literature and reference sequences of endemic and invasive mosquito species were selected from public databases. Phylogenetic trees using neighbour-joining and bootstrap algorithms were created to determine the suitability of genetic region for identification purposes. Based on these results, the nuclear rDNA internal transcribed spacer region (ITS) was chosen. Interestingly, the ITS region consists of two short but higly conserved framing regions and an extremely variable central region. In this variable region, substitutions of nucleotides but also deletions and insertions of several nucleotides are frequent, resulting in a sequence that is unique not only in nucleotide composition but also in its length.

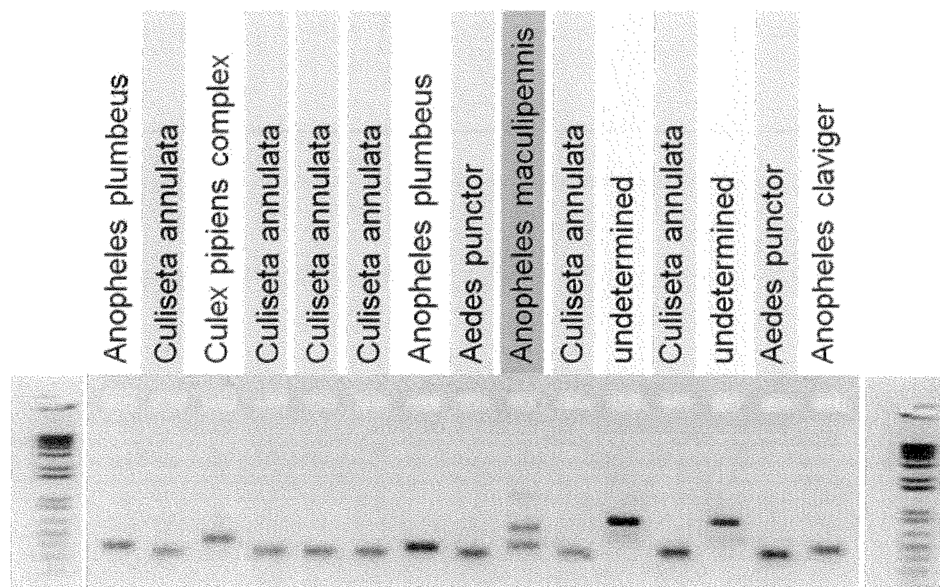

Figure 2. Visualisation of PCR products by size using agarose gel electrophosesis. Fragments of different size can be combined into three groups. Group A: *Culiseta annulata* and *Aedes* (*Ochlerotatus*) *punctor* (red); group B: *Anopheles plumbeus*, *Anopheles claviger* and *Culex pipiens* complex (yellow); group C: *Anopheles maculipennis* (green).

Due to long machine time of our in-house capillary sequencer, it was not possible to generate sequences of all collected mosquito-like insects yet. Obtained sequencing results for 204 specimen show, that so far *Culiseta annulata* is the predominant mosquito species (26.5%; n=54), followed by the *Culex pipiens* complex (17.2%; n=35), *Anopheles claviger* (12.7%; n=26) and *Anopheles plumbeus* (10.8%; n=22). Only occasionally *Aedes* (*Ochlerotatus*) *punctor* (3.4%; n=7) and *Anopheles maculipennis* (1.5%; n=3) were collected in Luxembourg. Currently, 57 sequences form distinct clusters in the phylogenetic trees used for mosquito identification and therefore cannot be identified. Once the reference sequence library is completed for the endemic European mosquito species, identification of these will follow. So far, no invasive mosquito species was detected in Luxembourg.

Identification of mosquito species based on PCR fragment length

As described above, also the length of each sequence differs between mosquito species allowing us to make assumptions on the overall prevalence of mosquito species based on the size of each PCR product. Although standard visualization methods have limitations in size resolution, we were able to identify three groups of mosquitoes containing one, two or three different species (Figure 2; Table 2). When applying these categories to the remaining PCR products of mosquito-like insects, we estimate overall prevalences for group A to be 24.7%, for group B 46.0% and for group C 6.3%. For 23.0% of cases, this species determination was not applicable because their PCR fragment lengths did not match any of our reference sequences.

The estimations based on PCR product size correspond well with the already obtained sequencing results. However, we expect that the predominant mosquito species *Culiseta annulata* will be replaced by members of the *Culex pipiens* complex, reflecting the seasonal adaptations of these species.

Table 2: Grouping of mosquito species according to the length of their ITS PCR product.

Group	Mosquito species	PCR fragment length (in bp)	Prevalence based on PCR fragment lengths (n=2349)	Prevalence based on sequence (n=204)
Group A	*Culiseta annulata* *Aedes (Ochlerotatus) punctor*	400	24.7%	29.9%
Group B	*Anopheles plumbeus* *Anopheles claviger* *Culex pipiens* complex	450-475	46.0%	40.7%
Group C	*Anopheles maculipennis*	400 and 500*	6.3%	1.5%
Unclear			23.0%	27.9%

*Due to an internal primer binding site, the PCR yields two products for *Anopheles maculipennis*.

Outlook

In the coming weeks, we will finalise the sequence analysis of mosquitoes, complete the reference sequence library for European mosquito species and will develop a new method for fast and reliable mosquito species identification. Based on the unique length of ITS region of each mosquito species, species-typing will be implemented to determine the exact length of ITS PCR products from each species as this technique provides the highest possible size resolution (one nucleotide difference measurable).

In addition, mosquito total nucleic acid extracts will be pooled by 10 to screen for the presence of West Nile virus (WNV). In 2010, several hundred cases of West Nile fever in humans have been reported from Greece, Romania and Hungary. In addition, the virus was detected in birds from Austria and Great Britain, suggesting further geographic spread and establishment of WNV in Europe.
In principal, the samples can be screened for other mosquito-borne pathogens like Chikungunya virus, Sindbis virus, Blue Tongue virus, Dengue virus, *Francisella tularensis*, *Babesia* sp., *Plasmodium* sp., *Leishmania* sp. and filarial nematodes. However, the cost-benefit of this strategy needs to be assessed for each pathogen individually.