Combination of PCR and electrical microarray allows rapid and sensitive multiplex detection of mosquito-transmitted pathogens

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Background: More than 300 million people are infected every year with pathogens transmitted by mosquitoes, with 3.3 billion at risk. The majority of cases are infections with different *Flaviviridae*, *Chikungunya Virus* or *Plasmodia*. Standard tests to detect these pathogens are serological assays like IgM and IgG ELISAs for viral infections and the blood smear test in malaria diagnosis for the detection of *Plasmodia*. An early, fast and specific diagnosis often is vital for efficient treatment of these infections, but serological tests usually remain negative during the viremic and early symptomatic phase and in addition antibodies show broad cross-reactivity between *Flaviviruses*. While smear tests are a cheap and rapid method for diagnosis of malaria the limited sensitivity of this test does not allow detection of low level *Plasmodia* infections.

Methods: Real-time RT-PCR is a diagnostic tool with high specificity and sensitivity but has limitations in the parallel detection of multiple pathogens. Micro arrays tend to be less sensitive but have the potential to detect and discriminate a brought range of pathogens.

Results: We developed and optimized a highly sensitive multiplex PCR approach designing a combination of PCR primers for parallel amplification of *Dengue viruses*, *Yellow Fever Virus*, *West Nile Virus*, *Japanese Encephalitis Virus*, *Chikungunya Virus* and *Plasmodia*. Amplicons are differentiated on an electrical microarray. Specificity and sensitivity of the multiplex assay was determined and compared to specific real-time RT-PCR assays.

Conclusion: Our results demonstrate that combination of a broad PCR approach with differentiation on the electrical microarray is a simple, specific, and sensitive tool for early differential diagnosis for the majority of mosquito-transmitted tropical fevers.

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Detection and differentiation of Dengue infections with highly sensitive real-time PCRs

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Background: Dengue is one of the most important arboviral diseases in tropical and subtropical countries caused by infection with one of the four dengue virus (DENV) serotypes. The disease is endemic in more than 100 countries around the globe and an estimated 50 to 100 million cases of acute febrile disease are caused by DENV each year. For treatment rapid, early diagnosis is a prerequisit for efficient treatment of infected individuals. Routine clinical diagnosis is based on serological assays as well as on RT-PCR.

Methods: We developed a real-time RT-PCR generic assay for highly sensitive detection of all four DENV serotypes. DENV-positive samples can be differentiated with a new real-time multiplex assay allowing differentiation of all 4 serotypes.

Results: The assay was validated with clinical samples. Serum from patients from the island of Saint Martin was collect for routine diagnosis of DENV infection with IgM and/or IgG ELISA or NS1 antigen capture assay and in addition RT-PCR at the hospital. DENV positive samples were re-analyzed with the new generic real-time RT-PCR assay and differentiated with the multiplex assay. Comparing these real-time RT-PCR results with the clinical data demonstrated a significantly increased sensitivity and specificity for the new assay. 6 out of 36 samples positive in ELISA but previously negative in RT-PCR were tested positive with the new real-time RT-PCR assays. In addition one sample, previously characterized as DENV1-positive was shown to be DENV2 infected. This was confirmed by sequencing data.

Conclusion: We could demonstrate that our new real-time PCR based approach resulted in improved detection and differentiation of Dengue viruses.

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An automated workflow for high throughput MLVA using the BioNumerics® software, able to deal with varying experimental settings

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Background: Multi Locus VNTR Analysis (MLVA) is a method for high -resolution typing of microbial isolates based upon Variable copy Numbers of Tandem Repeats (VNTR). The use of capillary sequencers for determining fragment lengths allows for a cost- and time-effective determination of repeat numbers, since multiple VNTRs with non-overlapping

size ranges can easily be pooled and loaded in a single capillary. However, the resulting complexity of the pooling schemes and the amount of data generated require an automated workflow for acquisition and processing of fragment files. Moreover, it is well-known that fragment sizes, compared between two genotyping laboratories using capillary electrophoresis (CE), can differ considerably because different laboratories may use different CE machines and running conditions.

Methods: Data processing: The software handles any pooling strategy (combination of dyes and size-compatible VNTRs) which can be parsed from the file name. Thus, each VNTR is defined by a pool, dye and expected size range, defined by the repeat length, offset and expected copy range. Using that information the software will automatically screen (in batch) all theoretical ranges for each VNTR and report on the peak(s) found (or not) in those ranges.

Results: The resulting VNTR information is stored in integer-type character sets where each VNTR represents one character. For comparisons within the same laboratory or between laboratories with compatible instruments and procedures, the software allows the user to handle calculations based on an expected band size range, depending on the known offset, repeat length, expected copy number variation and a user-defined tolerance. In order to deal with a possible experimental size shift linked to the CE system used, a custom 'mapping' tool was developed, allowing observed sizes for a specific instrument to be mapped to real sizes and exact copy numbers.

Conclusion: Data analysis: VNTR data can be analyzed as categorical characters (each different copy number is a different allele) or as quantitative characters. In the latter case, the larger the difference between copy numbers, the less related the organisms are considered. The Minimum Spanning Tree algorithm applied on VNTR data in BioNumerics has proven to be extremely useful for epidemiological study and population genetics.

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Navigating Dante's inferno: Creation of signatures for the rapid detection of heamorraghic fever agents

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Background: Heamorraghic fevers remain some of the most horrific diseases to impact human beings due to their deadly symptoms, limited treatment options and lack of successful vaccines. Heamorraghic fever agents include Ebola, Marburg, Machupo, Lassa, Junin, Rift Valley Fever, and Crimean Congo Heamorraghic Fever viruses, Yellow Fever virus and the bacterial agent *Rickettsia prowazekii*. Infection by a Heamorraghic fever agent leads to an acute illness where multiple organs of the body are affected and can result in death. Today outbreaks of the disease are sporadic and occur only within the agent's host species territory. Humans are not the natural reservoir for any of these agents but once infected, we can spread the disease through personal contact. Since we have no vaccines for these agents early detection remains the most important factor in preventing a localized outbreak from spreading internationally. Since outbreaks are unpredictable, a cost effective way of screening human populations for the disease would enable quick identification of new outbreaks. Furthermore, once an outbreak of Heamorraghic fever is identified, correct identification of the responsible agent is essential for treatment. Unfortunately the responsible agent is difficult to distinguish in a clinical setting by symptoms alone. A system for rapid identification of Heamorraghic fevers is required for quick containment and response to these diseases.

Methods: At the request of the Department of Homeland Security (DHS), Lawrence Livermore National Laboratory (LLNL) has developed signatures for one-step TaqMan RTPCR detection of seven Heamorraghic fever viral agents [Ebola, Marburg, Machupo, Lassa, Junin, Rift Valley Fever, and Crimean Congo Heamorraghic Fever viruses], Yellow Fever virus and one bacterial agent [*Rickettsia prowazekii*]. These signatures were designed by finding conserved and specific sequences belonging to each disease agent. They were then tested and validated using Taqman RT-PCR.

Results: Testing against near neighbors and over 2500 background samples eliminated potential cross-reactivity and increased specificity of the assay. Signatures that showed no cross reactivity were then tested for their Limit of Detection (LOD) value against their target virus.

Conclusion: These signatures will allow the specific and sensitive detection of Heamorraghic fever agents essential for a timely response.

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Usefulness of Montenegro skin test for the diagnosis of Mucosal Leishmaniasis

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Background: Mucosal leishmaniasis (ML) represents the hyperergic pole of American Tegumentary Leishmaniasis (ATL). This behavior makes its diagnosis difficult due to the scarce parasite load as a consequence of a strong local TH1 immune response. Only PCR has a good performance in comparison to traditional techniques like direct examination, culture and histopathology for the diagnosis of ML. Even when ATL is endemic in our country, only two centers can perform PCR for *Leishmania* detection in Peru. Since ML is a neglected disease, patients with mucosal involvement belong to poor, rural and remote areas. Montenegro Skin Test (MST) measures TH1 response, and theoretically ML patients have a longer skin reaction compared to patients with cutaneous leishmaniasis (CL).

Methods: A retrospective study was performed. Only medical records of patients with confirmed ML or CL, through scrapping, culture, PCR or an adequate clinical response, coming from jungle areas endemic in *L. (V) braziliensis,* and