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**Descoberta e Caracterização de Vírus Emergentes e Reergentes
em Áreas Peri-florestais**

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A fragmentação e a invasão de florestas tropicais e a crescente concentração de assentamentos humanos aumentaram exponencialmente as chances de exposição a vírus emergentes e emergentes. Dado o grande potencial de espalhamento de patógenos em população humanas, a identificação e caracterização de agentes patogênicos circulantes podem melhorar a atenção primária e as capacidades de diagnóstico para um agente emergente futuro. As abordagens moleculares e metagenômicas que utilizam as tecnologias de sequenciação da próxima geração levaram a descoberta e caracterização de muitos vírus emergentes na última década. Além disso, as abordagens *in silico* também podem ajudar a identificar vírus emergentes usando apenas dados de sequenciamento publicamente disponíveis. Além disso, estimar a ascendência filogenética e até mesmo analisar as mudanças no uso de codons são ferramentas adicionais que podem melhorar a nossa compreensão de vírus emergentes ou reemergentes. Este projeto visou aplicar essas ferramentas em ambos os vírus que poderiam estar circulando no Brasil: Parvovírus B19 e vírus da Febre Amarela. Também exploramos as aplicações de modelos ocultos de Markov e índice de adaptação de codons usando dados publicamente disponíveis. Esperamos que este trabalho forneça uma prova de conceito para futuros projetos metagenômicos e demonstre a utilidade das várias técnicas moleculares e bioinformáticas no estudo de vírus emergentes.

Palavras-chave: Vírus emergente. Vírus da febre amarela. Parvovírus B19. Uso do codão. Evolução viral. Filogenia. Modelos Ocultos de Markov.

ABSTRACT

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Fragmentation and encroachment of tropical rainforests and the growing concentration of human settlements have exponentially increased chances of exposure to re-emerging and emerging viruses. Given the large potential for pathogens to spillover and spread in a population, identifying and characterizing circulating human pathogens could improve the readiness and diagnostic capabilities for a future emergence. Molecular and metagenomic approaches using next-generation sequencing technologies have led to the discovery and characterization of many emerging viruses over the last decade. In complement, *in silico* approaches can also help identify emerging viruses using only publicly available sequencing data. Moreover, estimating the phylogenetic ancestry and even analyzing changes in codon usage are additional tools that can improve our understanding of an emerging or re-emerging virus. This project aimed to apply these tools to two viruses that could be circulating in Brazil: Parvovirus B19 and Yellow Fever virus. We also explored the applications of Hidden Markov models and codon adaptation index using publicly available data. We expect this work to provide a proof-of-concept for future metagenomic projects, and demonstrate the utility for several molecular and bioinformatics techniques in the study of emerging viruses.

Keywords: Emerging virus. Yellow Fever virus. Parvovirus B19. Codon usage. Viral Evolution. Phylogeny. Hidden Markov Models.

CHAPTER 1 – RESEARCH

1.1 Where there is no overlap, there is a gap

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Over the past 60 years, Brazil has seen extensive rural-urban migration, leading to human settlements encroaching on tropical ecosystems. Persistent human interaction with vectors from diverse tropical environments has led to the emergence of zoonotic pathogens varying in pathogenicity and infectivity. Over time, zoonotic viruses adapt to humans, eventually leading to secondary transmission in some cases. However, most zoonotic pathogens result in asymptomatic and/or dead-end cases, rarely leading to sustained human-human transmission. In very rare cases, a single transmission can explode into an outbreak with sustained networks of human-human transmission. This is the current situation in West African nations, initiated by a single emergence of the Zaire Ebola virus (EBOVZ) in Guéckédou in Guinea's eastern rainforest region (2). Although EBOVZ is a known virus, this was the first record of a case in this region, surprising unprepared health authorities with no experience of encountering Ebola viruses. Owing to ideal conditions, the 25th Ebola virus outbreak quickly spread to the neighboring countries, Sierra Leone and Liberia.

As of December 2, 2014, the CDC and WHO had conjunctly reported 16,933 cases and 6,002 deaths. Critically, the virus itself is not necessarily the main factor explaining the current high EBOVZ case count, as it is behaving similarly to the ones isolated during the first fully recorded outbreak of EBOVZ in 1976. This time, the virus is not experiencing restrictions with respect to the availability of susceptible hosts, as it shows high transmission in densely populated metropolitan settings. Accordingly, the basic reproductive rate (R) has been estimated to be around 2.56 in areas with the highest transmission rates, which is comparable to fomite-transmitted viruses such as small pox and influenza (3). Indeed, as we cannot predict the success of a pathogen or the time or location of emergence, we should focus on what we can control, *i.e.*, setting up efficient health systems to report and isolate cases and contacts and maximizing

surveillance efforts and resources to prevent future outbreaks. The arrival of all dengue virus serotypes (DENV-1, -2, -3, and -4), West Nile virus, and more recently, the Chikungunya virus (CHIKV) in the Americas is a reminder that even with a strong healthcare foundation, a novel emerging or re-emerging virus can cause morbidity and mortality in major metropolitan settings if it is not identified and reported quickly.

A recent review in *Revista da Sociedade Brasileira de Medicina Tropical*/Journal of the Brazilian Society of Tropical Medicine discussed the introduction of the Mayaro virus (MAYV) and more recently, CHIKV in Brazil (4). Both have the potential to cause increased morbidity and mortality in Brazil because of arbovirus infections, and therefore, require our attention.

Originally isolated in the 1950s, CHIKV has migrated through the Caribbean and Americas since then, with imported cases reported in Brazil since 2010. Autochthonous transmission was first recorded in September 2014 in the state of Amapá. CHIKV has already experienced sustained transmission in the states of Bahia and Minas Gerais and may have spread widely already. Both *Aedes aegypti* and *Aedes albopictus* are competent mosquito vectors of CHIKV in an urban cycle, and they are widespread across Brazil. Moreover, the three genotypes of CHIKV can be transmitted efficiently by all 35 strains of *Aedes aegypti* and *Aedes albopictus*, justifying the suggestion that outbreaks in other states are inevitable (4).

MAYV is another pathogen responsible for sporadic cases in Brazil, mainly in the Amazonian region. With MAYV residing in primate reservoirs, *Haemagogus* and *Aedes aegypti* mosquitos initiate transmission via a pseudo-sylvatic cycle, similar to yellow fever (5). Although there have been no large outbreaks, health authorities should preemptively prepare for a re-emergence in unexpected localities.

Symptoms of CHIKV and MAYV are similar to those of other common infectious agents, such as the DENV, and less common agents, such as Rocio, Oropouche, Saint Louis encephalitis, and Yellow Fever viruses (6). Importantly, the availability of rapid and efficient assays precisely identifying viral agents is crucial to diagnose patients and analyze epidemiological data. In addition, for medical personnel to follow proper treatment protocols, diagnostics should be readily available, accurate, sensitive, and economical to differentiate between these agents without a “coincidental” assumption

(*i.e.*, during a dengue outbreak, patients with similar symptoms may be infected by other arboviruses but not subjected to formal serological assay). Interestingly, this mirrors initial diagnostic scenarios in Guinea. According to the CDC, in the early stages of EBOVZ infection, patients could present with fever, severe headache, fatigue, muscle pain, vomiting, diarrhea, abdominal pain, or unexplained hemorrhaging. In West Africa, this symptom collection could indicate a number of differential diagnoses including malaria, typhoid fever, leptospirosis, rickettsiosis, African trypanosomiasis, Lassa fever, cholera, Marburg virus, and Crimean-Congo hemorrhagic fever (7). Therefore, in the early stages of an outbreak, lack of positive confirmatory serological tests could delay health authorities' responses, depending on the disease severity. Logically, health structure, resources, and sociological qualities differ between West African nations and the Americas. However, we must continuously remind ourselves that a similar scenario can occur in Brazil. Are Brazil's diagnostic and response capabilities prepared for the emergence of a novel pathogen?

It has previously been argued that many arbovirus infections have been neglected in Brazil, with few epidemiological studies conducted to understand their distribution. For instance, dengue fever is difficult to distinguish from other acute febrile illnesses, which emphasizes the importance of a differential diagnosis (6,8). Indeed, most diagnoses in the country are made on clinical and epidemiological grounds. In 2014 (as of November 2), 547,612 clinical cases of dengue fever were reported, but 8,423 (1.54%) were laboratory confirmed (9,10). These numbers do not represent the countless mildly symptomatic or nonspecific febrile cases. Therefore, the true rates of infection and transmission are underestimated, and the possibility of infection from other pathogens is ignored. With respect to the CDC Ebola case definition, DENV, CHIKV, and MAYV could cause overlapping symptoms with the above diagnoses, supporting the need for broad-spectrum, sensitive, and economic diagnostic assays to identify infectious agents without concerns regarding cross-reaction of closely related agents. This does not imply that an Ebola outbreak is a given in Brazil but exemplifies a complication that could arise in such a scenario.

The emergence of a novel pathogen should always be preconsidered. Moreover, if it has the potential for establishing itself within a human transmission network, having

diagnostic tools to confirm its presence and prompt notification of national health authorities should be priorities. Depending on the pathogen's transmissibility, contact tracing and quarantine should be established rapidly. Although EBOV is not novel in the sense of being unknown, it was novel in the locality of its zoonotic emergence in West Africa. Once a country undergoes a zoonotic-related emergence event leading to morbidity and mortality, the aftermath leads to preemptive measures to ensure that the scale of the next outbreak is significantly diminished. Continuous sentinel and active surveillance are costly and time consuming from a resource perspective. However, considering resource limitations, early diagnostic efforts and surveillance of emerging pathogens are the best ways to reduce mortality, morbidity, and the enormous health care costs associated with epidemics (11).

Brazil has thousands of communities surrounded by tropical ecosystems teeming with potentially pathogenic agents. Are there countries with limited resources whose healthcare system has successfully prevented large outbreaks efficiently and strategically, which who we can learn from? Uganda could be a good model; similar in respect to climate and ecological diversity, also limited in resources disposed towards surveillance and epidemic management. Uganda presented a model system of emerging infectious disease control, demonstrating from previous EBOV outbreaks that continuous surveillance, that dedicated teams, devoted to contact tracing, case isolation, and cooperating with local communities and bordering countries, successfully limits case counts and spread (12). Brazil and other nations could learn from Uganda's experience, applying the approach to CHIKV, MAYV, DENV, and other pathogens. Although our ability to diagnose, treat, trace, and contain pathogen outbreaks has improved over the last decade, it has not been sufficient to prevent large outbreaks of new serotypes of dengue, and there is no indication that CHIKV will be halted. Therefore, one has to conceive that what is ongoing in West Africa could happen in Brazil. *Where there is no overlap, there is a gap*; therefore, we should establish much-needed constructive interactions between governments, academia, and the private sector to continue searching for novel methods of diagnostics, improve surveillance, strategize, and maximize resources to prepare for any future emergence. The question is not one of whether an emergence will occur but when and whether we will be ready.

1.2 Objectives

The overall research goal is to identify and characterize emerging and re-emerging viruses through molecular and bioinformatics (*in silico*) approaches in São Paulo, Brazil. The specific objectives are:

- (i) To identify highly divergent viruses in metagenomic sequencing data using a Hidden Markov Modeling approach
- (ii) Monitoring changes in a virus' codon usage over time in relation to a host's codon usage using codon adaptation index
- (iii) Using next generation sequencing to identify the etiological agents in clinically suspected dengue fever patient's that were negative for the Dengue virus and antibody
- (iv) To sequence and characterize Yellow Fever virus isolated from a convalescent patient's urine and semen

1.3 Materials and Methods

1.3.1 Profile HMM construction and HMMER3

Complete amino acid (aa) sequences from human pathogenic viral genera and families, 70 in total, were downloaded from GenBank. Sequences were then aligned and manually fragmented into smaller alignments ranging from 70-200 aa. Cuts were based on the level of conservation between sequences: highly conserved regions were made the central “foundation” of a fragmented alignment and then were extended to adjacent regions that were less conserved regions and highly varied between species. Next, alignments were built into profile hidden Markov models (pHMM) using *hmmbuild* from the HMMER3 package (13).

To use pHMMs against sequencing databases, DNA and RNA reads were first downloaded from NCBI’s SRA database. Next, reads were trimmed using Trimmomatic 2.0, converted to fasta format using *fastx*, and then translated to amino acids in all 6 reading frames using an in-house script. Using *hmmsearch*, pHMMs were quarried against the translated dataset. To prevent false-positive matches, we set the search e-value to 10^{-5} . Hits were identified and individually extracted into Geneious 9.0.5 (www.geneious.com) for downstream assembly and analysis.

1.3.2 Codon Adaptation Index Sequence Databases.

For each virus (Yellow Fever virus, Dengue 2 virus, Tobacco Mosaic virus, West Nile virus), all available complete genome sequences with collection date and country of isolation information were downloaded from Genbank. Open reading frames of sequences were exported aligned using MAFFT 7.222 (14) and then manually curated using the Geneious 9.0.5 software (www.geneious.com).

1.3.3 Codon Adaptation Indices of Viral Coding Genes.

To investigate if our selected viruses have evidence for codon adaptation in humans, we calculated the codon adaptation indices (CAI) for each coding region of each respective virus. For Yellow Fever virus, Dengue 2 virus, and West Nile virus, the complete polyprotein was used to calculate CAI. For Tobacco Mosaic virus, coding regions were concatenated into frame and then used to generate CAI values.

To calculate normalized CAI, we first used the CAIcal program (15) to obtain a “raw” CAI (rCAI). Next, an “expected neutral CAI” (eCAI) value was calculated by generating 1000 random sequences using similar length, codon composition and GC-content. Normalized CAI values were then compared among different time points and viral lineages using a non-parametric rank test because central tendencies trend varied throughout time more than each time point variance. To obtain our normalized CAI threshold, rCAI/eCAI values were calculated. Values greater than 1 were taken as evidence for codon adaptation to the reference set of codon preferences (15). Values lower than 1 was taken as evidence that mutational bias are driving codon selection.

1.3.4 Codon Usage Tables.

The codon usage tables for *Culex pipiens*, *Aedes aegypti*, *Macaca mulata*, *Columba livia* and *Rhipicephalus microplus* were downloaded directly from the publically available Codon Usage Database (www.kazusa.or.jp/codon).

We used 3 in-house codon usage tables that aimed to represent human house-keeping genes, highly expressed human antiviral immune genes, and *Nicotiana tabacum* (tobacco) house-keeping genes. The human codon usage table available on the Kazusa Codon Usage Database was last updated in 2007. Since highly or constitutively expressed genes will manifest greater codon preference biases (16–18), we first identified which genes would be potential “targets” for viral codon adaptation. The 3804 identified human house-keeping genes by Eisenberg and Levanon in 2013 (19) were selected for the human house-keeping gene codon usage table.

To identify and select highly expressed human antiviral genes, we looked for studies that quantified innate immunity gene expression during infection, specifically differences between mice and human immune responses (20,21), various protein

functionalities of the innate immune system during viral infection (22–25), and immune responses during flavivirus infection (26,27). Ultimately, we selected 25 genes that we believed to represent highly expressed antiviral immune genes. To address the concern that we may be incorrectly comparing a population's (virus) versus an individual's genetic components, we cross-referenced all human coding sequences used with haplotype data from the 1000 Genomes Project to look for single-nucleotide polymorphisms in coding regions of interest (28). Variants in house-keeping and immune genes were very rare and did not alter the overall codon frequencies of our generated codon usage tables. Codon usage tables are made available at Github (<https://github.com/CaioFreire/CUB>).

1.3.5 WNV Phylogenetic inference and Malthusian fitness estimate.

First, 790 West Nile Virus complete polyprotein sequences from Genbank were downloaded. Sequences were aligned and curated by coding sequences using Muscle and Geneious respectively. Using FastTree.v2 (29), a maximum-likelihood tree was inferred, and allowed us to partition sequences by previously described lineages (30,31). Using 61 lineage 2 sequences, the rate of each substitution type under the general reversible model (GTR) substitution model, the proportion of invariant sites (I), and shape parameter of a gamma distribution with four rate categories (Γ_4) was estimated from the data. Tip times corresponding to the year of virus sampling, a lognormal uncorrelated relaxed clock using continuous quantile parameterization (32), and a GMRF Bayesian Skyride coalescence model (33) was used and run using Beast 1.8.3 (34). The MCMC analysis was run for 100 million chains to ensure convergence (achieved by a single run, with 10% of the run, discarded as representing “burn in”). A MCC was produced and annotated by the use of TreeAnnotator in the BEAST package. To infer the recent demographic history of WNV in Europe, we employed the Bayesian skyride method (33) and previously described methods to estimate the temporal dynamics of effective population size (N_e) of WNV, which approximates the number of infections in time. To reveal the dynamics of viral population size growth, we calculated

the Malthusian fitness (Wm) for the polyprotein, which was approximated by the ratio of the population size in sequential time points ($Wm = Ne_{gt}/Ne_{gt-1}$) (35).

1.3.6 Sample Collection of Guarujá

Samples were collected from November 10th, 2013, to February 25th of 2014. We collected 182 serum samples from patients who showed signs of acute-febrile illness, but tested negative for all dengue-specific ELISA and PCR assays. Patients of any age with symptoms and clinical signs of dengue disease were examined in Primary Health or Emergency Care Units. Patients were included who presented with the following symptoms and complications: fever, joint-pain (arthralgia), headache, rash, retro-orbital pain, muscle pain (myalgia), difficulty swallowing (odynophagia), vomiting, diarrhea, and thrombocytopenia. These patients reside in the coastal city of Guarujá (23°59'37"S 46°15'23"W), which is situated in the southeastern state of São Paulo, Brazil.

Patient records were retrospectively collected with the kind aid of the Guarujá Secretary of Health. From the 182 total serum samples collected, only 111 records that included basic patient information were available. Only 70 contained diagnostic information and 56 contained a physician's medical records from all Guarujá public healthcare clinics. Only 25 had usable hematology data. All adult subjects included in this study provided an informed written consent, and a parent or guardian of any child participant provided the written informed consent on their behalf.

1.3.7 Illumina Sequencing, Trimming & Contig Assembly

Viral RNA was extracted from the nasopharyngeal aspirates using the QIAamp Viral RNA Mini Kit (Qiagen; Valencia, CA), and purified with DNase I and concentrated using the RNA Clean & ConcentratorTM-5 Kit (Zymo research; Irvine, CA). The paired-end RNA libraries were constructed and validated using the TruSeq Stranded Total RNA HT Sample Prep Kit (Illumina; San Diego, CA) was used to construct and validate paired-end RNA libraries. Sequencing was done at the Core Facility for Scientific

Research – University of São Paulo (CEFAP-USP/GENIAL) using the Illumina NextSeq platform. Each sample was barcoded individually, which allowed separation of reads for each patient. Short, unpaired reads/bases, and low-quality reads were removed using Trimmomatic v0.36 (36). Paired-end reads (Qphred>33) were assembled *de novo* with SPAdes 3.10 using default parameters (37). Contigs over 500 nts were searched against the refseq Genbank repository using BLAST.

1.3.8 Phylogenetic Reconstruction: Maximum-likelihood estimation

To infer the phylogenetic relationships of our sequences with other partial or complete genome sequences, we downloaded all available sequences from Genbank that had country and date of the samples isolation. Sequences were aligned using Clustal Omega version 1.2.1 (<http://www.clustal.org/omega>) or Muscle v3.8.31 (38) and manually curated by using JalView version 1.18-β8 (<http://www.jalview.org/download>) or Se-AI v2 (39). A maximum-likelihood tree was estimated in the nucleotide general time-reversible with gamma-distribution rate variation and invariant sites model. Support for the tree was accessed after 10,000 nonparametric bootstrap replicates with FastTree version 2.1.8 (29).

1.3.9 Molecular Testing for Parvovirus B19

The detection of the B19V IgM and IgG antibodies were done using the Serion ELISA classic IgM and IgG test kits. Serum samples were extracted on the NUCLISENS easyMag platform (bioMerieux). The eluate, containing RNA and total DNA, was stored in a freezer at -70 °C until the time of its use. PCR, followed by a Semi-NESTED reaction was performed targeting the VP1/VP2 region using primers designed by Durigon *et al.*, 1996 and Erdman *et al.*, 1993 (40,41). The two primers used in the PCR were P12f (nt 4127-4148; 5'- CAGCCATACCACCACTGGGACA-3') and P17r (nt 4824-4803; 5'- TTACGCATCCTGGCTGAGGGCA-3'), with a fragment of approximately 697bp; semi-NESTED primers were P13f (nt 4214-4237; 5'-

GACAAAGAGTATCAGCAAGGAGTG-3') and P17r (nt 4824-4803; 5'-TTACGCATCCTGGCTGAGGGCA-3'), with final product of approximately 610 bp. The amplification was performed in a thermocycler with the following conditions: a cycle of 94 °C for 5 min for the activation of Hf, followed by 40 cycles of denaturation at 94 °C for 30 seconds, Hybridization at 55 °C for 30 seconds and extension at 72 °C for 45 seconds and finally a final extension cycle at 72 °C for 7 minutes. Semi-NESTED reaction was performed using the same PCR conditions. As control, a well-known sample was used, and DNA/RNase free water was used as the negative control. Detection of the PCR/semi-NESTED amplified product was performed by 2% agarose gel electrophoresis (Life Technologies) in TAE buffer [0.5x], stained with 0.5µg/ml ethidium bromide. In 3.0µL of bromophenol blue, 10.0µL of the sample was mixed and submitted to electrophoresis in a horizontal vessel for 60 minutes at 100V.

1.3.10 Sanger sequencing of Parvovirus B19

Samples that were found positive for B19V using a semi-NESTED PCR were selected for downstream sequencing. Purification of the PCR product was performed with the ExoSap kit (exonuclease I - Amersham Pharmacia Biotech), according to the manufacturer's instructions. After purification of the Semi-NESTED product, the amplified VP1/VP2 region using P13f/P17r primers was sequenced using the "ABI PRISM Dye™ Terminator Cycle Sequencing Ready Reaction kit" ("Big Dye" - Applied Biosystems, Inc., USA), using 3.0µL of BigDye® Terminator v1.1/3.1 Sequencing Buffer (5X), 1.0µL of Terminator Ready Reaction Mix and 1.0µL of the primer. The enzymatic extension was performed on GeneAmp PCR System 9700 thermocycler for 25 cycles of 96 °C for 10 seconds for denaturation of template DNA, 50 °C for 10 seconds for primer annealing and 60 °C for 4 minutes for extension. The obtained product was further purified with the X-terminator kit (Applied Biosystems Inc., USA) according to the manufacturer's instructions. The purified product was subjected to POP7 polymer electrophoresis using ABI model 3100 automatic sequencer (Applied Biosystems, Inc., USA).

CHAPTER 5 – CONCLUSIONS

- (i) Identified a highly divergent dicistrovirus in metagenomic sequencing data from sewage using an HMM approach
- (ii) Demonstrated that codon adaptation index can be applied to flavivirus subgroups and individual flavivirus species across a number of hosts
- (iii) Discovered and characterized an outbreak of Parvovirus B19 in the summer months of 2013/2014 in Guarujá, Brazil
- (iv) Sequenced and characterized a Yellow Fever virus isolate from a convalescent patient's urine

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