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DNA BARCODING AS A MOLECULAR TOOL FOR VECTORS IDENTIFICATION – REVIEW

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ABSTRACT

Vector-borne diseases pose a serious threat to public health and animal husbandry and require special attention. Knowledge of the ecology of different insect species acting as vectors of multiple infectious agents will be crucial for the development and implementation of appropriate and effective vector control strategies to reduce the impact of the diseases they transmit. However, the main limitation is that morphological methods for identifying insect species are time consuming and require taxonomic expertise. Inaccurate identification of vector species can have a significant impact on attempts to control their populations.

One of the remarkable achievements of modern biology and molecular diagnostic methods is the development of accurate and reliable technologies for rapid screening of DNA sequence variations. Molecular identification and DNA barcoding is a relatively new taxonomic method that can be used to identify a species, including an unknown one. Closely related or unknown species can be differentiated. DNA barcoding is a standardized approach to identifying plants and animals through minimal DNA sequences called DNA barcodes.

Key words: DNA barcoding, mosquitos, culicuides, sand flies, vectors.

Introduction

Vector-borne diseases and the identification of competent vectors in the European region according to the WHO is a serious problem. Factors such as globalization, increasing trade and travel of the population, transport and animal trafficking, continuing urbanization and changes in the environment and climate contribute to the spread of competent vectors. Aedes aegypti and Aedes albopictus are examples of competent vectors that are responsible for severe infectious diseases such as Dengue and Chikungunya fever. Travelers from countries endemic for such infectious diseases are increasingly the reason for the isolation of these competent vectors in European regions and the presence of these infectious diseases in Europe. Over the past five years, the WHO has seen a growing number of Dengue and Chikungunya outbreaks in the region.

Vector-born diseases account for a significant percentage of all infectious diseases (> 17% of all infectious diseases and 1 million deaths per year). Controlling and limiting competent vectors is often the best strategy, and sometimes the only way to protect humans and animals from these destructive diseases. It is becoming increasingly clear that effective vector control requires a multidisciplinary, community-based and environmentally sustainable, country-specific approach.

DNA barcoding is a standardized approach to identifying plants and animals through DNA sequences called DNA barcodes. Methods based on DNA identification of species can be applied at all stages of life, including old or damaged samples, as well as processed samples (Sperling et al., 1994; Wells and Stevens, 2008). There have been many attempts to use molecular taxonomic techniques to identify insects with good results, published by Xiang and Kochar, (1991); Kambhampati, (1995); Tang et al., (1996).

DNA sequencing is a far more informative and useful technique than other molecular methods (for identifying and studying interspecific and intraspecific variations). This method is very useful in identifying and classifying the largest and not well-studied group – insects.

DNA barcoding is a molecular method that is becoming increasingly popular for identifying animal species based on partial mitochondrial DNA sequences (Hebert et al., 2003a; Hebert et al., 2003b). This method is based on the concept that each species has a unique genetic identity (Rueanghiran et al., 2012). The DNA barcode is a short standardized DNA sequence that can be used as a genetic marker to identify species (Paz et al., 2012). Early studies on DNA barcoding used internal transcribed spacer 2 (Merget et al., 2012), cytochrome b oxidase (Sevilla et al., 2007; Shen et al., 2013), 12S rRNA (Vences et al., 2005; Chu et al., 2006) and nicotinamide adenine dinucleotide dehydrogenase (Rach et al., 2008; Webster et al., 2012) as target genes. In recent years, however, the cytochrome C oxidase subunit 1 (COI) mitochondrial gene has gained popularity, mainly due to the ease of use of a universal set of primers to enhance the gene and its ability to provide higher sequence variation in interspecific than intraspecific level. Therefore, a genetically based COI DNA barcode is an alternative type of identification method that can be easily standardized to obtain comparable results from different sources.

DNA barcoding mosquitos

Morphological identification is the conventional gold standard for identifying species of vectors based on their external features. However, it requires experienced specialists, and the method itself is time consuming, especially performed by more inexperienced researchers. Moreover, incomplete identification often occurs when important morphological features located on the body of insects are damaged as a result of improper handling of specimens. In addition, similar morphological features are shared by members of species complexes, making identification a difficult task based solely on taxonomic keys (Jinbo et al., 2011). Most taxonomic keys for example are limited to adult female mosquitoes and fourth-stage larvae, as many of the morphological characteristics are not well developed in the early stages of larvae.

In addition, many species of Culex Lophoceraomyia are identified by differences in the antennae, palps, proboscis, and genitals of the male, which makes it difficult to morphologically identify females (Sirivanakarn, 1977). These restrictions affect the applicability of existing taxonomic keys to identify certain mosquito species. Therefore, there is a need for an alternative, universally applicable method to support existing mosquito identification methods.

Herbert et al., 2003a, b, suggest using the 658 (bp) region of the COI gene as a universal marker for animal species barcodes. Previous studies have shown that the COI gene is an effective and useful barcode for identifying mosquitoes (Laurito et al., 2013; Khamis et al., 2012). However, the COI barcode may not be universally applicable to the identification of all animal species. For example, COI-based barcoding is not promising in the identification of fungi and plant species (Roy et al., 2010; Schoch et al., 2012). Similarly, COI barcoding fails to distinguish some mosquito species from Anopheles and Culex (Laurito et al., 2013; Wang et al., 2012). Kumar et al., 2007 report that two closely related species of mosquitoes of the genus Ochlerotatus cannot be differentiated using their COI barcodes. On the other hand, DNA barcoding has other limitations. Its success depends on the availability of representative sequences for comparison. DNA barcoding fails if there are not enough reference sequences in the databases for comparison and analysis (Jinbo et al., 2011). In cases where the COI barcode fails to identify specific mosquito species, a multilocus approach is

recommended (Bourke et al., 2013). By using other gene markers and combining data sets, identification accuracy can be increased. Therefore, these observations indicate the need to use integrated databases, including genomic, morphological and ecological, to better understand the species diversity of the animal kingdom (Rubinoff., 2006).

Mosquitoes belong to the genus Diptera, family Culicidae and are represented by 3490 recognized species, grouped in 44 genera.

The identification of the genus and species can be performed by standard entomological features such as phenotype, eggs, larvae, habitat, etc. Newer species determination techniques include biochemical techniques such as MALDI-TOF and DNA barcoding. They are successfully used to distinguish morphologically similar species in a species complex (eg Anopheles gambiae-complex, Culex pipiens-complex).

Most traditional morphological identification keys are organized in a series of alternative features, which can be dichotomous (each feature / parameter has two alternatives) or polytomic (two or more options at each choice). In practice, most of them are a mixture of dichotomous and polytomic sequential choices.

Within these two types of keys, the dichotomous keys of Becker et al. and computer programs such as MosKeyTool are among the most commonly used to identify mosquitoes in the European region.

DNA barcoding Culicuides

Culicoides (order Diptera, family Ceratopogonidae) cover more than 1300 species distributed worldwide (Borkent., 2017) and are vectors of a number of pathogens of veterinary importance. Some species of Culicoides are biological vectors of important animal arboviruses worldwide, such as African horse sickness virus (AHSV), bluetongue virus (BTV), epizootic haemorrhagic virus (EHDV), equine encephalosis virus (EEV) and Schmalenberg virus (SBV)). (Purse et al., 2015). The African horse sickness virus is from the family Reoviridae of the genus Orbivirus, which is biologically transmitted by competent vectors of the genus Culicoides (Carpenter et al., 2017). This disease is prevalent mainly in Africa and is classified as one of the deadliest viral infections in horses with a mortality rate of about 80–90% (Mellor et al., 2000). Massive epizootic outbreaks of AHS were reported in Senegal in 2007 (Akakpo et al., 2011; Diouf et al., 2012). Despite their economic importance, identifying species only morphologically and considering only the evolutionary relationships between species of this genus remains problematic. In recent years, the DNA barcoding has contributed significantly to the identification of species of the genus Culicoides spp. in the numerous entomological research projects initiated in many European countries after the bluetongue epidemic in 2006-2009. These studies and projects help to identify, potentially new species or classify them into "species complexes/groups" with great genetic and morphological variability. Most of them have a black-brown body color with characteristic white and dark spots on the wings.

- C. obsoletus group: the most common species at low altitude. The C. obsoletus group includes 4 morphologically similar species (C. obsoletus, C. scoticus; C. dewulfi and C. shiopterus).

- C. pulicaris group: very common and widespread and predominant at high altitudes. The C. pulicaris group includes mainly two distinct species: C. pulicaris and C. punctatus).

- C. impunctatus group: common in northern Europe, where it is very common and in large populations. The species of the C. impunctatus group are responsible for the transmission of animal BTV, AHSV, EHDV.

According to Campbell and Pelham-Clinton, 1960**,** the subgenus Culicoides is morphologically characterized by wings with dark spots on a light background, and the apical third of the second radial cells are included in a pale area. A dark spot in the shape of a clock glass (r5) is present in the wing cell. The cell in the form of a watch glass can be broken or continuous. The cubital cell (cu) can be with or without a dark spot. In some species, which usually do not have a spot in the cubital cell, a spot may appear in some specimens of species, eg: C. impunctatus and C. deltus.

However, morphological determination of Culicoides at the species level is difficult even for the specialists. It has been proved that the related species in the species complexes are difficult to distinguish morphologically. To overcome this, molecular methods (COI-barcoding or other molecular markers) are used to identify species of the genus Culicoides, especially those responsible for the spread of diseases in domestic animals.

DNA barcoding sand flies

Sandflies (Diptera: Psychodidae, Phlebotominae) are essential for public health and in many parts of the world play a role as vectors for leishmaniasis, bartonellosis and sand fly fever. Sandflies are the only proven vectors of leishmaniasis, with approximately 800 species registered in five main genera: Phlebotomus, Sergentomyia, Lutzomyia, Brumptomyia and Warileya. Leishmaniasis remains one of the most neglected diseases in the world. It is endemic in 98 countries, putting 350 million people at risk, with about two million new cases each year (WHO., 1990). Studies on phlebotomes (Diptera: Psychodidae) show that there are mainly two genera circulating: Phlebotomus and Sergentomyia, comprising 44 species. Therefore, the identification of sandflies in order to vector control and control of leishmaniasis and other infectious diseases is critical. The correct identification of the sand fly species provides a better understanding of the sand fly-pathogen relationship as well as the planning of ecological studies on populations, the sensitivity of risk analysis on sand fly-borne diseases and ultimately for the success of disease control measures. The presence of 14 sand fly species of two genera was recorded in eight Balkan countries by a large-scale field survey that thoroughly updated the knowledge of sand fly fauna in this important and yet understudied region (Dvorak et al., 2020).

DNA barcoding procedures

It is becoming increasingly clear that in order to distinguish phlebotomies by species and to identify them in separate taxa, it is necessary to use multidisciplinary approach combining modern techniques (DNA barcoding) and traditional ones (morphological identification). The use of statistical approaches, such as models based on discriminant or multivariate analyzes used in morphometric studies, can also contribute to the identification of intra- and inter-specific differences.

With regard to the identification of species using methods other than traditional dichotomous keys, the development of cybertaxonomic determinants can facilitate this task for non-specialists or when it is necessary to identify many specimens or in the field.

Frequently used morphological parameters to identify adults should be constantly updated, taking into account other data such as behavioral, biochemical, environmental and molecular data. The use of molecular techniques for species determination of catches, such as the barcoding method, is strongly recommended. It is desirable that the markers used in molecular analysis be standardized and that gene sequences be deposited in freely accessible databases in order to extend and enrich the

morphological identification of species and to achieve phylogenetic analysis and determination of their origin.

Hebert et al., (2003a), b, demonstrated the benefit of the gene encoding cytochrome C oxidase I (COI), first used by Folmer et al., (1994) (DNA primers published by Hebert as a tool for phylogenetic analysis at the species level were included in the analysis).

In general, the DNA barcode is a short sequence of genes that are part of the mitochondrial DNA that is used to identify species. This standardized short sequence of DNA is in the range of 400-800 bp, which should be easily generated to characterize all species on the planet.

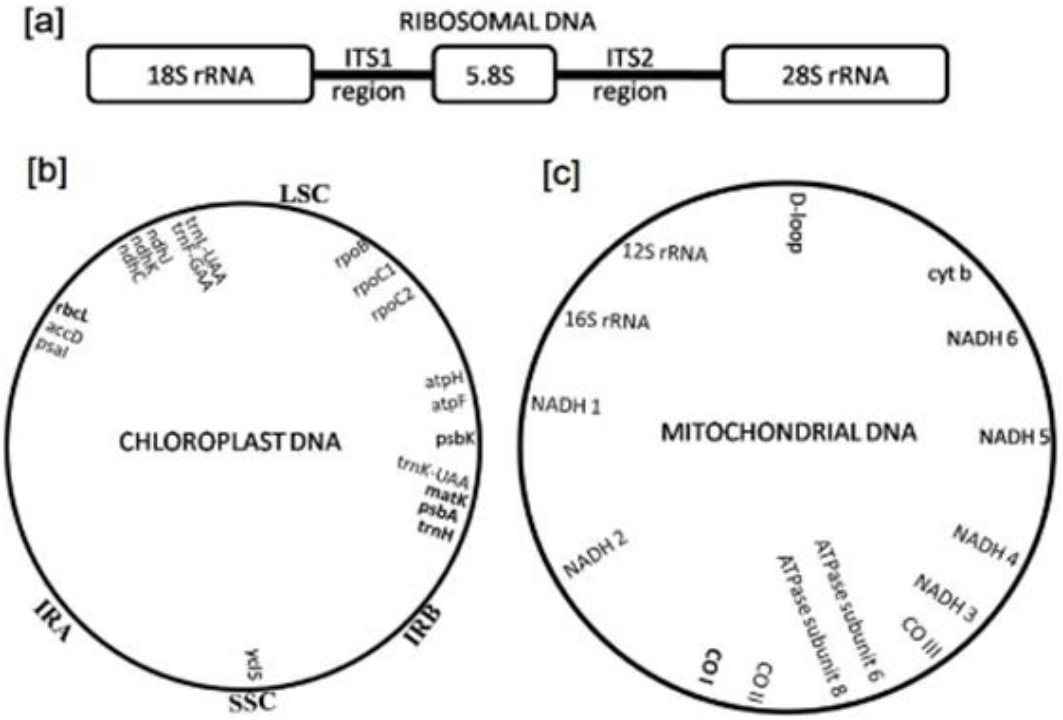

Figure 1: Specific loci of chloroplast/mitochondrial DNA involved in the DNA barcoding.

It is no coincidence that DNA coding was developed in conjunction with genomics-based research. DNA barcoding and genomics share one ultimate goal, namely the large-scale collection of genetic data, which offers new answers to questions that have so far been beyond the scope of traditional disciplines. A huge online barcode database serves as a standard to which the DNA barcode sequence of an unidentified sample can be assigned. Like genomics, DNA barcoding will make it possible to categorize known species and determine the taxonomic affiliation of species yet to be discovered in the wild. One of the purposes of DNA barcoding is to use information from one or more gene regions to taxonomically identify competent vectors that carry various diseases in animals and humans.

The "Global Bioidentification System", so called by Hebert et al., Is managed through the Barcode of Life Data Systems (BOLD) database, which contains more than 5,500,000 generated barcodes of over 265,000 species animals, plants and fungi. The BOLD system is a web-based work environment and database covering the storage, analysis and publication of DNA barcodes and can

serve as a reference library for DNA barcodes. BOLD is the most prominently used barcode software and is freely available to any researcher with interests in the field.

It is a taxonomic identification tool, alternative or complementary to morphology. DNA sequencing is a fast and relatively inexpensive technique. It can process a large number of specimens at once, which is useful, for example, in biodiversity studies. Once a reference database has been created, it can be applied even by nonspecialists.

The DNA barcoding involves two main stages:

- Collection of a database of barcodes in taxonomically known species to serve as a reference.
- Comparison of reference DNA barcodes of species from a database with data that are unidentified or unknown species of animals, plants and other organisms.

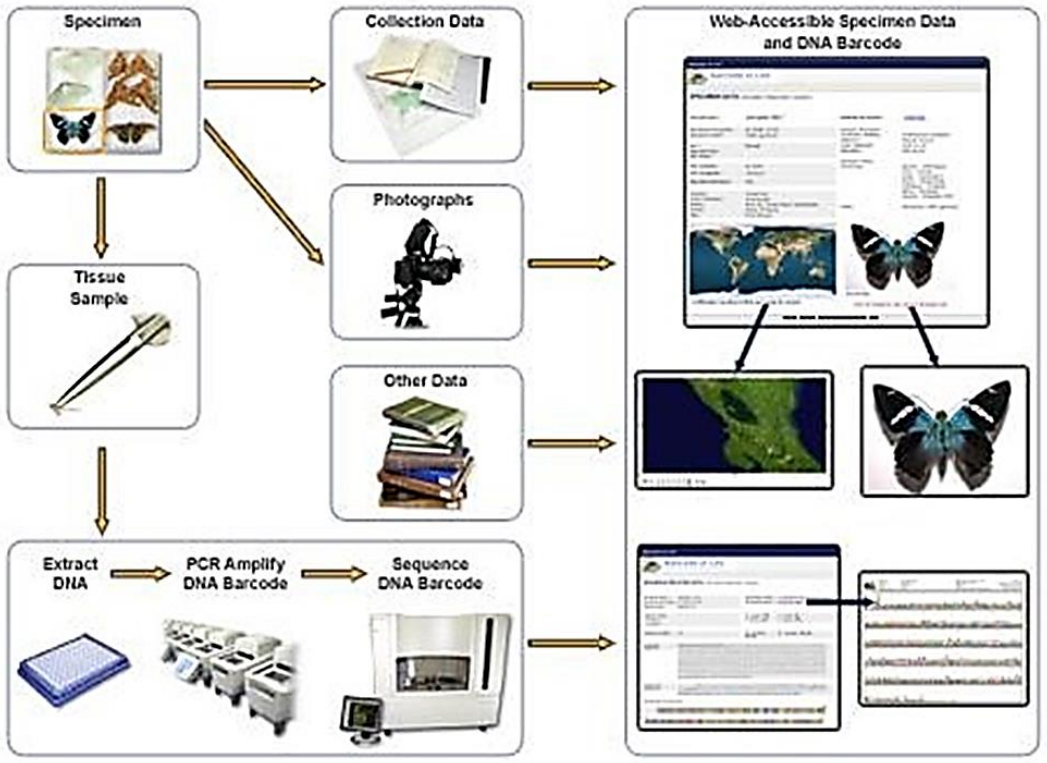

Figure 2: Step by step process of barcode generation.

The first stage includes taxonomic expertise in the selection of several individuals of the species, serving as reference samples in the DNA barcode database. These samples can be taken from herbariums and museum exhibits, as well as from live animals and plants in the field. Once the reference library for DNA barcodes has been prepared for the studied organisms, whether information on the geographical region from which they were collected, taxonomic group or target group (eg culicuides, mosquitos, sandflies etc.) is included, then DNA barcodes generated for the unidentified test samples are compared with the reference barcodes using a matching algorithm. Most algorithms involve comparing two DNA sequences to measure the distance between

sequences. In DNA barcoding, an algorithm is usually used to find the closest to the unknown sequence in the database. BLAST (Basic local alignment search tool) is a nucleotide or peptide sequence matching tool provided by the GenBank database for matching matches between two sequences, in this case two DNA barcodes. Two other commonly used algorithms are the Kimura-2-Parameter Distance and the Smith-Waterman Algorithm, similar to BLAST.

1. Sampling. The capture of the main vectors is done by setting traps. It is good to start betting before sunset, and the catches to be collected after sunrise the next day. Culicoides can be captured using several methods. The adults are most easily captured using various light-trap models (these vary in power, in the use of different colours, with or without an additional attractant), truck traps, aspirators, bait-traps and also emergence traps for the sampling of larval habitats. The most commonly used traps for culicuides are OVI traps. Adults are stored in sample containers filled with 70% ethanol to avoid contamination or damage to the collected samples. The catches are then stored at + 4 ° C until further processing.

Mosquito traps are set in the same way, a lot of different traps are availeble, Biogents Sentinel trap (BG trap), Heavy Duty Encephalitis Vector Survey trap (EVS trap), Centres for Disease Control miniature light trap (CDC trap) and Mosquito Magnet Patriot Mosquito trap (MM trap), accompanied by dry ice containers or light (the CO2 that is released when the ice melts and the lights serves as an attractants). Mosquitoes are caught in the net located at the bottom of the traps. The net is then transferred to a freezer at -20 °C. CDC, EVS traps and so-called sticky traps are used to catch sand flies.

2. Preparatory stage of the sample and process of lysis of the sample are very important. Catches must be sorted by morphological characteristics and species. The morphological determination of the competent vectors was standardly performed using taxonomic key, including the morphological characteristics of each taxonomic species of insects and observation of samples under a stereoscope. There are a lot of procedures and Standart operating procedures for cathes analisys (M. Goffredo& R. Meiswinkel) and taxonomic key for Mosquitoes (Becker et al., 2010) Culicuides (Mathieu et al., 2010); Sand fly samples should be sorted only to the genus level, as further identification by species requires additional dissection.

3. DNA extraction. Prior to extraction, pre-homogenization of the sample is required, after which various extraction kits are used following the protocols of the manufacturing companies (DNeasy Blood and Tissue Kit Qiagen) or following a previously documented extraction protocols (Kasap et al., 2013).

4. Measurement of the amount of DNA.

5. Polymerase chain reaction – amplification of the target region encoding cytochrome oxidase I (COI or COX1); The polymerase chain reaction (PCR) of the loci encoding mitochondrial cytochrome C oxidase I (COI) is very similar for most groups of animals. The main difference is the choice of specific primers for individual taxa. For example, for sand flies the universal LCO1490 and HCO2198 primers (Folmer et al., 1994) are used to amplify the ~650 bp barcoding region of the cox1 gene, using previously defined PCR reaction (Gunay et al., 2015). Numerous PCR protocols for the determination of these three types of vectors have been described in the literature (Hernández-Triana et al., 2012; 2014; 2017; Hebert et al., 2003 a, b).

6. Product purification;

7. Sequencing;

8. Data analisys. The resultant cox1 sequences have to be aligned using different programs for example the ClustalW Multiple Alignment algorithm, as implemented in the BioEdit v.7.2.5 (Hall 1999) alignment editor. Neighbor Joining (NJ) analysis on 1000 replicates under the assumptions of Kimura's two parameter (K2P) substitution model in MEGA v.6.0 (Tamura et al., 2013) in order to be obtain a robust taxon identity tree with bootstrapped branch support. The K2P distances generated through pairwise sequence comparisons using the default settings of P-values (0.1–0.001) in the Automatic Barcode Gap Discovery (ABGD) web-interface program (http://wwwab i.snv.jussieu.fr/public/abgd/) (Puillandre et al., 2012) is used to assign each barcode sequence into molecular operational taxonomic units (MOTUs). ABGD splits the sequences into groups through two subsequent partitioning. Using a user-defned range of prior intraspecifc distance divergence threshold values (P), this method frst infers a barcode gap and uses this gap value to partition the data into clusters. In the second step, these clusters are repeatedly partitioned into hypothetical species until no more splits take place (Puillandre et al., 2012). Species boundaries among closely related taxa within each subgenus in some cases have to be evaluated by Maximum Likelihood (ML) analysis in MEGA v.6.0, using only the unique representative haplotypes, as identifed by DnaSP v.5.10.01 (Librado et al., 2009).

The consortium responsible for the BOLD platform (CBOL) was launched in May 2004 and now includes more than 120 organizations from 45 countries. CBOL encourages the development of international research unions tasked with the difficult task of building an online barcode library for all eukaryotes over the next 20 years. The Animal Kingdom Barcode Library will be much larger, about 100 million records – almost twice the current size of GenBank (52 million recorded sequences as of March 7, 2006). The BOLD platform – www.barcodinglife.org – supports all phases of the analytical path from sample collection to a strictly validated library with generated barcodes.

The BOLD platform is written in Java, C ++ and PHP. It works with Linux and with all the data that is in the relational database PostgreSql (www.postgresql.org). The BOLD platform is available at: www.barcodinglife.org.

Conclusion

In this multidisciplinary context, molecular biological studies of vector pathogens play a key role in elucidating the epidemiology of infectious vector-borne diseases, the development of successful control methods. Phylogenetic analyzes of the main species of vector pathogens have helped to identify, categorize and taxonomically identify newly discovered species and subspecies.

Molecular genetics and newly developed tools for genomic and proteomic analysis are promising methods in understanding the genetics and capacity of vectors, including characterization: preferred habitats and hosts, innate immunity, drought resistance, insecticide resistance, and in the development of new species.

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