Species Determination of Brazilian Mammals Implicated in the Epidemiology of Rabies Based on the Control Region of Mitochondrial DNA

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Identification of animals that are decomposing or have been run over or burnt and cannot be visually identified is a problem in the surveillance and control of infectious diseases. Many of these animals are wild and represent a valuable source of information for epidemiologic research as they may be carriers of an infectious agent. This article discusses the results obtained using a method for identifying mammals genetically by sequencing their mitochondrial DNA control region. Fourteen species were analyzed and identified. These included the main reservoirs and transmitters of rabies virus, namely, canids, chiroptera and primates. The results prove that this method of genetic identification is both efficient and simple and that it can be used in the surveillance of infectious diseases which includes mammals in their epidemiologic cycle, such as rabies.

Key-Words: Mitochondrial DNA, sequencing, identification, mammals, rabies.

Different causative agents of infectious diseases are transmitted by wild or domestic mammals. The transmitting species are very often also wild reservoirs for the pathogen, and identification of these species is essential for an understanding of the epidemiologic cycles of the disease so that effective surveillance and control can be put in place [1]. Rabies is an example of a zoonosis in which the causative agent, the *Rabies virus*, can be transmitted by many species of mammals, some of which, such as chiroptera and wild canids, are wild reservoirs [2]. A large number of other zoonoses, such as hantavirus diseases, yellow fever and dengue, in which various mammals act as wild reservoirs for the etiologic agent, are also known [1].

As methods for controlling mammals that transmit infectious agents in urban centers are now well understood, efforts can be concentrated on developing effective and ecologically correct methods to handle and control wild vectors and reservoirs. If this objective is to be achieved, it is important to know which animals are involved in the disease and which are the terminal hosts.

For various reasons, including principles related both to ethics and animal conservation, obtaining wild animals is both difficult and expensive. However, many studies can be carried out with dead animals, particularly those that are run over on highways, and many researchers have taken advantage of this possibility.

In recent decades, great strides have been made in the use of molecular biology methods based on nucleic acids, and sequencing of mitochondrial DNA (mtDNA) continues to be used intensively for the genetic identification of species [3-5].

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Certain mtDNA genetic markers, such as the mtDNA control region (D-loop) and the cytochrome b gene, are the most frequently used. Many of the genetic sequences of these two areas of mtDNA can be found in public domain websites, such as GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez). Analyzing the mtDNA D-loop region and epidemiologic data, Carnieli et al. (2008)[6] found that the main wild canid involved in rabies in Northeast Brazil is the crab-eating fox (*Cerdocyon thous*). In the same study, the authors discussed the importance of analyzing mtDNA to identify wild mammals found run over or decomposing, as definitive and unequivocal visual identification of the animal is not possible in these situations.

The objective of this study was therefore to determine the sequence of the mtDNA control region in a number of animal species from different orders of mammals found decomposing or not and associated or not with infectious diseases, with a view to anticipating situations related to human and animal health in which determination of the species of the transmitter or host is essential for surveillance and control of a zoonosis. Fourteen species of mammals that had been sent to the Pasteur Institute (IP) for diagnosis of rabies between 2005 and 2007 were used to analyze the mtDNA D-loop region. Following are the species from which samples were obtained and their common name in English and Portuguese: *Bos taurus* (cattle = gado); *Equus caballus* (horse = cavalo); *Mus musculus domesticus* (mouse = camundongo); *Procyon cancrivorus* (raccoon = crab-eating raccoon = mão-pelada = guaxinim); *Eira barbara* (cabeza de Viejo = tayra = Irara = papa-mel); *Felis catus* (cat = gato); *Cebus albifrons* (capuchin monkey = white-fronted capuchin = macaco-prego = caiarara); *Callithrix jacchus* (common marmoset = sagüi = sagüi-de-tufos-brancos = mico-estrela-de-tufo-branco); *Cerdocyon thous* (*Dusicyon thous* = crab-eating fox = cachorro-do-mato = graxaim-do-mato); *Lycalopex vetulus* (*Pseudalopex vetulus* = *Dusicyon vetulus*, hoary-fox = raposinha-do-campo); *Canis lupus familiaris* (dog = cão), *Tamandua tetradactyla* (southern tamandua = lesser anteater = tamanduá-mirim); *Myotis nigricans* (little brown bat = black myotis = pequeno morcego

marrom) and *Artibeus lituratus* (great fruit-eating bat = morcego das frutas). All the samples used consisted of fragments of central nervous system (CNS). The common name of each animal is usually given on the shipping docket for the sample, and the animals are identified morphologically by the person sending the sample, who is often a professional in the field of biology. Apart from the chiroptera, the two wild canids and the *Eira barbara*, all of the wild animals had been run over and were therefore in varying stages of decomposition. The classification used for the mammals referred to in this study follows the standards laid down in Wilson & Reeder (2005) [7].

PCR, DNA Sequencing and Phylogenetic Analysis are the same used in Carnieli et al. (2008) [6]. Data from raw sequencing were edited using CHROMAS software (Version 2.24 Copyright© 1998-2004 Technelysium Pty Ltd), and the final sequences were submitted to BLAST/n [11] at www.ncbi.nlm.nih.gov for species identification.

A number of criteria were used to present the results obtained in this study. The sequence deposited in GenBank that had the greatest identity with each of the sequences for the species studied in this work and listed previously was chosen for comparison. The scientific name of the species studied is given first (numbered). This is followed by the scientific name of the species retrieved from GenBank using BLAST/n (preceded by the letters GB) that had the greatest identity with the sample studied, bibliographic reference, accession number, identities (in numbers and as a percentage), number of gaps (in numbers and as a percentage), and the query coverage (as a percentage). When further information is required, this can be found under "Comments".

1- *Cebus albifrons* – GB: *Cebus albifrons* [12], Accession Number: AJ309866, Identities = 555/632 (87.8%), Gaps = 7/632 (1.1%), Query coverage = 86.0%. Comments: Capuchin monkey is the generic name for the species *Cebus apella*, which has several subspecies. The genus Cebus currently has eight species, which were previously considered subspecies.

2- *Bos taurus* – GB: *Bos taurus* [13], Accession Number: AF517824, Identities = 562/566 (99.9%), Gaps = 2/566 (0.3%), Query coverage = 99.0%.

3- *Equus caballus* – GB: *Equus caballus* [14], Accession Number: X79547, Identities = 640/690 (92.7%), Gaps = 27/690 (3.9%), Query coverage = 99.0%.

4- *Mus musculus domesticus* – GB: *Mus musculus domesticus* [15], Accession Number: AM182717, Identities = 600/609 (98.5%), Gaps = 4/609 (0.6%), Query coverage = 100%.

5- *Procyon cancrivorus* – GB: *Procyon lotor* [16], Accession Number: AB291073, Identities = 405/460 (88.0%), Gaps = 6/460 (1.3%), Query coverage = 77.0%. Comments: there are no sequences in GenBank of mtDNA from *Procyon cancrivorus*, the species from the genus *Procyon* in Brazil.

6- *Eira barbara* – GB: *Mustela erminea* [17], Accession Number: AB049777, Identities = 478/528 (90.5%), Gaps = 3/528 (0.5%), Query coverage = 100%. Comments: there are 17 sequences of the *Mustelidae Eira barbara* in GenBank, although none of them contains the mtDNA D-loop.

7- *Felis catus* – GB: *Felis catus* [18], Accession Number: AF348642, Identities = 456/469 (97.2%), Gaps = 5/469 (1.0%), Query coverage = 97.0%.

8-*Tamandua tetradactyla* – GB: *Tamandua tetradactyla* [19], Accession Number: AJ421450, Identities = 452/466 (96.9%), Gaps = 10/466 (2.1%), Query coverage = 100%. Comments: two samples of *Tamandua tetradactyla* were sequenced, but as the results were practically the same, only one is shown in the Results section.

9- *Callithrix* – GB: *Callithrix jacchus* [20], Accession Number: AY196775, Identities = 181/188 (96.2%), Gaps = 2/188 (1.0%), Query coverage = 99.0%. Comments: *Callithrix jacchus* is found in two Brazilian biomes – the caatinga (a savannah-like habitat) and the Atlantic forest. It is typical of Northeast Brazil, but was introduced into and is now found in the states of Minas Gerais, São Paulo, Santa Catarina, Rio de Janeiro and Espírito Santo, all states in South and Southeast Brazil [21]. As the sample used in this study was from Southeast Brazil, we can only affirm that it was from the genus *Callithrix*.

10- *Cerdocyon thous* – GB: *Dusicyon thous* [10], Accession Number: EF107015, Identities = 509/525 (96.9%), Gaps = 12/525 (2.2%), Query coverage = 100%. Comments: the sequence analyzed in this study (EF194179) is one of the 28 sequences of *Dusicyon thous* included in Carnieli et al., 2008 [6].

11- *Lycalopex vetulus* – GB: *Lycalopex vetulus* [10], Accession Number: EF107032, Identities = 510/523 (97.5%), Gaps = 10/523 (1.9%), Query coverage = 100%. Comments: the sequence analyzed in this study (EF194201) is one of the two sequences of *Pseudalopex vetulus* included in Carnieli et al., 2008 [6].

12- *Canis lupus familiaris* – GB: *Canis lupus familiaris* [22], Accession Number: AF531666, Identities = 513/513 (100%), Gaps = 0/513 (0%), Query Coverage = 93.0%. Comments: the sequence analyzed in this study (EF194197) is one of the four sequences of *Canis lupus familiaris* included in Carnieli et al., 2008 [6].

13 – *Artibeus lituratus* – GB: *Artibeus jamaicensis* [23], Accession Number: NC002009, Identities = 460/477 (96.4%), Gaps = 6/477 (1.2%), Query Coverage = 100%. Comments: there are no mtDNA D-loop sequences from *A. lituratus* in GenBank.

14 - *Myotis nigricans* - *Myotis myotis* [24], Accession Number: U95332, Identities = 451/466 (96.7%), Gaps = 8/466 (1.7%), Query Coverage = 100%. Comments: there are no mtDNA D-loop sequences from *Myotis nigricans* in GenBank.

We also analyzed mtDNA from a mule, a hybrid animal that was the offspring of a mare (*Equus caballus*) and an ass (*Equus asinus*). As mtDNA is transmitted to the next generation by extrachromosomal inheritance and is therefore of maternal origin, the result of the analysis of the mtDNA from this hybrid is similar to those for *Equus caballus* described earlier.

The great diversity of animals currently found in the infraclass *Placentalia* (formerly *Eutheria*), in the class *Mammalia* [7], first started to develop approximately 53 million years ago during the Eocene epoch. However, orders such as *Primates*, *Chiroptera* and *Pilosa* (formerly *Edentata*), whether extinct or not, diverged from the remaining orders even before the beginning of this geological epoch [25]. Using the same pair of primers, we determined the mtDNA D-loop sequences of animal species from three of the four superorders in the infraclass *Placentalia*: *Xenarthra* (lesser anteater), *Euarchontoglires* (capuchin monkey, common marmoset and mouse) and *Laurasiatheria* (canids, cat, cattle, horse, raccoon, bats and tayra); the only superorder not represented here is the *Afrotheria*. As comparison of mtDNA identity is used extensively to determine the relationship between species in the animal kingdom, it is not unreasonable to expect that the results obtained could be extended to the other species of the three superorders of the class *Mammalia* studied here.

The methodology used in this study, together with epidemiologic data and traditional methods for identifying animals, can be used to solve a number of cases involving zoonoses and non-zoonotic infectious diseases. In addition, mtDNA is the only way of identifying an animal that is decomposing after being badly run over or burnt.

One problem associated with the samples sent to diagnostic laboratories involves the extent to which the identification of the animals can be relied upon. This identification is often done by technicians who have insufficient experience to identify wild species. The problem is further complicated because the name of a particular species may differ from region to region and its phenotype may vary; however, a comparative study of mtDNA can help solve this problem. The method is safe, speedy and inexpensive and can be readily implemented in molecular biology laboratories that perform DNA sequencing.

The results described here enabled us to achieve our objective, namely, to identify the species using molecular techniques. The results for *Eira barbara*, *Procyon cancrivorus* and the genus *Callithrix*, which were at first the subject of some doubt, can and should be considered correct. The reasons for the discrepancies in the results are clarified in the comments contained in the Results section. The results for the genera *Procyon* and *Callithrix* are in complete agreement with the zoogeography of these animals [7]. In the case of *Eira Barbara*, which is the only genus in the subfamily *Mustelinae*, the mtDNA D-loop region had 90% similarity with that of *Mustela erminea*, also in the subfamily *Mustelinae*.

The results for the bats *Artibeus lituratus* and *Myotis nigricans* were completely satisfactory and are explained in the Results section. Identification of the genera *Myotis* and *Artibeus* is important because of the epidemiology of rabies. These genera, together with *Lasiurus* and the species *Tadarida brasiliensis*, are genera and species of chiroptera that are of great epidemiologic importance for rabies in South America [2].

It should be stressed that, according to the same authors, the strategies advocated in Latin America by the Pan American Health Organization and adopted by the Department of Health in Brazil led to a change in the epidemiologic profile of rabies in this country, namely, a reduction in canine and human rabies transmitted by dogs.

Currently, the *Rabies virus* in Brazil is maintained in populations of wild canids, especially the species *Cerdocyon thous*, 36 species of bats with distinct feeding habits and, in some limited regions of Northeast Brazil, common marmosets (*Callithrix jacchus*).

The main objective of this study was to identify those species of mammals that can transmit the *Rabies virus* in Brazil. This objective was completely achieved, as the two orders that include the main wild reservoirs of the virus – chiroptera and canids – were analyzed and identified, as were animals from the genus *Callithrix*.

Although rabies has not been identified to date in *Tamandua tetradactyla*, we analyzed mtDNA from this species because it was the only species from the superorder *Xenarthra* and, in addition to providing an opportunity to analyze genetic material from this species threatened with extinction, it allowed us to evaluate the primers MTL-PRO2 and CCR-DR1 [6] more thoroughly.

The method described is neither suitable for, nor intended to be used for, replacing formally recognized methods for species identification or even for determining the relationship between individuals and species. Researchers proposing to carry out a similar analysis should bear this in mind and should also retain a critical sense in relation to zoogeography.

The method for species identification tested in this study is useful for diagnostic laboratories in which the source of infection must be determined, both to investigate the case and to establish control measures.

Decomposition is a limiting factor when identifying the species of a small animal and can at times render identification impossible. Small mammals, such as bats and common marmosets, can potentially transmit the *Rabies virus*. These animals are very often found dead and decomposing but even so are sent for diagnosis.

Public health laboratories, such as the Pasteur Institute, where this study was planned and carried out, must anticipate any and every kind of problem related to a disease so that

epidemiologic surveillance and control measures can be put in place. The results of this study have shown that identification of mammals by analysis of the mtDNA D-loop region using the primers MTLPRO2 and CCR-DR1 is feasible, indicating that this technique is suitable for use in epidemiologic surveillance.

In conclusion, the continued development of techniques to identify pathogenic microorganisms in poor-quality samples or samples that are in an advanced stage of decomposition, as described in Oliveira et al., 2006 [26], allows the host species to be associated with the agent of an infection, while the present study allows host mammals to be identified and associated with the pathogen.

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References

1. Brasil. SVS/MS - Ministério da Saúde. Secretaria de Vigilância em Saúde. Doenças infecciosas e parasitárias: guia de bolso. Ministério da Saúde, Secretaria de Vigilância em Saúde. 5ª ed. ampliada. Brasília: Ministério da Saúde, **2005**. 320 p. – (Série B. Textos Básicos de Saúde). Disponível em URL: http://portal.saude.gov.br/portal/saude.
2. Kotait I., Carrieri M.L., Carnieli P. Jr., et al. Reservatórios silvestres do vírus da raiva: um desafio para a saúde pública. BEPA **2007**; 40: 04-10. Disponível em URL: http://www.cve.saude.sp.gov.br/agencia/bepa40_raiva.htm.
3. Farrell L.E., Roman J., Sunquist M.E. Dietary separation of sympatric carnivores identified by molecular analysis of scats. Mol Ecol **2000**;9(10):1583-90.
4. Vilà C., Sundqvist A.K., Flagstad Ø., et al. Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant. Proc R Soc Lond B **2003**;270:91-7.
5. Dragoo J.W., Matthes D.K., Aragon A., et al. Identification of Skunk Species Submitted for Rabies Testing in the Desert Southwest. J Wildl Dis **2004**;40(2):371-6.
6. Carnieli P.Jr., Fahl W.O., Castilho J.G., et al. Characterization of *Rabies virus* isolated from canids and identification of the main wild canid host in Northeastern Brazil. Virus Res **2008**;131(1):33-46.
7. Wilson D.E., Reeder D.M. Mammal Species of the World. Johns Hopkins University Press, **2005**. 142 pp. (Available from Johns Hopkins University Press, 1-800-537-5487 or (410) 516-6900. Disponível em URL: http://www.press.jhu.edu/.
8. Ward R.H., Frazier B.L., Dew-Jager K., Pääbo S. Extensive mitochondrial diversity within a single 875 Amerindian tribe. Proc Nat Acad Sci **1991**;88:8720-4.
9. Sambrook J., Fritsch E.F., Maniatis T. Molecular cloning: a Laboratory Manual, 2nd edition, New York: Cold Spring Harbor Laboratory, **1989**.
10. Tchaicka L., Eizirik E., Oliveira T.G., et al. Phylogeography and population history of the crab-eating fox (*Cerdocyon thous*). Mol Ecol **2007**;16:819-38.
11. Altschul S.F., Madden T.L., Schäffer A.A., et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **1997**;25:3389-402.
12. Arnason U., Gullberg A., Burguete A.S., Janke A. Molecular estimates of primate divergences and new hypotheses for primate dispersal and the origin of modern humans. Hereditas **2000**;133(3):217-8.
13. Miretti M.M., Pereira H.A.Jr., Poli M.A., Contel E.P., Ferro J.A. African-Derived Mitochondria in South American Native Cattle Breeds *Bos taurus*: Evidence of a New Taurine Mitochondrial. J Hered **2002**;93(5):323-30.
14. Nilsson M.A., Arnason U., Spencer P.B., Janke A. Marsupial relationships and a timeline for marsupial radiation in South Gondwana. Gene **2004**;340(2):189-96.
15. Ihle S., Ravaoarimanana I., Thomas M., Tautz D. An analysis of signatures of selective sweeps in natural populations of the house mouse. Mol Biol Evol **2006**;23(4):790-7.
16. Yonezawa T., Nikaido M., Kohno N., et al. Molecular phylogenetic study on the origin and evolution of *Mustelidae*. Gene **2007**;396(1):1-12.
17. Kurose N., Abramov A.V., Masuda R. Comparative phylogeography between the *Mustela erminea* and the least weasel *M. nivalis* of Palaearctic and Nearctic regions, based on analysis of mitochondrial DNA control region. Zool Sci **2005**;22(10):1069-78.
18. Freeman A.R., MacHugh D.E., McKeown S., et al. Sequence variation in the mitochondrial DNA control region of wild African cheetahs (*Acinonyx jubatus*). Heredity **2001**;86(Pt 3):355-62.
19. Arnason U., Adegoke J.A., Bodin K., et al. Mammalian mitogenomic relationships and the root of the eutherian tree. Proc Natl Acad Sci U.S.A. **2002**;99(12):8151-6.
20. Faulkes C.G., Arruda M.F., Monteiro da Cruz M.A. Matrilinial genetic structure within and among populations of the cooperatively breeding common marmoset, *Callithrix jacchus*. Mol Ecol **2003**;12(4):1101-8.
21. IBAMA - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. Ministério do Meio Ambiente. [citado em 06 de fevereiro de **2008**]. Disponível em URL: http://www.ibama.gov.br/cpb.
22. Savolainen P., Zhang Y.P., Luo J., et al. Genetic evidence for an East Asian Origin of Domestic Dogs. Science **2002**;298(5598);1610-3.
23. Pumo D.E., Finamore P.S., Franek W.R., et al. Complete mitochondrial genome of a neotropical fruit bat, *Artibeus jamaicensis*, and a new hypothesis of the relationships of bats to other eutherian mammals. J Mol Evol **1998**;47(6):709-17.
24. Wilkinson G.S., Mayer F., Kerth G., Petri B. Evolution of repeated sequence arrays in the D-loop region of bat mitochondrial DNA. Genetics **1997**;146(3):1035-48.
25. Bininda-Emonds O.R., Cardillo M., Jones K.E., et al. The delayed rise of present-day mammals. Nature **2007**;29:446(7135):507-12.
26. Oliveira R., Takaoka N., Brandao P., et al. Postmortem confirmation of human rabies source. Emerg Infect Dis **2006**;12(5):867-9.