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Experiments on intramuscular inoculation and feeding domestic cats (*Felis catus*) with brains of mice previously infected by rabies viruses

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Abstract

Nineteen kittens divided into four groups were fed with brains of mice infected with rabies viruses. Each four kittens (group I) received four brains infected with the PV fixed strain; nine kittens (group II) ingested 4-5 brains infected with the field isolate T-9/95, isolated from the *Desmodus rotundus* vampire bat; two kittens (group III) fed ten T-9/95-infected brains, and four cats consumed 32-37 PV strain-infected brains. One adult male, inoculated into masseter muscle with a 20% T-9/95-infected brain suspension, presented rabies after an incubation period of six days, followed with 8 days of clinical evolution, and died thereafter and this cat was considered as the rabies "positive standard". After observing for 20-230 days, all the cats feeding the rabid brains were submitted to euthanasia, by using Acepran®, Zoletil®, and T-61®. At necropsy, samples of brain, heart, lung, kidney, submaxillary salivary gland, and cervical medulla were collected from all the cats and further submitted to the direct fluorescence antibody test (dFA), mouse inoculation test (MIT) and to the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Brain, cervical medulla, and the submaxillary salivary gland of the positive standard cat were dFA-positive, and brain and cervical medulla were positive for MIT. All specimens of this cat tested by the RT-PCR were found positive. No animals ingesting PV or T-9/95 virus-infected brains developed clinical signs and all materials tested were negative by dFA and MIT. Several specimens, however, showed positive reactions by the RT-PCR technique, but cats were resistant to rabies through the viruses administered orally.

Key words:
Rabies virus.
Cats.
Inoculation.
Feeding.
Diagnosis.

Introduction

Domestic cats (*Felis catus*) are occasionally affected by rabies. The disease in this species occurs as a result of "spillover" infections from underlying foci in domestic dogs and wildlife. In areas where dog rabies has been controlled, but wildlife rabies is still rampant, the cat may be the most commonly reported domestic species rabid, causing most of the human exposures.[1] In countries where mass dog rabies vaccination campaigns are effective, dogs do not act as the main reservoirs in the rabies transmission cycle.[2] However, due to higher human population density in heavy urbanized areas and consequent to less available individual spaces, cats are increasing in the preference as a companion animal.[3]

Cats are more independent than dogs and their survival in nature has increased the number of the ownerless cats, i.e., cats readapted to wildlife, the so-called feral cats [4] and concerned to some zoonoses like toxoplasmosis and rabies, those cats can act as the potential source of infection to other

domestic animals and humans.[5,6,7] Although the exact number of feral cats is not known in the USA, Levy, Gale and Gale[8] estimated that there were almost 73 million feral cats in the USA. Another study, also made in the USA, indicated that there were approximately 30 million feral cats in 1970's, and in 1990's, the population had increased to 60 million. This population of feral cats survived by eating human leftovers or by hunting birds, small mammals, and other animals like fish, lizards, insects and bats.[9]

Cats are conspicuous predators of bats and the ingestion of a rabid carcass might be a potential risk of transmission[10], however, the natural transmission of rabies virus through the oral route has been considered very unusual.[11] Bell and Moore [12] failed to infect cats by the oral route when administered a field rabies virus isolated from a bat, but they succeeded in infecting skunks. Negative results were obtained after feeding experimentally infected mice to susceptible dogs and foxes, although later it was possible to infect these animals through parenteral routes. There are no reports in the medical literature of human infection through the alimentary tract, and this is not possible unless there are open abrasions of the gastro-intestinal mucosa.[13]

Transmission experiments through oral route in dogs, foxes, sheep and horses did not show any successful results.[14] Oral transmission in mice and rats, however, was reported by Remlinger[15] and Correa-Giron, Allen and Sulkin[16] demonstrated that rabies virus could be transmitted in mice after feeding rabid brains and concluded that the infection initiated through the cells of buccal and lingual mucosa, lungs and small intestine.

The question involving the transmission of rabies virus by means of cannibalism on animals dead of rabies was presented by Johnson[17] and Soave[18], but for Fishman and Ward III[11], there is a lack of scientifically proved data and the subject is purely speculative, and before considering the oral transmission as an important via of transmission in the epidemiology of rabies, more experiments would be repeated by using field isolates with different viral challenge doses. As oral infection is reproducible in laboratory animals, there is a necessity to study if this route of transmission occurs also in domestic and wildlife animals, by using field isolates. Rabies isolates of vampire bats were found to be viscerotropic and the presence of the virus was described in several non-nervous tissues, and usually the virus is found in low titers.[19,20]

The exact site of primary replication and the way run by the ERA or SAD-type attenuated vaccinal rabies virus administered orally in form of bait is not known.[21] The attenuated virus first infect the cells of buccal and lingual mucosa, but its dynamics is still unclear.[22]

Transmission experiments of rabies in cats are scarce and due to their behavior as the extraordinary predator, risks in acquiring the virus from other wild reservoirs exist, through fights or ingesting rabid carcasses.[10] In regions where the population of feral cats is increasing, the possibility of cats acquiring rabies virus from infected bats is always considered. In 2000, rabies in a 52 year old woman in Dracena, a municipality 647 Km distant from the São Paulo city, Southern Brazil, was linked to a family-owned cat that used to hunt bats.[23]

The aim of this work was to study rabies in cats, simulating a condition of "predator-pray", by feeding the cats once or several times with rabid brains of mice which had previously been inoculated with rabies virus. Cats ingesting rabid brains of mice were compared to a cat inoculated intramuscularly and the viral viscerotropism was assessed by using the direct fluorescent antibody test (dFA), mouse inoculation test (MIT), and the reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

Material and Method

Cats

Twenty kittens, at ages varying from 30 to 80 days old, without any defined breed, divided into four groups and separated in

males and females were maintained in individual wire cages. A week before initiating the experiment, the cats received an antihelminthic and antibiotic treatment. During all the experiment, the cats were fed by using a commercial feed and water at *ad libitum* intake. The use of the animals was authorized by the Bioethics Commission of the Faculty of Veterinary Medicine and Zootechny of the University of São Paulo, on April 17th 2002, under the protocol number 112/2002.

Mice

Swiss albino mice of the breed CH-3 – Rockefeller, for the isolation and titration of rabies virus, were provided by the animal facility of the Department of the Preventive Veterinary Medicine and Animal Health of the Faculty of Veterinary Medicine and Zootechny of the University of São Paulo, São Paulo, Southern Brazil. Mice were divided into males and females, in groups of 10 animals each, weighing 14-17 grams. The original colony was provided from the now extinct Foot and Mouth Disease Laboratory of the Brazilian Ministry of Agriculture, Barretos-SP, in 1970's. The use of mice was approved by the Bioethics Commission of the FMVZ-USP, protocol number 112/2002.

Rabies viruses

Pasteur fixed virus (PV strain) was provided from the Butantan Institute, São Paulo-SP. This strain was maintained in liquid Nitrogen and after thawing; it was passed seven times in mice through intracerebral inoculation, and stored again at –20ºC. The virus was passed twice in mice before administrating orally to the cats. This strain was genetically characterized at the College of Bioresource Sciences of the Nihon University, Fujisawa, Kanagawa, Japan and showed 100% identity to that available in GenBank, PV-Brazil (M13215).

Field isolate T-9/95, from a vampire bat *Desmodus rotundus*, passed once in mice through intracerebral inoculation and stored at –20ºC. It was reactivated twice in mice and titrated in mice before administration to the cats. The isolate was genetically characterized at the College of Bioresource Sciences, Japan, and it belonged to the genotype 1 of rabies virus, a variant commonly found in *D. rotundus* bat in Brazil.

Challenge Virus Standard (CVS /31/ 2) fixed strain was used for the absorption of the direct fluorescent antibody (dFA) conjugate.

Rabies conjugate

Rabies hyperimmune serum was prepared in hamsters, according to the procedures described by the Centro Panamericano de Zoonoses[24], with little modification, i.e., the antigen used was substituted by a commercial rabies vaccine. After purification, the IgG fraction of the rabies immunoglobulin was conjugated to fluorescein isothiocyanate (Fluorescein isothiocyanate, isomer I – Sigma Chemical Company) and the final working dilution was established to 1:100.

Drugs

Endal Gatos® (Praziquantel and Pyrantel pamoate, Schering-Plough • Avenida Sir Henry Wellcome, 335 - • Moinho Velho - Cotia – SP) was used to control the intestinal worms, at an oral dose of 2 pills for each 5kg of body weight) and Synulox® (amoxicillin and clavulanic acid, Pfizer Saúde Animal, Guarulhos-SP) (10mg/kg of body weight, oral intake twice a day), for treatment against rhinotracheitis. Acepromazine (Acepran® Univet S. A. Indústria Veterinária) and tiletamine-zolazepam (Zoletil® Virbac do Brasil Ind. e Comércio) were used for sedation of animals, at a dose of 0.1mg/kg and 10mg/kg by intramuscular injection, as indicated by the manufacturer. Association of embutamide, mebezone and tetracain (T61® Intervet S.A.) was used for euthanasia of the cats at a dose of 0.1 mg/kg/ intravenously, according to the instruction of the manufacturer.

Mouse Inoculation Test (MIT)

This test was performed in young

adult mice, with inoculation of 0.03mL of 20% (weight/volume) suspensions prepared from several specimens of cats and mice (for viral titration). The procedure of the test was according to that described by Koprowski [25].

Direct fluorescent antibody test (dFA).

Impression smears were taken in duplicate from the fragments of brain, lung, heart, kidney, cervical medulla, and submaxillary salivary gland. The procedure used was according to that described by Dean, Abelseth and Atanasiu[26]. The smears were examined using the Olympus trinocular microscope, model BX60F5.

Virus titration

After the results of MIT using 10 mice per viral dilution, the titers were calculated according to Reed and Müench[27] and expressed in $\log_{10}ICLD_{50}$/0.03mL, using a confidence interval of á = 0.05and Z = 1.96, according to Pizzi[28].

RT-PCR and hemi-nested PCR

For amplification of the coding region of the nucleoprotein N of rabies virus, the primers used were: sense primer 505 (5'ATAGAGCAGATTTTCGAGACAGC3'), anti-sense primer 779 (5'CCTCAAAGTTCTTGTGGAAGA3'), and anti-sense primer 937 (5'CCCATATAACATCCAACAAAGTG3'). RT-PCR tests were performed with the PTC-200® Peltier Thermal Cycler (MJ Research). For the execution of hemi-nested PCR technique, the primer used for re-amplification was the 779, and performed in distinct laboratory to avoid contamination and the procedures were described by Soares et al.[29].

Procedures

Rabies "Positive Standard" Cat

After sedating one adult male cat with Acepran® and Zoletil®, a 20% brain suspension of T-9/95 isolate in a volume of 0.3mL was inoculated into masseter muscle and thereafter observed for manifestation of rabies signs. After the death, samples were collected from the heart, brain, lung, kidney, and cervical medulla and submaxillary salivary gland. These materials were first submitted to dFA and MIT, and afterwards to hemi-nested RT-PCR. This cat was named as the rabies "positive standard".

"Experimental Groups"

Following a 12 hour restriction of food intake, 19 kittens were divided into four groups (I-IV), respectively constituted by 4, 9, 2, and 4 individuals, and they were fed with different quantity of brains of rabid mice, which had been previously inoculated either with the fixed PV or T-9/95 field isolate of rabies virus. Each kitten of the group I were fed four brains of mice inoculated with PV strain; each cat of the group II received 4 to 5 brains of mice inoculated with T-9/95 isolate; kittens of the group III received 10 brains of mice inoculated with the T-9/95 isolate; and cats of the group IV were fed repeatedly in different times, a total of 32 to 37 brains inoculated with PV strain. At the time of ingestion of rabid brains, the age of the kittens of the group I were in a range of 30-35 days, group II varying from 45-60 days, group III estimated to 45 days, and group IV varying from 35-80 days old in age. The rabid brains were given to the cats in different occasions and after the end of observation period, all surviving animals were submitted to euthanasia using Acepran®, Zoletil®, and T-61® and brain and other non-nervous tissue fragments were collected, as described previously. After submitting to dFA and MIT in parallel, all the specimens were further examined by hemi-nested RT-PCR.

Results

The titrations of the rabid brains, performed on the same day of the feeding of the cats, indicated a titer of $5x10^{4.2\pm0.6}ICLD_{50}$/0.03mL for the T-9/95

Table 1 – Distribution of groups of kittens according to the feeding of rabid brains of mice either inoculated with a fixed PV strain or T-9/95 isolate; approximate age at the time of ingestion, sex, times and number of brains ingested, period of observation and results of positive specimens examined by hemi-nested RT-PCR. São Paulo - Brazil, 2003

Groups	Animal No. And Sex	Age at first feeding in days/ No. times fed	Virus/ (No. brains fed)	Observation period (in days)	Specimens positive by hemi-nested RT-PCR[1]
I	1♂	30/1	PV strain/(4)	20	Lung
	2♀	30/1	PV strain/(4)	28[2]	Heart
	3♀	30/1	PV strain/(4)	44	Heart
	4♂	45/1	PV strain/(4)	75	Lung and heart
II	5♀	45/1	T-9/95/(5)	62	Kidney
	6♀	45/1	T-9/95/(4)	100	Brain, lung and heart
	7♂	45/1	T-9/95/(5)	99	Lung and kidney
	8♂	45/1	T-9/95/(5)	99	-
	9♀	45/1	T-9/95/(5)	99	Kidney
	10♀	60/1	T-9/95/(4)	87	Brain and lung
	11♂	60/1	T-9/95/(4)	87	Kidney and heart
	12♀	60/1	T-9/95/(4)	87	Brain, lung and kidney
	13♀	70/1	T-9/95/(4)	87	Brain
III	14♂	45/1	T-9/95/(10)	88	Lung, kidney and heart
	15♂	45/1	T-9/95/(10)	88	-
IV	16♀	35/4	PV strain/(37)[4]	230	Lung, kidney and heart
	17♀	75/3	PV strain/(32)[3]	94	Lung and kidney
	18♀	80/3	PV strain/(32)[3]	94	Kidney
	19♀	75/3	PV strain/(32)[3]	94	Kidney and heart

[1]Specimens submitted for RT-PCR examination: brain; lung, kidney and heart; only hemi-nested RT-PCR-positive specimens are indicated, [2]Natural death due to rhinotracheitis, [3]Fed in three occasions with 9, 8, and 15 rabid brains at the 1rst, 29th, and 62nd day from the first day of observation, [4]Fed in four occasions with 4, 10, 8, and 15 rabid brains at the 1st, 84th, 165th, and 198th day from the first day of observation, - = All specimens tested, with hemi-nested RT-PCR negative results.

isolate, and for the PV fixed strain, $5x10^{5.5\pm0.7}ICLD_{50}$/0.03mL. The male cat inoculated through intramuscular route with T-9/95 isolate received an infective dose of $10^{5.2\pm0.6}$ $ICLD_{50}$/0.03mL and died of rabies with symptoms and signs characteristic of rabies, without showing any aggressiveness.

The prodromal phase was observed on the 6th post inoculation day with subtle change of temperament, hypophagia, hyponuria, and decreased movement. As the progression of the clinical course, the cat showed dilation of pupils, sluggish corneal reflex, restlessness and hyperesthesia. Increase in vocalization, abundant salivation, dyspnea, ocular secretion, spasms and paralysis were observed with the progression of the disease and death occurred at the 15th post inoculation day.

Among the materials submitted to MIT, brain and cervical medulla were found positive and by the dFA examination, fragments of brain, cervical medulla, and submaxillary salivary gland were positive. And all specimens submitted to RT-PCR were also positive.

Kittens of the group I, which ingested four PV strain-infected brains, were administered viral dose of $10^{6.8\pm0.7}ICLD_{50}$/ 0.03mL and they were submitted to euthanasia at 20th, 44th and 75th post inoculation days, except one cat which died of rhinotracheitis at 28th day of observation. Cats of the group II, which ingested four brains, infected with T-9/95 isolate received a viral dose of $10^{5.5\pm0.6}ICLD_{50}$/0.03mL and those ingesting five brains received $10^{5.6\pm0.6}ICLD_{50}$/0.03mL. The cats were observed for varying period of 62-100 post inoculation days and after euthanasia, all carcasses were submitted for necropsies. Similarly, cats of the group III received a dose of $10^{5.9\pm0.6}ICLD_{50}$/0.03mL and were observed for 88 days, and then submitted to euthanasia. Cats of the group IV were fed with PV strain-inoculated brains, with

viral dose of $10^{7.7\pm0.7}ICLD_{50}$/0.03mL, and observed for varying period of 94-230 days and all cats were sacrificed. None of the examined specimens collected from cats feeding on rabid brains proved to be positive for rabies by means of dFA and MIT. Several specimens of cats feeding on rabid brains inoculated either with PV strain or T-9/95 field isolate of rabies virus presented hemi-nested RT-PCR positive results as indicated in table 1.

Discussion and Conclusion

During the period of observation, none of the cats feeding the brains of mice experimentally inoculated either with a fixed PV virus or an isolate T-9/95 of rabies virus developed signs of the disease similar to those presented by the positive standard cat. This fact corroborates the results of Bell and Moore[12], i.e.; cats are resistant to rabies virus given by the oral route. However, the intramuscular inoculation of the isolate T-9/95 reproduced promptly the disease, after an incubation period of 6 days and the cat died at the 15th post inoculation day. After inoculating cats through intramuscular route with a field isolate of an insectivorous bat, Trimarchi, Rudd and Abelseth [30] reported the recovery of virus from the brain, salivary gland, and urinary bladder. The urinary bladder was the only one non-nervous specimen that showed positive result by the dFA examination. In our experiment, brain, cervical medulla and submaxillary salivary gland of the positive standard cat were found with dFA-positive results and only brain and cervical medulla were positive for the virus isolation. The salivary gland of the positive standard cat was positive by the dFA, and this result can be interpreted as an evidence of viscerotropic capacity of the isolate, although being negative by the MIT. In respect to the results of the hemi-nested RT-PCR, the test indicated 100% positivity for all the specimens examined.

The oral administration of the PV strain-infected brains to the cats did not reproduce the disease, although the same PV strain used in this experiment was described to be pathogenic in mice and hamsters through oral route and highly viscerotropic.[31] Similarly, the T-9/95 isolate, obtained from a *D. rotundus* vampire bat from the State of São Paulo did not provoke rabies in cat. This isolate showed genetic identity >89%, when compared to other field rabies isolates from *D. rotundus* bats from Brazil. [32]

The specimens collected from cats feeding on the rabid brains inoculated either with the PV strain or T-9/95 field isolate also were found with high frequencies of positive results through RT-PCR examination. The RT-PCR is a very sensitive test and it can detect fragments of virus circulating inside an organism, but this does not mean obligatorily that the infection was in course (Rupprecht, C. E. Information provided during the Virológica Congress, held in Águas de Lindóia – SP, Brazil, 2002). In our experiment, RT-PCR-positive results were found in dFA and MIT-negative materials. The results are intriguing and difficult to interpret; these results could be related to the detection of fragments of RNA circulating in the body before the "clearance" of the administered virus takes place. In this work, the amplification procedure and the hemi-nested RT-PCR were performed in two distinct rooms and the results were checked twice or more times, depending on the specimens, especially those collected from cats feeding the PV strain. Although RT-PCR positive results were found in clinically healthy cats, we have to be conservative in interpreting these findings and it is worthy to recall that rabies specialists collaborating to World Health Organization maintain the recommendation against the use of RT-PCR technique for routine post mortem diagnosis of rabies.[33]

Soulebot et al.[34] reported that cats are less susceptible than dogs in acquiring rabies through intramuscular inoculation of the virus. Murphy et al. [35] reported the occurrence of "chronic rabies" in two cats, inoculated with a street rabies virus strain, and survived with only some progressive debility and atrophy of musculature in the injected limb

for 136 weeks. Virus was isolated from two brain specimens of one cat obtained at necropsy; isolation was successful only by explant culture and inoculation of explanted tissue into mice. Virus antigen was detected in eight sites in the brain and spinal cord of the same cat by frozen-section immunofluorescence. These experimental cases of chronic progressive rabies resembled more closely subacute sclerosing panecephalitis of man than the usual subacute fatal rabies encephalitis of man and other mammalian species.

In this experiment, however, the viral dose of the T-9/95 isolate used to inoculate the male cat was somewhat excessive and was sufficient enough to reproduce rabies and to cause death in only 15 post inoculation days.

The PV fixed strain or the T-9/95 rabies field isolate administered repeatedly at high viral doses through oral route could not promptly reproduce rabies in cats at least during the observed time interval and the results found in this experiment corroborate previous reports made by other researchers [11,12], i.e., cats are resistant to rabies through ingestion of rabid tissues. And the epidemiology of rabies involving domestic cat as a reservoir still deserves more investigation.

Ensaios sobre inoculação intramuscular e alimentação de gatos domésticos (*Felis catus*) com cérebros de camundongos préviamente inoculados com vírus da raiva

Resumo

Dezenove gatos, divididos em quatro grupos, foram alimentados com cérebros de camundongos infectados com vírus de raiva. Cada um dos quatro gatos (grupo I) receberam quatro cérebros infectados com vírus fixo PV; nove gatos (grupo II) ingeriram 4-5 cérebros infectados com uma amostra de campo T-9/95, isolada do morcego *Desmodus rotundus*; dois gatos (grupo III) ingeriram 10 cérebros infectados com T-9/95 e quatro gatos (grupo IV) ingeriram 32-37 cérebros infectados com vírus PV. Um macho adulto, inoculado no músculo masséter, com uma suspensão cerebral a 20% da amostra T-9/95, desenvolveu raiva após período de incubação de seis dias, seguidos por oito dias de evolução clínica, morrendo em seguida. Este gato foi denominado de "padrão positivo". Após observação por um período de 20-230 dias, todos os gatos que receberam cérebros foram submetidos à eutanásia, utilizando Acepran®, Zoletil® e T-61®. À necropsia, foram colhidas amostras do cérebro, coração, pulmão, rim, glândula salivar submaxilar e medula cervical e submetidas à prova de imunofluorescência direta (IFD), inoculação em camundongos (IC), e reação em cadeia pela polimerase-transcriptase reversa (RT-PCR). No "padrão positivo", cérebro, medula cervical e glândula salivar foram positivos à IFD e à IC, cérebro e medula cervical foram os positivos. Todos os espécimes do "padrão positivo" foram positivos à RT-PCR. Nenhum animal que ingeriu cérebros contendo amostras de vírus PV ou T-9/95 apresentou sinais clínicos e todos os espécimes testados foram negativos à IFD e IC, no entanto, alguns espécimes reagiram positivamente à RT-PCR, porém, os gatos foram resistentes à raiva com vírus administrados oralmente.

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Palavras chave:
Vírus da raiva.
Gatos.
Inoculação.
Ingestão.
Diagnóstico.

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