Cervical medulla as laboratory diagnosis material for Rabies

Medula cervical como material para diagnóstico laboratorial da Raiva

RIALA6/1201

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Recebido: 30.07.2008 – Aceito para publicação: 30.04.2009

ABSTRACT

Rabies is a contagious, neurotropic zoonosis associated with abandoned street dogs and low immunity. The disease has a reduced laboratory diagnosis rate because it is difficult to gather and transport sample material (brain). Based on this challenge, we studied the cervical medulla (CNS) as the pathway of the Rabies virus from the body to the brain. The cervical medulla was an ideal candidate for our study because its anatomy and location make it an easy material to gather. Our objective was to analyse the use of cervical medulla in the laboratory diagnosis of Rabies. Rabies viruses were intramuscularly inoculated into five *Rattus* species. After death, the brain and cervical medulla of each animal were intra-cerebrally macerated and inoculated. Five *Rattus* species were used in the study (a total of twenty-five brains). Twenty-five of the medullas were 100% positive for Rabies using the direct immunofluorescence (DIF) test and intracerebral inoculation. Overall, there was agreement between the analyses of the brains and the cervical medullas. Therefore, we propose the use of cervical medulla as a material for the laboratory diagnosis of Rabies.

Key words. rabies, diagnosis, laboratory, cervical medulla.

RESUMO

A Raiva é uma zoonose neurotrópica infecto-contagiosa, cuja persistência está associada: aos cães abandonados nas ruas, baixa imunidade, reduzida procura pelo diagnóstico laboratorial devido a difícil coleta e transporte do material (cérebro). Portanto, justifica-se o estudo da medula cervical (SNC), por ser via obrigatória dos vírus rábicos para o cérebro e deste para todo corpo, cuja anatomia e localização, facilitam a coleta. Assim, objetivou-se testar sua eficácia como material para o diagnóstico laboratorial da Raiva. Para tanto, inoculou-se vírus da raiva intramuscular em cinco *Rattus*. Após o óbito, o cérebro e a medula cervical de cada animal foram macerados e inoculados intracerebral em cinco *Rattus* (repetições). Os 25 cérebros e 25 medulas foram 100% positivo para Raiva, pelas provas de imunofluorescência e inoculação intracerebral. Apesar da concordância entre o cérebro e a medula cervical, este estudo propõe a utilização da medula cervical como material para o diagnóstico laboratorial da Raiva.

Palavras-chave. raiva, diagnóstico, medula cervical.

INTRODUCTION

Rabies is a contagious zoonosis caused by a neurotropic virus that acts on the Central Nervous System (CNS). The virus produces an acute and deadly encephalomyelitis because its replication leads to nervous system cell destruction[1]. The virus often causes behavioural and motor alterations, including restlessness, rage, aggressiveness and hind limb paralysis[2].

Rabies infection in humans is associated with domestic animals of low immunity, an overpopulation of abandoned cats and dogs, and a failure or lack of epidemiological monitoring. According to OPS[3], 0.1% of annual samples from the canine population should undergo Rabies laboratory diagnosis. Despite these recommendations, there are challenges related to the gathering, preservation, and transport of these samples (brain, head, or whole animal).

According to the literature, the encephalus is the sample of choice for post-mortem diagnosis of Rabies in animals, but the diagnostic procedure must be completed quickly. The sample should be preserved on ice and transported to the laboratory[4] in a rapid fashion. Animals suspected of having Rabies often arrive whole, or with their head inside an appropriate container. These methods lead to an increased infection risk to personnel during transportation to the laboratory. In the state of Ceara, samples for Rabies examination are received 24 hours a day, including weekends and holidays. After long holidays, the freezer used to store the samples (whole animals or heads) often gets full. Frequently, the cover to the freezer is left half-open. Consequently, the samples are inappropriately conserved, leading to the loss of samples and serious consequences for the victims.

A study of the cervical medulla is justifiable because it is a part of the CNS. It is an obligatory pathway of the Rabies virus to the brain (where the virus multiplies). Once the virus is in the brain, it disseminates to the rest of the body. This study aims to test the efficiency of the cervical medulla as a sample for Rabies diagnosis.

The anatomy and location of the cervical medulla make it easy to gather, store, and transport. The use of the cervical medulla may reduce the risk of infection due to mishandling of materials. This reduction may lead to a decrease in human prophylactic treatments. A decrease in prophylactic vaccination would strongly contribute to epidemiological monitoring and disease control. The objective of this study was to test the effectiveness of the cervical medulla as an alternative material for the laboratory diagnosis of Rabies.

METHODS AND MATERIAL

The experiment was carried out at the Virology Laboratory of the Post-Graduate Program in Veterinary Science at the State University Ceara and at the Laboratory of Medical Entomology of the state of Ceará Secretariat of Health. Work was also completed at the Rabies Diagnostic Laboratory in the Zoonosis Control Center at Crato-CCZC.

▪ Viruses used in the experiment

A sample of dog brain that was positive for Rabies was kindly donated by the Rabies Diagnostic Laboratory in the Zoonosis Control Center at Crato-CCZC. To create samples with easily identifiable anatomy and increased reliability, the virus from this sample was replicated. To accomplish this, 5 g of the sample was macerated in 95 mL of sodium chloride solution (NaCl 0.9%) and filtered through gauze. After this filtration, 0.3 mL was inoculated by intra-cerebral (IC) into three organisms of *Rattus norvegicus wistar.* The first animal to die was confirmed to have Rabies. Laboratory corroboration was completed throughout the experiment.

▪ Animals used in the experiment

To perform this experiment, 65 *Rattus norvegicus winstar* from the Central Animal House – BIOCEM of the Federal University of Ceará-UFC were used. All of the protocols used were in accordance with the ethics committee for the use of animals-UECE, number 07175988-5.

Thirty-day-old rats of both genders with live weights between 85 g and 100 g were used. The rats were assigned randomly into two groups and eleven subgroups (each subgroup contained five animals). The rats remained confined in 13 cages appropriate for *R. norvegicus* during the entire experiment.

▪ Experimental Design

The experiment was conducted in three stages:

1st Stage (two groups with five animals each)

Group I: served as the control group. Five *R. norvegicus* were inoculated with 0.5 mL of a sodium chloride physiological solution (NaCl 0.9%) by an intramuscular

route (IM). This administration corresponded to the same dose and solution as the diluent in the experiment.

Group II: served as the challenge group and was infected by the Rabies virus. The first rat brain that evolved to obit was macerated in 95 mL of sodium chloride physiological solution (NaCl 0.9%) and filtered through gauze. This treatment resulted in a suspension containing first passage Rabies virus. A half millilitre of the solution was inoculated intramuscularly into the inner surface of the thigh of each of the five *R. norvegicus* in Group II. According to Germano[5], an observation time of 30 days was set. After death, the cervical medulla and the brain of each rodent were gathered separately using clean utensils. The laboratory diagnoses were 100% positive by direct immunofluorescence (IFD) and biological proofs (IC).

2nd Stage

Every *R. norvegicus* in experimental group II tested positive for Rabies. Throughout the experiment, the samples were stored in individual containers. The brain and medullar samples were macerated separately in 10ml sodium chloride physiological solution (NaCl 0.9%) and filtered through gauze. This process resulted in five brain filtrates and five medullar filtrates. Five *R. norvegicus* were inoculated with 0.3 mL of brain filtrate by intracerebral administration (IC). The five subgroups (C1; C2; C3; C4; C5) were repeated five times. A total of 25 rodents were challenged with the Rabies virus derived from the brain. Similarly, the medullar filtrates were inoculated by the IC route in five *R. norvegicus*. These five subgroups (M1; M2; M3; M4; M5) underwent five replications, leading to a total of 25 rodents challenged with Rabies virus from the medullar source.

The control subgroup was inoculated by the IC route with 0.3 mL sodium chloride physiological solution (NaCl 0.9%). This dose correlated with the solution used as diluent in the other experimental groups. Overall, there were a total of eleven subgroups.

3rd Stage

The clinical term and the infection onset were monitored in the *R. norvegicus*. The clinical characteristics of the animals that received brain and medullar inoculate were compared to the sham group.

RESULTS

Rattus norvegicus belonging to **Group I** and the control subgroups did not go to obit. Every *Rattus norvegicus winstar* belonging to experimental **Group II** evolved to obit. These rats had a mean incubation time of 15.2 days and a clinical term of 4.2 days.

The rats had clinical findings compatible with Rabies, including isolation from the group, loss of appetite, acute weight loss, bristled hair, agitation, lack of coordination, paralysis, death, and aggressive rage. These findings demonstrate the pathogenic power of the inoculated suspension.

The laboratory results of the brain and cervical medulla from *Rattus* belonging to experimental Group II were 100% positive for Rabies. This finding demonstrates the presence of the virus in the cervical medulla and brain. There was perfect conformity of the techniques

Table 1. Clinical observation terms and results of brain and cervical medulla samples using IFD and IC proofs

Group II Challenge	Clinical observation		Brain		Medulla	
	Incubation T. (I.T.)	Clinical T. (C.T.)	DIF	IC	DIF	IC
R. norvegicus I	14	3	+	+	+	+
R. norvegicus II	14	4	+	+	+	+
R. norvegicus III	15	5	+	+	+	+
R. norvegicus IV	16	4	+	+	+	+
R. norvegicus V	17	5	+	+	+	+
Total	**76**	**21**	**100%**	**100%**	**100%**	**100%**

DIF – Direct Immunofluorescence; IC – intracerebral inoculation.

of direct immunofluorescence (DIF) and intra-cerebral inoculation (IC) (Table 1).

Laboratory diagnoses of 25 brain samples and 25 cervical medulla samples (repetition subgroups) were 100% positive for Rabies using DIF. These results corroborated the presence of the Rabies virus inside both regions of the Central Nervous System (Table 2).

The mean incubation terms in the subgroups inoculated with brain and medulla filtrates were 6.36 and 6.2 days, respectively. The mean clinical terms of the subgroups inoculated with medullar filtrates were 2.88 and 2.8 days, respectively.

DISCUSSION

Comparison of the results of the brain and medullar samples in this experiment demonstrated agreement between these two areas of the nervous system. These results are in agreement with those reported by Ito[4], who detected 100% Rabies antigen in the brain and spinal medulla of naturally infected dogs using direct immunofluorescence (DIF) and intra-inoculation (IC). This group utilised intramuscular inoculation (IM) at the masseter with a Rabies virus suspension.

In this experiment, Rabies virus was detected in the brain and medullar tissue of every *Rattus norvegicus winstar* inoculated with Rabies virus by the intramuscular route. This finding was in agreement with the results of Germano[5], who analysed viral dissemination through different organs (brain, medulla, tongue, heart, lungs, kidneys and liver). Germano used three groups of mice infected by the intramuscular route with three different Rabies strains. Jales and Nigéria used two canines and one desmodine (DR19). Both groups detected Rabies antigen inside the brain and medulla of every animal, regardless of the inoculated strain. This consistency did not occur with the rest of the researched organs. These results disagree with those found by Silva et al.[6], who reported hydrophobic dogs with the virus in their salivary glands and absent in their encephalus. However, Heuschele[7] stated that the neurotropism of the Rabies virus was not controversial. The studies of Fishbein, Robinson[8] and Tsiang[9] demonstrated that the Rabies virus reaches the spinal medulla via a centripetal mechanism after reaching the peripheral nerves. Using this mechanism, it invariably reaches the brain and replicates with great intensity.

In the first stage of this study, a natural infection (infected animal bite) was simulated in *Rattus norvegicus winstar*. Brain filtrate containing Rabies virus was inoculated intramuscularly, leading to the death of all animals. These results are in agreement with Tsiang[9]. After inoculation with an animal bite, the Rabies virus reaches sensory and/or motor nerve endings, or remains for an unidentified time in the affected muscle cells. At these locations, the process of viral amplification occurs, leading to propitious nerve infection.

According to Germano et al.[5], the material of choice for the laboratory diagnosis of Rabies is the

Table 2. Consolidated diagnostic and clinical monitoring results from five repetitions of each subgroup

Subgroups	Repetitions	Inoculated Material	Diagnostic and Clinical monitoring		
			DIF	I.T.	C. T.
C1	5	Brain filtrate	+	7	2,8
M1	5	Medullar Filtrate	+	6,4	2,6
C2	5	Brain filtrate	+	5,8	3
M2	5	Medullar Filtrate	+	5,8	2,6
C3	5	Brain filtrate	+	6,2	3
M3	5	Medullar Filtrate	+	6,4	2,8
C4	5	Brain filtrate	+	6,6	2,8
M4	5	Medullar Filtrate	+	6	2,8
C5	5	Brain filtrate	+	6,2	2,8
M5	5	Medullar Filtrate	+	6,4	3,2

DIF – Direct Immunofluorescence; I.T. – incubation time; C.T – clinic time.

Central Nervous System (hippocampus, cerebral trunk, thalamus, cortex, cerebellum and medulla oblongata). This rule does not apply to *equidae* species (horse, ass, and donkey), except in cases where the material gathered in these animals is the medulla. In this paper, we conclude that Rabies viruses are present in the cervical medulla of animals killed by Rabies.

In this study, agreement was found between the tests on the brain and the cervical medulla. The sensitivities of immunofluorescence and intracerebral inoculation were similar to those reported by Smith[10]. Smith showed that the brain and cervical marrow are the best areas for the laboratory diagnosis of Rabies. These areas have sensitivities that are greater than the hippocampal, cortical and cerebellar areas.

Charlton[11], cited by Carrieri[12], observed that human paralytic Rabies is characterised by the destruction of nerve cells, microglial proliferation, and perivascular infiltration. These findings are mainly observed in the brain and cervical marrow. In classic Rabies (furious), the inflammatory reaction, vascular modifications, and inclusion bodies are more diffuse in the thalamus, hippothalamus, cerebellum and cervical medulla. Perl and Good[13] confirmed that paralytic Rabies lesions are always found in the cervical medulla and brain. The results of these studies showed the inevitable presence of large amounts of viral antigen in the brain and cervical medulla. Therefore, these two CNS regions should be targeted for laboratory diagnosis.

In this experiment, Rabies antigens were present in all medullar samples of animals that died. This finding is in agreement with those of Lee and Becker[14], Ito[7], Germano et al.[5], Binghan and Van der Merwe[15], and Rolim et al. [16]. Due to its location and anatomy, several features of the medulla make it an ideal sample. The medulla can be gathered without contamination, is easy to contain, is easy to transport and store, has a reduced infection risk during manipulation, and has greater resistance to decomposition. Overall, these qualities paired with credible and incontestable results make the routine use of this organ feasible in the laboratory diagnosis of Rabies.

CONCLUSION

The cervical medulla is the region of the CNS that is most suitable for the laboratory diagnosis of Rabies using direct immunofluorescence (IFD) and intracerebral inoculation (CI).

Rabies viruses, regardless of the inoculation site, are present in the cervical medulla and brain of animals killed by Rabies.

Agradecimentos

The authors thank the Graduate Program in Veterinary Sciences at the State University of Ceará (Veterinary Sciences Pos-Graduation Program of the Ceara State University), the Central Biotery of the Universidade Federal do Ceará (Federal University of Ceará), the Medical Entomology Laboratory of the Ceará State Secretariat for Health, and the Ceará Foundation for Support for Scientific and Technlogical Development. The authors would also like to thank the teachers, employees, friends, family and others that directly or indirectly contributed to this study.

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