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Diagnosis of Rabies via RT-PCR on Skin Samples of Wild and Domestic Animals

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Abstract

In developing countries, brain tissues from rabies suspect animals are not always available for diagnosis for a variety of reasons, such as lack of transport to submit a carcass or the difficulty of removing an animal's head or brain under field conditions. To enable diagnosis in such cases, there is a need for a reliable method, using an alternative non-neural tissue, which can be removed and submitted to the diagnostic laboratory without special training or equipment. In human medicine, skin is used successfully for the detection of rabies virus antigen using RT-PCR technology. Little work has been done in animals using RT-PCR on skin or extracted hair follicles. The current study was conducted in Grenada on skin from 36 wild and domestic animals, in which rabies virus infection had been confirmed in brain tissue via the direct fluorescent antibody (DFA) test, and in 31 negative control animals. RT-PCR on skin yielded a sensitivity of 97.2% (35/36) and a specificity of 100% (31/31). It is concluded that the examination of skin samples via RT-PCR provides a valuable diagnostic alternative in those cases where brain tissue is not readily available.

Keywords

Rabies Virus, Diagnosis, Skin, Animals, RT-PCR

1. Introduction

Rabies is a globally distributed infectious disease of the central nervous system of mammals, caused by members of the genus *Lyssavirus* in the family *Rhabdoviridae*. The rabies virus (RABV) is the type species of the genus *Lyssavirus* and causes most of the human rabies cases. Transmission typically occurs via the bite of an infected animal [1]. RABV antigen is detected in stained brain sections using the direct fluorescent antibody (DFA) test, which is the diagnostic gold standard [2] [3].

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In Grenada, a small island in the Caribbean Sea, rabies is endemic. It is maintained in a wildlife host, the small Indian mongoose (*Herpestes auropunctatus*), with spillover into other animals or humans [4]-[7]. Unfortunately, many rabies suspects are never submitted and carcasses are burned or buried without laboratory confirmation. Reasons given include the lack of adequate transport, especially where livestock is involved, or the inaccessibility by car of the area where an animal has died, quick decomposition of a carcass in the tropical climate, or lack of expertise to safely remove the head or brain. At other times, brain removal is delayed leading to late diagnostic reporting.

To overcome some of the above limitations, it would be of advantage if even laymen could collect an easily accessible non-neural tissue without having to open or transport an entire carcass for the reliable diagnosis of rabies virus infection. RABV moves centripetally along nerves from the site of entry to the brain, where it multiplies and begins to cause clinical signs. Then, it moves centrifugally along nerves to peripheral tissues [8]. Among these tissues, saliva, tear fluid, or corneal impressions have been widely used [9], especially in human medicine, but viral shedding in these fluids is too inconsistent to give reliable results, unless taken as serial samples [10]. In contrast, examination of skin yielded very promising results: serial cryosections of skin from a variety of animals showed a sensitivity of 98% in the post-mortem rabies diagnosis using DFA when compared to the DFA test performed on brain tissue [11]. Limitations of using skin for DFA are that at least 20 sections need to be examined, the expensive equipment, and that decomposition renders tissues unsuitable for DFA testing. PCR can be used even on decomposed tissues, and a highly reliable PCR protocol was described using skin biopsies in human rabies cases [12]. Some data are available using PCR on animal skins [13]-[16], yet reports are not promising: For example, Wacharapluesadee *et al.* [13] examined extracted whisker follicles and hair follicles of dogs that had died of rabies in Thailand and concluded that sensitivities of 81.8% using real-time PCR, and 66.7% using RT-PCR were "...not sufficient to help physicians (decide) whether to administer post exposure prophylaxis" in bite victims.

In the current study, we determined whether skin samples taken from animals after death and using conventional RT-PCR technology would be sufficiently sensitive in the diagnosis of RABV infection to serve as an easily obtainable alternative to brain tissue.

2. Material and Methods

2.1. Animals

A total of 36 confirmed rabid animals and 31 negative control animals were used. All cases had been submitted for rabies diagnosis to the Pathobiology Department, School of Veterinary Medicine, St. George's University, Grenada, West Indies, between March 2011 and May 2015. Carcasses were processed within 24 hours of an animal's death, or when this was not possible, carcasses were kept at −20˚C until processing.

2.2. Samples

To reduce the potential risk of saliva contamination of the skin, carcasses were thoroughly cleaned under running water. A 2 - 4 cm^2 section of skin was removed from the muzzle area, using a fresh sterile blade and taking care that the underlying muscle tissue or oral mucosa were not included. For a subset of animals, skin sections were also taken from the shoulder and hip area. All skin sections were individually stored dry at −20˚C in 50mL plastic containers until processing. Sampling and processing of saliva and brain samples from these animals including total RNA extraction, RABV antigen detection using DFA, RT-PCR, real-time PCR and sequencing have been described [7].

2.3. RNA Extraction

For the extraction of total RNA, a skin sample was cut into sections of approximately 1 mm^3 using fresh sterile blades. Care was taken to cut the skin at oblique angles to increase exposure of hair follicles. Excess hair and fat were cut off and removed. Total RNA was extracted from approximately 30 mg of skin, using RNeasy Mini Kit spin column extraction (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions. Skin was lysed in 0.6 mL RLT/beta-mercaptoethanol lysis buffer containing 1 mm zirconia/silica beads at a bead volume equal to skin volume (Biospec Products, Inc. Bartlesville, OK, USA), and milling samples in a beadbeater (Mini Beatbeater™, Biospec Products) for 20 sec, resting for 60 sec, and milling again for 20 sec. Samples were cen-

trifuged after lysis at 13,000 rpm for 3 min and 0.5 mL of the clear lysate was used in the spin column protocol. The final elution volume was 30 µL.

Total extracted RNA was measured using Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). The minimum concentration used in this study was 2.3 ng RNA/µL and the maximum was 99.7 ng RNA/µL. Any sample containing more than 100 ng RNA/µL was diluted to a final concentration of 40 - 50 ng RNA/µL. Samples, which contained less than 1 ng RNA/µL or with a 260/280 ratio below 1.90 were discarded and the extraction repeated.

2.4. RT-PCR

RABV antigen was detected via RT-PCR as recently described [7]. Briefly, the one-step iScript protocol (Biorad Laboratories, Hercules, CA, USA) was used on 1 - 50 ng total RNA per 25 µL reaction mix. The primers used for amplification targeted a 110 bp fragment of the highly conserved region of the nucleoprotein (N)-gene: JW 12 (5'-ATGTAACACCYCTACAATG-3') and N 165-146 (5'-GCAGGGTAYTTRTACTCATA-3'). An Eppendorf Mastercycler ProS (Eppendorf AG, Hamburg, Germany) was used and RT-PCR products were visualized using 3 % agarose gel electrophoresis and ethidium bromide staining.

2.5. Statistical Methods

Results obtained from the new method, *i.e.* RABV antigen detection in skin by RT-PCR, were compared to the gold standard of rabies diagnosis, the viral antigen detection in brain tissue by DFA. Sensitivity, specificity, Positive Predictive Value (PVP), Negative Predictive Value (NPV) and confidence limits at 95% were calculated using MedCalc statistical software.

3. Results

Skin samples were taken from the muzzle area of 36 rabid animals and 31 negative controls. The animal species and numbers are listed in **Table 1**. All cases had previously been confirmed as RABV positive, respectively negative, using DFA on brain tissue [7]. RT-PCR results from skin samples taken from the head are also presented in **Table 1**. In relation to the DFA test performed on brain tissue, the RT-PCR test performed on skin samples taken from the head of animals showed a sensitivity of 97.2% (95%Cl: 85.47% - 99.93%) and a specificity of 100% (95%Cl: 88.78% - 100.00%). The Positive Predictive Value (PVP) was 100% (95% Cl: 90.00% - 100.00%) and the Negative Predictive Value (NPV) was 96.88 (95% Cl: 83.78% - 99.92%).

Of the animals that tested RABV positive in skin taken from the head, only eight were also available for the collection of skin from their shoulder and hip areas: 3 dogs, 3 mongooses and 2 cats. In seven of these animals (87.5%), RABV was detected in all three skin areas. In one dog, only the skin from the head tested RABV positive, whereas skin taken from the shoulder and hip areas were RABV negative.

Table 1. Comparison of RABV detection via RT-PCR in skin taken from the head to DFA in brain tissue in rabies-infected and uninfected animals.

Animal species	Numbers	
	RT-PCR positive (skin)/DFA positive (brain)	RT-PCR negative (skin)/DFA negative (brain)
Mongoose (*Herpestes auropunctatus*)	20/20 [100%]	5/5 [100%]
Dog	10/11 [90.9%]	17/17 [100%]
Cat	4/4 [100%]	3/3 [100%]
Goat	1/1 [100%]	1/1 [100%]
Cow	-	1/1 [100%]
Bats (2 *Glossophaga longirostris*; 1 *Molossus molossus*)	-	3/3 [100%]
Opossum (*Didelphis marsupialis*)	-	1/1 [100%]
TOTAL	**35/36 [97.2%]**	**31/31 [100%]**

Testing skin for RABV antigen via spin column extraction and RT-PCR as described here took three hours from tissue lysis to gel visualization.

4. Discussion

The current study tested the use of RT-PCR on skin samples as an alternative to DFA testing on brain tissue for the post mortem diagnosis of rabies. Skin samples collected from animals' heads showed a sensitivity of 97.2% and a specificity of 100% compared to the gold standard.

Skin as a potential non-neural tissue for rabies diagnosis has long been investigated. In human medicine, immunofluorescence (IF) techniques on skin biopsies typically taken from the nape of the neck have been successfully used in the post-mortem [17] and ante mortem rabies diagnosis [18] [19]. PCR technology has meanwhile replaced IF-staining of skin sections, and the collection of skin biopsies along with saliva and CSF samples and testing by PCR technology is now recommended as a routine measure in the ante-mortem diagnosis of rabies in humans [12] [20]-[23]. Indeed, Dacheux *et al.* [12] reported a sensitivity of 98% using hemi-nested PCR applied to human skin and proposed to use skin as an alternative to brain DFA testing for the confirmation of rabies before and after death.

In veterinary medicine, IF techniques on skin specimens of rabid animals showed promising results [8] [11] [24]-[26], but were never routinely used, probably due to the expensive equipment needed for cryosectioning and the number of sections to be stained. Few reports are available which examine the use of skin using PCR techniques, possibly because the ante-mortem diagnosis is not as important in animals as in humans or because PCR with its inherent contamination issues has not been accepted as a primary method in rabies diagnosis. Wacharapluesadee *et al.* [13] concluded that their sensitivity of 81.8% using real-time PCR on extracted dog whisker follicles was insufficient. Studies conducted in India used skin from various animals before they died of rabies and detected RABV antigen in 9 of 13 (69%) by RT-PC and in 11 of 13 (84%) by real-time PCR [14] [15]. Kaw *et al.* [16] only observed a sensitivity of 57.1% for ante-mortem skin samples using RT-PCR and 71.4% using real-time PCR in animals.

It is interesting to note that the false negative case in the current study was a dog, which had died of paralytic rabies. Wacharapluesadee *et al.* [13] also found that most of their false-negative results originated from dogs that had died of paralytic rabies. Similarly, a high false-negative rate was reported for human paralytic cases when examining non-neural tissues [27]. This supports the idea that viral spread from the infected brain to peripheral tissues differs between paralytic and furious forms.

Considering the future possibility to use skin for ante-mortem diagnosis in animals, collection of biopsies from the head of a rabies suspect would only be safe under very tight physical restraint, with heavy sedation and/or brief anesthesia. Samples collected from the shoulder or hip area of an animal would be easier and safer to take. Although the sample size for comparing skin results was small (n = 8), the current study showed that RABV antigen could be detected in the skin of the head, shoulder and hip of a rabid animal. One of the 8 animals examined tested rabies positive using head skin, but had no detectable RABV antigen in its skin from the shoulder and hip regions. Contamination of the head skin with saliva in this case could be excluded, as its saliva tested RABV negative. The hair follicles of the head, lips, and especially whiskers are particularly well innervated [17], and being closest to the brain, it is reasonable to assume that the virus is detected here earlier and perhaps in higher numbers than elsewhere.

5. Conclusion

In the post-mortem diagnosis of rabies in animals, testing skin tissue via RT-PCR provides a highly sensitive and rapid alternative to the examination of brain tissue via DFA in those situations where brain is not readily available, or where the removal or examination of brain is delayed. The method described here may also be useful for the ante-mortem diagnosis of rabies during an animal's quarantine or clinical work-up stage.

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Competing Interests

The author declares that there is no competing interest.

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