An anatomical aspect on masseteric muscles in cattle rabies by Real-Time PCR

Atilla Yoldaş¹, Ramazan İlgün²*, Nevin Tuzcu³, Akın Yığın⁴, Erdal Özmen⁵

¹Veterinary Research Institute, Adana, ²Department of Anatomy, Faculty of Veterinary Medicine, Cumhuriyet University, Sivas, ³Cumhuriyet University Cutfam, Sivas, ⁴Department of Biology, Faculty of Arts and Sciences, Cukurova University, Adana, ⁵Department of Anatomy, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay, Turkey

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*rilgun1980@hotmail.com

Özet

Amaç: Bu çalışmada sığırlarda kuduz virüsünün masseter kaslarında izlediği patho-anatomik lokalizasyonun Real-Time PCR ile belirlenmesi amaçlanmıştır.


Öneri: Bu çalışmada sığırlarda nöronal bağlantıların ve sinaps geçişlerinin virüs nükleik asitlerinin RT-PCR yöntemi ile ortaya konulmasıyla anatomi olarak izlenebileceği belirlendi.

Anahtar kelimeler: Masseter kasları, kuduz, Real time-PCR

Abstract

Aim: In this case, the anatomical pathways on masticatory muscles of cattle infected rabies virus have been determined by Real Time PCR (RT-PCR).

Materials and Methods: A 1.5-year old male Brown Swiss cattle which was bitten in face by dog was used. The cattle was died 15 days post exposure to the dog rabies suspected. The areas where the tissue samples to taken. The origin, course and branches of the trigeminal nerve were exposed by standard dissection method. Rabies virus was investigated on 32 different parts of the cattle’s head by means of TaqMan Probe-Based Real-Time PCR and FAT.

Results: The rabies virus specific nucleic acid was detected in masseter muscles motor nerves; N. mandibularis branch of N. trigeminus, N. masticatorius, N. massetericus and Nn. temporalis profunda which are two extra branches of N. masticatorius, unilaterally ganglion trigeminaile, and pons by Real Time-PCR. However, no rabies virus has been detected in the samples obtained from other different parts of the brain.

Conclusion: Contrary to immunohistochemical methods, it is not possible to trace neuronal connections across synapses within the brain. RT-PCR method could be helpful to detect pathways of the neurotropic agents suspected cattle.

Keywords: Masseteric muscle, rabies, real time PCR
**Introduction**

Classical rabies virus is classified in the genus *Lyssavirus* of the family *Rhabdoviridae*, has a genome consisting of single stranded RNA. Rabies is a cause of fatal neurologic infection in all mammals. It was found that replication of virus in skeletal muscle might be necessary to generate sufficient virus for entry into the peripheral nervous system (Charlton et al 1997). But, some authors indicated that rabies virus could also be entered into nerve terminals directly without replication in muscle (Baer and Cleary 1972, Ugolini 1995, Lewis et al 2000). On the other hand, Rabies virus is a kind of virus that affinity to the neuron cells therefore there are several researches in the neuoroanatomy field that displays the relationship between the periferic nervous system and the central nervous system (Theerasurakarn and Ubol 1989, Astic 1993).

Previously, neurons infected with virus were identified using by histological methods, conventional polymerase chain re-action (PCR) and immunohistochemical labelling techniques (Ugolini 1995, Kelly ve Strick 2000, Lanciego et al 2000, Morcuende 2002, Katherine et al 2005). Recently, a highly sensitive molecular diagnostic method based on quantitative PCR (Real Time PCR), have been used for diagnosis of the rabies (Sacramento et al 1991, Wakeley et al 2005, Yoldas et al 2010). Moreover, the real time PCR, conventional PCR, histopathological methods immunofluorescence technique was used for examining transneuronal transport of neurotropic viruses. Innervation of some motor muscle was revealed to be via these methods (Lewis et al 2000, Espy et al 2006, Mackay 2007). We inspired from these methods used for examining transneuronal transport of neurotropic viruses (Lewis et al 2000, Espy et al 2006, Mackay 2007).

The primary goal of the study is to investigate the patho-anatomical pathways on masticatory muscles of cattle infected rabies virus using by Real Time PCR (RT-PCR).

**Materials and Methods**

**Animal**

A 1.5-year old male Brown Swiss cattle which was bitten in face by dog was used in this study. The cattle was died 15 days post exposure to the suspect dog. The places, from where the tissue samples are taken, are shown in the Table 1. It was determined by the Twining Project TR 02/IB/AG-01 (Anonymous 2002). The origin, course and branches of the trigiminal nerve was a revealed by using standard dissection method.

**Real Time PCR**

In order to increase the reliability of the test, two samples were taken from the same tissue. The weights of issues were calculated as 1 g for analysis. These tissues were homogenized in the MagNA Lyser (Roche). Viral RNA from the tissues was directly extracted using a MangNA Pure LC Total Nucleic Acid Kit and MagNA Pure LC (Roche) isolation instrument (Roche, Germany) according to the manufacturer’s instructions.

RT-PCR was performed using oligonucleotide primers and probe based on specific region of nucleoprotein gene of rabies virus, as described elsewhere (McElhinney et al 2002). Primers and probe used in the research are given in following: Sense 5’-ATGTAACACCYCTACAATG-3’ and antisense 5’-CAATTCGCACACATTTTGTG-3’ (GenBank X03673), TaqMan probe 5’-6-FAM CCC AAT TCC CGT CTA CAT CAG TAC GT- TAMRA. The RT-PCR was performed using a LigthCycler RNA Amplification Hybridization Probes kit (Roche, Germany) and amplified by RT-PCR assay procedure was performed as described by McElhinney et al (2002), with minor modifications. The PCR reaction mixtures were prepared by adding 1 µL of RNA to 19 µL master mix of containing a final concentration of 2 µL of Light Cycler Real Time PCR reaction mix.

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<th>Table 1. The places from where the tissue samples are taken.</th>
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hybridization prob, 2.4 µL of 25 mM MgCl2, 0.4 µL of RT-PCR enzyme mix, 1.6 µL of each primer, 0.6 µL 5 µM TaqMan prob, and then all reaction mixtures added to each capillary sealed capillaries were spun (1000 g for 15 s) and placed in Light Cycler instrument 2.0 (Roche, Switzerland) and using the following reaction conditions: All dilutions were made in sterile molecular biology grade water. The amplification conditions were shown Table 2.

Fluorescent Antibody Test (FAT)

FAT was performed as described by Valleca and Forrester (1981) and Dean and Albeseth (1973). Impression smear preparations of the tissue were fixed in acetone at -20°C overnight before being stained with fluorescein-labelled anti-rabies immunoglobulin (BBL Microbiology Systems, USA) absorbed with 10% normal mouse brain. The slides were incubated for 20 minutes at 37°C in humid chambers, and washed with phosphate buffered saline (PBS) in three successive wash at 10 minutes intervals. After rinsing with distilled water, the slides were air-dried and mounting buff ered glycerine (Oksilab, Turkeye) was applied before the slides were examined under cover slips at 400xUV microscope (Olympus R, Japan). Rabies antigen in infected tissues appeared as bright (3+ to 4+), dull (2+) or dim (1+) apple -green or yellow-green, round to oval intracellular accumulations. In addition to the larger stained bodies, infected tissues contained smaller collections of antigen, which appeared as granules or dust-like fluoresecent particles or threads (Yoldas et al 2010). Positive and negative controls (mouse brains) were carried out synchronous with the test specimens.

Results

Dissection

In material, it was observed that mandibular nervi of nervi trigeminal was exited the cranial cavity via the foramen ovale. Mandibular nerve was indicated to distribute branches on the opening of the foramen ovale. One of these branches that masticatorius nerve coursed dorsally and reached articulation temporomandibulaire. After a course of 1.5 cm, the masticatorius nerve bifurcated into two branches, massetericus nerve and temporal nerve. Masseter muscle was innervated by massetericus nerve. The second branch, temporal nevre was shown to supply temporal muscle.

PCR and FAT

In the dissection of head, there were bite marks on the middle part of the musculi masseterica of the regio masseterica and the ventral part of the musculi masseterica of the regio masseterica. According to the crossing point values, the smallest amount of nucleic acid was detected in superficial pars of masseter muscle. Moreover, the viral nucleic acid was not found in regio coli ventralis, regio parotidea, regio articulatio temporo mandibuleris, regio mandibuleris, regio baccalis, regio infraorbitalis of the head. By using FAT, the virus was not shown on bite site and head of cattles. RT-PCR was found a high specificity than FAT technique on peripheral neural system for rabies virus.

In present study, the rabies virus was found in masticarious nerve, mandibular nerve and trigeminus nerve, pons and trigeminal ganglion by RT-PCR, but the virus was not demonstrated on the peripheral nerve fibers and part of brain. Moreover, any viruses were also not detected on the peripheral nerve fibers and brain by FAT apart from pons.

Rabies virus was not detected on Regio naris, Regio infraorbitalis, conjuctiva, Regio mandibularis, Regio buccalis, Regio articulationis temporomandibularis, Regio parotidea, Regio coli ventralis, Temporal nerve, Lingual nerve, Mylohyoideus nerve, Chorda tympani profunda, Auriculapalpebralis nerve, Buccal nerve of Facialis nerve, Facial nerve, Medulla oblongata, Cerebellum, Inferior colliculi, Superior colliculi, Pineal gland, Thalamus, Hypothalamus, Hypophis, Frontal lobe of the cerebrum, Parasitoid of the cerebrum, Occipital lobe of the cerebrum. But, it found on Regio masseterica, Masticatorius nevre, Mandibular nerve, Masseteric nerve, Trigeminal

| Table 2. Amplification conditions used by RT-PCR. |
|----------------------------------|-------------------------|
| **Parametre**    | **Value** |
| Cycles           | 45         |
| Type             | Quantification |
| Target temperature (°C) | Segment 1 | Segment 2 |
| Incubation time (s)  | 1         | 30         |
| Temperaturetransition(°C/s) | 20        | 20         |
| Acquisition mode   | None       | Single     |
| Gains             | F1=1;      | F2=15      |

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made by histopathologically, that represents inflammatory effect on skin area, tissue structure and immune response. The diagnosis of rabies is either from the related motor nerves or ganglions, depend on the virus ascending rate. The viral nucleic acid could be obtained approaches such as quarantine or vaccine application (Jackson et al 1973), and the virus was not detected at 6-30 hours after the injection into the muscle in rat (Shankar et al 1991). Coulon et al (1989) also found that rabies virus penetrates both sensory and motor routes directly with equal efficiency, without prior multiplication in muscle cells. Long incubation periods in muscle of rabid animals were reported by researchers (Baer et al 1972, Charlton et al 1997). Viral antigens were persistently detected in muscle at the inoculation site and no spread directly from one muscle fiber to another (Park et al 2006). Our finding contributed the information that the replication of the virus in the inoculated into muscles (Baer et al 1972, Charlton et al 1997) and no spread the other regions of head.

We found as described by some authors (Tecirlioğlu 1977, Karadağ and Nur 1989, Tipırdamaz et al 2000, Budras et al 2007), noted that after masticatorius nevre originated from mandibularis nerve, it extended towards dorsal and reaches to the level of temporomandibularis articulation and give off separated two branchs on level temporomandibular articulation. In the material, masticatorius nerve contrary to Karadağ and Nur (1989), the prophund temporale nerve of masticatorius nerve was seen one root as described Tecirlioğlu (1977) and Tipırdamaz et al (2000). Rabies’s viral nucleic acid was found on motor nerve, massteric nerve and masstericus nerve. Contrary to Kucer et al (1985), we didn’t viral nucleic acid on sympatic nervous fiber and mix fiber, buccal nerve (the branch of facial nerve), lingual nerve, inferior alveolar nerve and on the tissues and nerves around. This indicated that the viral RNA distributes just after it reaches in the central nervous system. In the current study, it was found that viral nucleic acids were not seen on the materials from it was contradicted with the data that the virus extend with sympatic nervous system and used the receptors out of acetylcholine. This may be attributed to either the environmental effects on skin area, tissue structure and immune response against the virus. The ordinary diagnostic methods used for rabies are mostly difficult on clinically suspicious cases or may result false results which leads to unnecessary approaches such as quarantine or vaccine application (Jackson and Reimer 1989). The viral nucleic acid could be obtained either from the related motor nerves or ganglions, depending on the virus ascending rate. The diagnosis of rabies is made by histopathologically, that represents inflammatory changes and negri bodies. However these changes are more insidious on herbivores than carnivores, which is supported by the inclusion bodies are not seen on %30 of bovine cases (Urman 1977). In our study, diagnosis was made before the incubation period by this way. The amount of the viral nucleic acid was higher in parts of the brain different part than head region in cattle (Yoldas et al 2009). A positive correlation was found between the viral load on the different areas of the head and viral load on origin of the nerves innerved these areas. This report is supported our findings.

Many studies tend to generalize the idea that RT-PCR has an improved sensitivity compared with conventional PCR and other histopathological methods. It was absolutely undeniable that RT-PCR has greatly improved our molecular diagnostic practices, and represented a considerable progress in molecular diagnosis of several virus infections. Indeed, it has many objective advantages over conventional PCR such as decreased costs, labor, time and contamination risk, broad dynamic range of target DNA quantitation. Contrary to immunohistochemical methods, it is not possible to trace neuronal connections across synapses within the brain. In this project the pathologic findings did not evaluated because of it was planned tracing molecular structure of viral genome in neuroanatomical studies.

In conclusion, the recently developed methods could be used for future studies on the pathogenesis and molecular diagnosis, especially on the motor function of peripheric neural system, ganglion and the brain’s various topographic regions.

Discussion

The rabies virus is only replicates in neural tissues (Murphy et al 1973), and the virus was not detected at 6-30 hours after the injection into the muscle in rat (Shankar et al 1991). Coulon et al (1989) also found that rabies virus penetrates both sensory and motor routes directly with equal efficiency, without prior multiplication in muscle cells. Long incubation periods in muscle of rabid animals were reported by researchers (Baer et al 1972, Charlton et al 1997). Viral antigens were persistently detected in muscle at the inoculation site and no spread directly from one muscle fiber to another (Park et al 2006). Our finding contributed the information that the replication of the virus in the inoculated into muscles (Baer et al 1972, Charlton et al 1997) and no spread the other regions of head.

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References


