RESEARCH ARTICLE

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

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Özet

Yoldaş A, İlgün R, Tuzcu N, Yığın A, Özmen E. Sığır masseter kaslarında kuduz virüsünün anatomik lokalizasyonun Real-Time PCR ile belirlenmesi. **Eurasian J Vet Sci, 2013, 29, 4, 211-215**

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

Gereç ve Yöntem: Çalışma kuduz bir köpek tarafından yüz bölgesinden ısırılmış 1.5 yaşında erkek sığırda yapıldı. Kuduz şüphesiyle karantinada tutulduktan 15 gün sonra ölen sığırın ilgili bölgesinden doku örnekleri alındı. Standart diseksiyon yöntemleriyle ısırılan bölgede N. trigeminus sinirinin dalları incelendi. Sığır baş bölgesinin 32 farklı kısmı Tagman probe esasına dayalı RT-PCR ve FAT yöntemiyle incelendi.

Bulgular: Kuduz virüsüne spesifik nükleik asitler masseter kasların motor sinirlerinde; N. trigeminus'un N. mandibularis dalında N. masticatorius, N. mandibularis, N. massetericus ve Nn. temporalis profunda unilaterally ganglion trigeminale ve ponsta Real Time-PCR ile belirlendi. Beynin diğer kısımlarında incelenen örneklerde tespit edilemedi.

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

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

Abstract

Yoldas A, Ilgun R, Tuzcu N, Yigin A, Ozmen E. An anatomical aspect on masseteric muscles in cattle rabies by Real-Time PCR. **Eurasian J Vet Sci, 2013, 29, 4, 211-215**

Aim: In this case, the anatomical pathways on masticatory muscles of cattle infected rabies virüs 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 standart 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, motor leaf of N. mandibularis, N. massetericus and Nn. temporalis profunda which are two extra branches of N. masticatorius, unilaterally ganglion trigeminale, 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 reaction (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 neourotropic viruses. Innervation of some motor muscle was revealed to via these methods (Lewis et al 2000, Espy et al 2006, Mackay 2007). We inspirited from these methods used for examining transneuronal transport of neourotropic 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 trigminal 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 Asid 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 GC-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

Table 1. The places from where the tissue samples are taken.

Regiones
Regio naris, Regio infraorbitalis, Conjuctiva, Regio mandibularis, Regio buccalis, Regio masseterica, Regio articulationis temporomandibularis, Regio parotidea, Regio colli ventralis.
Nerves
N. mandibularis, N. masticatorius, N. massetericus, N. temporalis N. lingualis, N. mylohyoideus, Chorda tympani profunda, N. auriculapalpebralis, N. facialis
Other parts of the brain
Medulla, Pons, Cerebellum, Inferior and superior colliculi, Pineal gland, Thalamus, Hypothalamus, Hypophis, Frontal and pariatel, occipital lobe of the cerebrum.

Table 2. Amplification conditions used by RT-PCR.

Parametre	Value	
Cycles	45	
Type	Quantification	
	Segment1	Segment 2
Target temperature (^{0}C)	95	58
Incubation time (s)	1	30
Temperaturetransition(^{0}C/s)	20	20
Acquisition mode	None	Single
Gains	F1=1; F2=15	

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 buffered 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 floureseent 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 trigemninal was exited the cranial cavity via the foramen ovale. Mandibular nerve was indicated to distribute branches on the opening of the foremen ovale. One of these branches that masticatorius nerve coursed dorsally and reached articulation temporomandibulaire. After a course of 1.5 cm, the masticatorius nevre bifarcuted into two branches, masstericus nerve and temporal nerve. Masseter muscle was innerved by masstericus 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 vental 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, mandbilar 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, Pariatel lobe of the cerebrum, Occipital lobe of the cerebrum. But, it found on Regio massetericai, Masticatorius nevre, Mandibular nerve, Masseteric nerve, Trigeminal

ganglion and Pons. It was obserbed that values of crossing point of pons and trigeminal gangilion were higher than peripheral nevre fiber. On other hand, according to the crossing point values, it suggests that pons have been higher concentration of the viral nucleic acid than other sites.

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.

We found as described by some authors (Tecirlioğlu 1977, Karadağ and Nur 1989, Tıpırdamaz 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 Tıpırdamaz et al (2000). Rabies's viral nucleic acid was found on motor nerve, masseteric nerve and masetericus 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 sympathic nerveous 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.

Conclusions

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.

References

Anonymus, 2002. Annex 3.5, Laboratory Method Collection Twinning Project-Twinning Project Between and Germany. Project No: TR0203.05, KKGM, Ankara, Türkiye, pp: 99-155.

Astic L, Saucier D, Coulon P, Lafay F, Flamand A, 1993. The CVS strain of rabies virus a transneuronal tracer in the olfactory system of mice. Brain Res, 619, 146-156.

Baer GM, Cleary WF, 1972. A model in mice for the pathogenesis and treatment of rabies. J Infect Dis, 125, 520-527.

Budras KD, McCarthy PH, Fricke W, Richter R, 2007. Anatomy of the Dog. An illustrated text, 5th edition, Hannover, Germany, pp: 57-96.

Charlton KM, Nadin-Davis S, Casey GA, Wandeler A, 1997. The long incubation period in rabies delayed progression of infection in muscle at the site of exposure. Acta Neuropathal, 94, 73-77.

Coulon PC, Derkin P, Kutera F, Lafay C, Prehaud A, 1989. Invasion of the peripheral nervous systems of adult mice by the CVS strain of rabies virus and its avirulent derivative. Av Ol J Virol, 63, 3550-3554.

Dean DJ, Abelseth MK, 1973. The fluorescent antibody test, in: Laboratory Techniques in Rabies, Eds: Kaplan MM, Koprowski H, third edition, World Health Organization, Geneva, Italy, pp: 74-78.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack JE, Rosenblatt FR, Cockerill III, Smith TF, 2006. Real-time PCR in clinical microbiology: Applications for routine laboratory testing. Clin Microbiol Rev, 19, 165-256.

Jackson A, Reimer DL, 1989. Pathogenesis of experimental rabies in mice: An immunohistochemical study. Acta Neuropathol, 78, 159-165.

Karadağ H, Nur İH, 1989. Kılkeçisinde somatoafferent ve özel visceroefferent beyin sinirlerinde üzerinde makro-anatomik bir araştırma. UÜ Vet Fak Derg, 36, 260-272.

Katherine H, Taber PD, Peter L, Strick PhD and Robin A, HurleyMD, 2005. Rabies and the cerebellum: New methods for tracing circuits in the brain neuropsychiatry clinic. Neuroscience, 17, 133-139.

Kelly RM, Strick PL, 2000. Rabies as a transneuronal tracer of circuits in the central nervous system. J Neurosci Methods, 103, 63-71.

Kelly RM, Strick PL, 2004. Macro-architecture of basal ganglia loops with the cerebral cortex: use of rabies virus to reveal multisynaptic circuits. Prog Brain Res, 143, 449-459.

Lanciego JL, Wouterlood FG Neuroanatomic tract-tracing methods beyond 2000: What's now and next. J Neurosci Methods, 103, 1-2.

Lewis P, Thomas FY, Lentz L, 2000. Rabies virus entry at the neuromuscular junction in nerve-muscle cultures. Muscle & Nerve, 23, 20-730.

Mackay IM, 2007. Real-time PCR in microbiology: From diagnosis to characterization. Caister Academic Press, Norwich, UK, pp: 458-481.

McElhinney L, Sawyer J, Finnegan CJ, Smith J, Fooks AR, 2002. Use of rapid cycle real-time PCR for the detection of rabies virus. In: Microbiology and Food Analysis; Rapid Cycle RT-PCR: Methods and Applications, Eds: Wittwer UC, Cockerill F, Springer-Verlag, Berlin, Germany, pp: 235-241.

Murphy FA, Bauer SP, Harrison AK, Winn JWC, 1973. Comparative pathogenesis of rabies and rabies-like viruses: viral infection and transit from inoculation site to the central nervous system. Lab Invest, 28, 361-376.

Shankar V, Dietzschold B, Koprowski H, 1991. Direct entry of rabies virus into the central nervous system without prior local replication. J Vir, 65, 2736-2738.

Tecirlioğlu S, 1977. Merkepte (Equus asinus L.) beyin sinirlerinin (Nn. encephalici) makroskobik anatomisi üzerine araştırmalar. Ankara Üniv Vet Fak Derg, 24, 269-295.

Tıpırdamaz S, Eken E, Beşoluk K, 2000. Holstein ırkı sığırlarda N. trigeminus üzerinde makro-anatomik araştırmalar. Eurasian J Vet Sci, 16, 45-50.

Ugolini G, 1995. Specificity of rabies virus as a transneuronal tracer of motor networks: Transfer from hypoglossal motoneurons to connected second-order and higher order central nervous system cell groups. J Comp Neurol, 356, 457-480.

Urman HK, 1977. Veteriner Patoloji. Cilt IV, Sinir Sistemi Patolojisi. Ankara Üniversitesi Veteriner Fakültesi Yayınları, Ankara, Türkiye, pp: 130-148.

Wakeley PR, Johnson N, McElhinney LM, Marston D, Sawyer J, Fooks AR, 2005. Development of a real-time, taqman reverse transcription-pcr assay for detection and differentiation of *lyssavirus* genotypes 1, 5, and 6. J Clin Microbiol, 43, 2786-2792.

Yoldas A, Tuzcu M, Özmen E, Yıgın A, Özmen M, Kul S, 2010. Sığırlarda kuduz hastalığının teşhisi ve kuduz virüsünün anatomik lokalizasyonunun real time PCR ile belirlenmesi üzerine karşılaştırmalı çalışmalar. Dicle Üniv Vet Fak Derg, 2, 42-50.