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AN INVESTIGATION OF RABIES VIRUS EXISTENCE ON RODENTS BY USING NEW PCR PRIMER PAIRS

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ABSTRACT. Zoonotic diseases are the infections to be carried between human and other vertebrates. Rhabdoviruses belong to a virus family which could infect a wide range of host organism. It is important to find new molecular diagnostic tools and primers for the identification of the viruses to be able to make the molecular identification process faster and reveal new forms of the virus. Rodents are the primary mammals group that can uncontrollably go in and out from quarantine regions. Therefore, in this study, 242 *Apodemus* spp. (wood mouse) and *Myodes glareolus* (bank vole) specimens collected from 16 localities in province Zonguldak, Çaycuma district were used to scan brain tissues to determine RABV using hemi-nested PCR. Also, to determine RABV a new primer pairs were designed using already published sequences. According to results, eight specimens were showed positive bands for RABV. Those eight sequences blasted. But the sequences did not match according to the Blast result. The designed primer pairs provide positive bands on electrophoresis for positive control so that the primer pairs are new and can be used for following studies. With this study it was also tested whether rodents are the potential carriers for RABV since they are primarily prey source for carnivores, and domestic animals.

1. INTRODUCTION

Zoonotic diseases are naturally transmitted infections and diseases between humans and other vertebrates. The reservoir host animal acts as a source of infection because it carries the pathogen virus. Important zoonotic diseases include, Crimean-Congo Hemorrhagic Fever, Hantavirus infections, Rabies and Tularemia diseases [1]. There

Keyword and phrases. Rodents, rabies, heminested PCR, sequence analysis, Apodemus, Myodes, Turkey

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are many animal groups with zoonotic agents including rodents. Rodents are extremely important in terms of zoonotic diseases because of their frequent contact with people and having the highest number of groups in mammals. For this reason, knowing the spread areas, the viruses they carry, the relationships with the living things around them and the diseases they spread are the primary topics for understanding of human infectious diseases.

Rhabdoviruses (Figure 1) are a large family of viruses that can infect a wide range of hosts [2,3,4]. More than 160 Rhabdovirus species have been isolated from plants, invertebrates and vertebrates to date with the discovery of new species [3,5,6,7]. Vesiculovirus, Lyssavirus and Ephemerrovirus species of rhabdoviruses cause various diseases in humans and animals [3,8]. Rabies is a disease with a history of 5000 years [4,9] showing a widespread distribution worldwide except Australia and some island countries [1,10]. Despite significant scientific developments, it still exists as a global disease [11]. There are two epidemiological forms of the disease, usually the urban form seen in domestic animals and the sylvatic form seen in wildlife [12]. It has been reported that 54% of animal rabies are caused by dogs, 42% by terrestrial wild animals and 4% by bats [13].

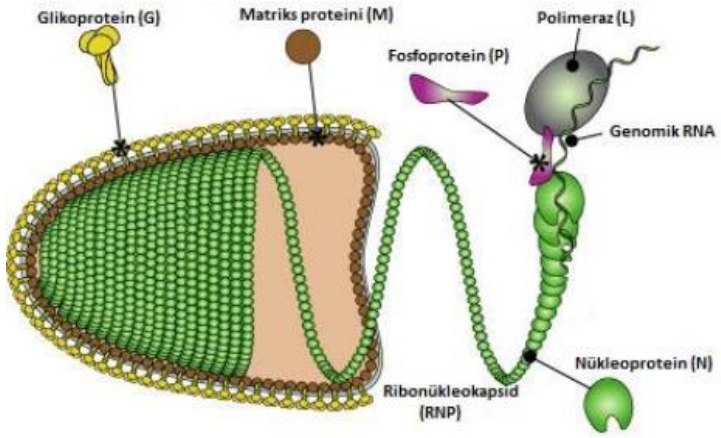

FIGURE 1. Schematic diagram of a Rhabdovirus (©ViralZone 2008, Swiss Institute of Bionformatics).

The screening and detection of viruses by molecular tools has been accepted and highly differentiated. Similarly, detection of rabies viruses can be done in different ways [14]. Each one has differences from the other. One of the most used is the real time PCR technique. In addition, considering that the endemic forms are quite high,

each primer designed for the diagnosis made by Real Time PCR will be important. Although this technique is reduced the time and the one of the most sensitive methods, false positives can also be observed due to the possibility of mixing with the genome of the host [14]. Serological assays are not suitable for routine testing used as identification. The test may lack sensitivity and specificity, and the interpretation of the test results may be difficult as the host response to infection varies substantially between individuals. As such, the negative predictive value of serological tests for rabies diagnosis is considered poor [14].

Small rodents, such as squirrels and mice, are rarely known to be natural reservoirs or vectors that cause human rabies [13,15]. Although rabies does not have a single host, the virus is spreading rapidly from one infected animal to another by inter-species interaction. Most mammalian species can be infected with rabies virus. According to Rabies surveillance data of the USA and Puerto Rico for the period 2005–2010 [16], 6,153 cases of rabies in animals and 2 in humans were reported [17,18]. Wild animals accounted for 92 % of reported cases in this study. Racoons were the most frequently reported rabid wildlife species (2,246 raccoons, 36.5 % of all rabid animals during 2010), followed by 1,448 skunks (23.5 %), 1,430 bats (23.2 %), 429 foxes (6.9 %), 303 cats (4.9 %), 71 cattle (1.1 %), and 69 dogs (1.1 %). Other wild animals included rodents and lagomorphs (1.8 %) [17]. Small rodents (squirrels, hamsters, guinea pigs, gerbils, chipmunks, rats, and mice) and lagomorphs (rabbits and hares) are rarely infected with rabies [16].

In another study, only one of the 57 *Apodemus agrarius* samples collected from the Zhejiang region of China was found positive for rabies [19] In addition, Özsoy et al. [20] with the suspicious animal bite Refik Saydam Hygiene Center of the rabies vaccination station, 92 (6%) of the patients reported to have applied to this unit [20].

A number of rabies cases have been seen with a high frequency in many villages in the province of Zonguldak Çaycuma district and they are originated from wild animals according to data from Zonguldak Provincial Directorate of Health. The Directorate have decided to take this region under quarantine several times and carnivores such as wolves and jackals were given as a source of rabies in the quarantine area. However, uncontrolled rodents' group in quarantine areas may have higher potential to be the bearer of many viruses due to their ease of travel between areas and also, they are primary food sources for carnivores. The existence of this uncontrolled group/animal might cause management studies fail.

The aim of this study is to prove that the rodents are the host for rabies and they should not be ignored during quarantine times, by screening for rabies virus on rodents and design new primer pairs to detect rabies in a host.

2. Materials and Methods

In this study, a total of 242 rodent specimens (197 *Apodemus* sp, 45 *Myodes* sp.) were collected from Çaycuma, Zonguldak province (Figure 2, Table 1). The individuals were anesthetized with ether. The research permission was taken from General Directorate of Nature Conservation and National Parks (01/12/2011- 83-703). After they were knocked out, they were killed quickly by breaking neck. For viral RNA study, the whole brain tissue of each animal is carefully removed from the foramen magnum with a pasteur pipette without damaging the skull [21]. Brain tissues were taken into the RNA preservation solution and stored at -80 ° C. RNA extraction was performed with Acide-guanidium-phenol-chloroform method [22].

Table 1. Distribution of species by locality.

	Species		
Locality	***Myodes glareolus***	***Apodemus* sp.**	**Total**
Akpınar	2	17	19
Temenler	13	7	20
Türkali	-	12	12
Derecikören	7	14	21
Çömlekçi	7	6	13
Yeşilköy	2	6	8
Çomranlı	-	1	1
Yeşilyayla	-	4	4
Y. İhsaniye	3	9	12
A. İhsaniye	1	5	6
Sazköy	2	18	20
Sarmaşık	-	27	27
Sarmasık countrysice	6	29	35
Olukyanı Village	-	11	11
Filyos	2	31	33
Total	45	197	242

FIGURE 2. Sample collected localities.

Two forward primers were designed, and Heaton et al. [23] reference primers were used (Table 2).

TABLO 2. Rabies (RABV) and rabies like viruses primers for (RRV) hnRT-PCR [23].

Primer	Sequence	Sense	Positon
JW12	ATGTAACACC(C/T)CTACAATTG	M	55-73
JW6 (DPL)	CAATTCGCACACATTTTGTG	G	660-641
JW6 (E)	CAGTTGGCACACATCTTGTG	G	660-641
JW6 (M)	CAGTTAGCGCACATCTTATG	G	660-641
JW10 (DLE2)	GTCATCAAAGTGTG(A/G)TGCTC	G	636-617
JW10 (ME1)	GTCATCAATGTGTG(A/G)TGTTC	G	636-617
JW10 (P)	GTCATTAGAGTATGGTGTTC	G	636-617
SB1	GATCA(A/G)TATGAGTACAAGTACCCTGC	M	140-165
SB2	GATCAATATGAATATAAATATCCCGC	M	140-165

Already published RABV sequences that derived from different taxon were downloaded from Genebank (Suppl. Table 8). The sequences were aligned by MEGA v.5.2.1 [24].

The forward and reverse primers were selected from the conserved region of the whole genome, the N gene, to be replicated to 300 nucleotide regions. To determine the suitability of the selected primers, we uploaded to the web page of Integrated DNA Technologies (http://eu.idtdna.com/) to review the melting temperature, GC percentage, self-dimer and hair-pin states of the primers, as well as BLAST (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). The software was used to check sequence and organism matches.

2.1. cDNA Synthesis

In order to replicate the DNA of RABV RNA virus by PCR, first of all, cDNA synthesis was performed. cDNA synthesis was performed according to the recommendations of the manufacturer (Fermentas #EP0352) Details were provided in the additional file.

2.2. PCR optimization with designed primers for RABV

In the optimization process of the designed primers, the sensitivity of the polymerase chain reaction was maximized and the lowest amount of RNA found in the tissue was targeted (Table 3). For this reason, positive control dilutions with a known initial amount (104 copies/ml) were used.

Reaction mixture concentrations, loops, and annealing temperatures were determined with Hemi-nested and Touchdown PCR. The most appropriate PCR mixtures and reaction conditions are given in the additional files to search the samples for both designed and reference primers.

TABLE 3. In this study the designed primers for the N gene of RABV for PCR.

Primer	Sequence	Polarity	Positon	Number of Nucleotide	%GC	Tm	Hairpin Tm	Self-dimer (kcal/mole)
RABV-F	5'-ATGGATGCYG AYAAGATTG-3'	Sense	1-19	19	% 42.1	50.0 °C	-	-
RABV-R1	5'-GTC ART TCC AWG CCT CCT G-3' Reverse: 5'- CAG GAG GCW TGG AAY TGA C -3'	Antisense	368-386	19	% 55.3	54.6 °C	-	-
RABV-R2	5'- ACG YTT TAT BTC YAC CAG AGA -3' Reverse: 5'- TCT CTG GTR GAV ATA AAR CGT -3'	Antisense	319-339	21	% 41.3	52.5 °C	-	-

2.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to view the PCR products. 2 µL of 10xDNA loading buffer was mixed into 10 µL of PCR product and loaded into wells drilled in the gel. A "ladder" was loaded in one of the wells. The electrophoresis power supply was set to 120V and the gel was run for 40 minutes. The gel was visualized in medium-wave UV (280-340nm) in a transluminator and photographed with the (Vilber Lourtmat Infinity-1000/26MX) device. The displayed band sizes were compared with the "ladder".

2.4. Sequence Analysis

Samples with positive bands were sent to Macrogen company for sequence analysis. After a second PCR of the purificated PCR products was performed, the sequences were created with the ABI device. the Sequences are controlled with Seqscape and Bioedit programs and aligned with MEGA and Bioedit programs; aligned sequences were blast-analyzed with sequences in GenBank.

3. Results and Discussion

In this study, RABV screening was performed with Hemi-nested and Touchdown PCR methods in 242 samples collected from 16 localities in Çaycuma district of Zonguldak province (Figure 3, Table 4).

Table 4. Positive samples and information.

Sample no	Species	Locality
6511	*Apodemus* sp.	Filyos
6517	*Apodemus* sp.	Türkali
6536	*Myodes glareolus*	Çömlekçi
6810	*Myodes glareolus*	Filyos
6686	*Apodemus* sp.	Yukarı İhsaniye
6867	*Apodemus* sp.	Olukyanı
6582	*Myodes glareolus*	Temenler
6677	*Myodes glareolus*	Yeşilköy

FIGURE 3. The distribution of Positive samples.

It was observed that the band size of the R + control was the same as expected (Figure 4). RNA extraction from the brain tissue of the specimens and PCR screening performed as a result of cDNA synthesis revealed a positive RABV in 8 samples of 8 localities. The result of PCR scanning with the reference primers is shown in Figure 5. Table 4 shows the information about the localities and samples where the positively identified samples were collected.

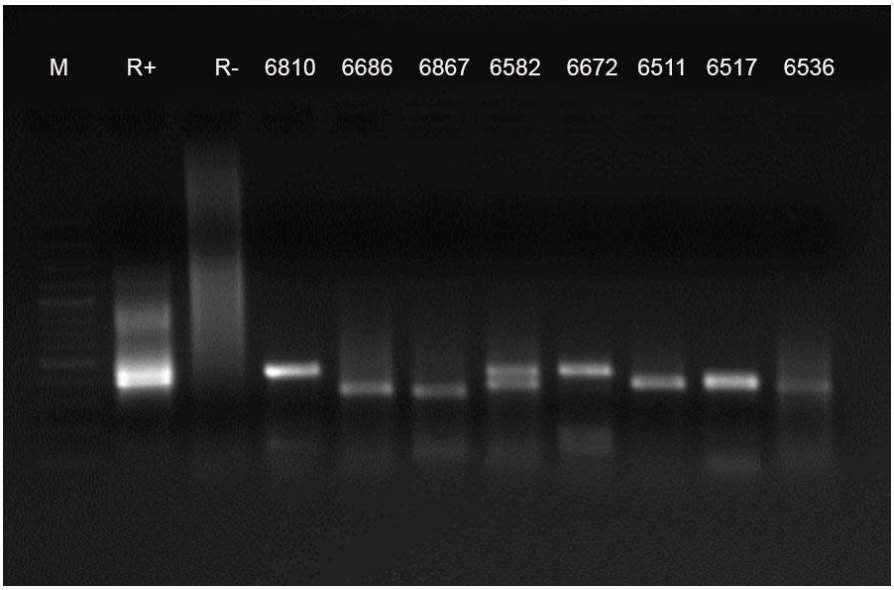

FIGURE 4. Gel images of positive samples from PCR results of the designed primers.

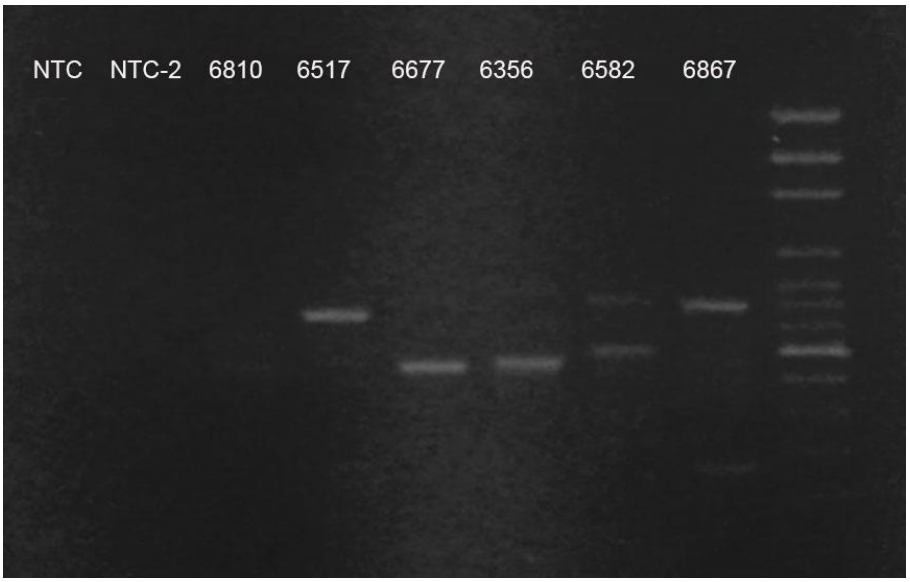

FIGURE 5. Gel image of the PCR result performed as a service delivery with the reference primers.

However, since these findings need to be verified at a higher DNA sequence level, PCR products of positive samples were sent to Macrogen for DNA sequence analysis. Sequences were obtained from 8 samples with an average of 340 bp. However, in blast analyzes, it was found that only two samples showed a low degree of similarity with the sequences of published Rabies viruses.

Because rabies has not a single host, the virus rapidly spreads from one infected animal to another by inter-species interaction. The existence of rabies in a host or in a specific area should be double check by investing among prey species that move faster, quicker than their predators. In the diets of carnivores, rodents have been assumed as an important member of their diets [25]. Due to ecological niches, rodents were the first place in the investigation of presence of rabies. In developed countries, rabies was a disease usually seen in wild animals and transmitted from these hosts to pets and humans [11, 26]. Human rabies suspected animal bites and rabies was still an important health problem in Turkey [27] and as seen as intense in these cases where the quarantine zone was formed. When we look at the study area, the distribution of the carnivores in the area was fragmented due to the fragmentation of the settlements and it was uncertain that they might spread throughout the area. From this point of view, it was likely that rodents might be a source that spread the rabies virus. Lei et al. [19] showed the presence of rabies virus in *Apodemus agrarius* and emphasized that they could play a role in human rabies. Winkler, Schneider & Jennings [27] described rabies rodent specimens at 31 out of 50 states in the United States between 1953-1970. It was also reported that rodent rabies were as common as other species [27]. Nel & Markotter [28] stated that rodents could be considered as carriers of rabies virus. However, in the Middle East, only squirrels were found among the rodents as carriers of the rabies [28]. Mukherjee et al [25] stated that rodents constitute a significant amount of carnivores and feline nutrients. In this study, positive band was obtained from 8 samples among 242 samples when the sequence results were examined, with a 23 bp similarity was found with the rabies virus in the sequences of only two out of the eight samples. According to these results, it can be stated that rodents in the study area were likely to carry rabies among fragmented habitats since carnivores could not travel among the habitats.

As a result of the rabies protection and control guidelines issued by the Ministry of Health in 2005, vaccination was applied as a result of contact with any species that was likely to be exposed to rabies [29]. However, as stated in the directive, no vaccination was performed for those who was bitten and contacted by other small rodents such as mice [27]. Lei et al. [19] reported that one of 57 *Apodemus agrarius* specimens collected from the Zhejiang region of China was positive for rabies. In addition, according to Özsoy et al. [20], 92 (6 %) patients who came to Refik Saydam Hıfzısıhha Center Rabies Vaccine Station with a suspicious animal bite applied to

this unit due to rat bites. However, we also suggest a final test to check each specimen using recent molecular diagnostic tool listed in Duong et al. [30].

In order to obtain a host of information about missing data for the study of rabies in Turkey, our study is based on investigating the presence of rabies virus in rodents in a quarantine zone was the first study. Even the study might be considered as a preliminary study We discovered some clue that rodents could carry the virus among fragmented area since carnivores couldn't travel among habitats so that during fighting back an epidemic disease, the screening of rabies virus on rodents might be examined by a bigger sampling. Our secondary finding was new primer pairs for detecting rodents. To design new primer pairs against a group which has highly mutational genome and capability to do would be crucial for further studies. Recent molecular diagnostic tools have a potential to give interesting results especially in quarantine site.

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Author Contribution Statement TG and FM - specimen collection, wet lab works Virus identification (TG), data analysis, manuscript writing. MAÖ-Virus identification, data analysis, manuscript writing.

Declaration of Competing Interests The authors declare no conflict of interest.

References

[1] Us, D., Moleküler, Klinik ve Tanısal Viroloji, Güneş Tıp Kitapevi, Ankara, 2008.
[2] Van Regenmortel, M. H. V., Fauquet C. M., Division M. S. V., Bishop, D. H. L., Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses, Academic Press, 2000.
[3] Fu, Z. F., The World of Rhabdoviruses, Springer Verlag, Berlin, 2005.
[4] Fu, Z. F., Rabies and rabies research: past, present and future. *Vaccine*, 15 (1997), 20-24. https://doi.org/10.1016/s0264-410x(96)00312-x
[5] Wagner R.R., The Rhabdoviruses, Plenum Press, New York, 1987
[6] Travassos Da Rosa, A. P. A., Mather, T. N., Takeda, T., Whitehouse, C. A., Shope, R. E., Popov, V. L., Guzman, H., Coffey, L., Araujo, T. P., Tesh, R. B., Two new rhabdoviruses (Rhabdoviridae) isolated from birds during surveillance for arboviral, *Emerging Infection Disease*, 8 (6) (2002), 616–618.

https://doi.org/10.3201/eid0806.010384

[7] Kuzmin, I. V., Orciari, L. A., Arai, Y. T., Smith, J. S., Hanlon, C. A., Kameoka, Y., Rupprecht, C. E., Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationships according to N, P and G gene sequences, *Virus research* 97 (2) (2003), 65-79. https://doi.org/10.1016/s0168-1702(03)00217-x

[8] Gönen, İ., Türkiye'deki Hekimlerin Kuduz Hastalığıyla İlgili Bilgi ve Tutumlarınının Değerlendirilmesi. Uzmanlık Tezi, Sağlık Bilimleri Enstitüsü Çocuk Sağlığı ve Hastalıkları Anabilim Dalı İstanbul. (2010).

[9] Lackay, S. N., Kuang, Y., Fu, Z. F., Rabies in small animals. Veterinary Clinics of North America, *Small Animal Practice*, 38 (4) (2008), 851-861. https://doi.org/10.1016/j.cvsm.2008.03.003

[10] Buzgan, T., Irmak, H., Yılmaz, G., Torunoğlu, M. A., Safran, A., Epidemiology of human rabies in Turkey: 1992-2007, *Turkish Journal of Medical Science*, 39 (4) (2009), 591-597. https://doi.org/10.3906/sag-0901-6

[11] Meslin, F., Fishbein, D., Matter, H., Rationale and prospects for rabies elimination in developing countries, *Current topics microbiology and immunology*, 187 (1993), 1-26. https://doi.org/110.1007/978-3-642-78490-3_1

[12] Özkütük, N., İnsan Diploid Hücresi Kuduz Aşısı (HCDV) İle İkinci Kuşak Hücre Kültürü Kuduz Aşılarının Etkinliklerinin Karşılaştırılması. Doktora Tezi, Sağlık Bilimleri Enstitüsü Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı Manisa, (2002).

[13] Mandell, G. L., Bennett, J. E., Dolin, R., Principles and Practice of Infectious Diseases. *Elsevier Churchill*, Livingstone, (2009).

[14] Fooks, A. R., Johnson, N., Freuling, C. M., Wakeley, P. R., Banyard, A. C., McElhinney, L. M., Marston, D.A., Dastjerdi, A., Wright, E., Weiss, R. A., Müller, T., Emerging Technologies for the Detection of Rabies Virus: Challenges and Hopes in the 21st Century, *PLOS Neglected Tropical Diseases*, 3(9) (2009), e530. https://doi.org/10.1371/journal.pntd.0000530

[15] WHO. WHO: Mediterranean Zoonoses Control Centre Information Circular, 33 (1994) 7-9.

[16] Gang, S. R., Rabies in Man and Animals. Springer Verlag, India, 2014.

[17] Blanton, J. D., Palmer, D., Dyer, J., Rupprecht, C. E., Rabies surveillance in the United States during 2010, *Journal of American Veterinary Medicine Association*, 239, (2011), 773–783. https://doi.org/10.2460/javma.239.6.773

[18] CDC. The burden of rabies. Centers for Disease Control and Prevention. http://www.cdc.gov/Features/ dsRabies/. Accessed 8 Apr 2012.

[19] Lei, Y., Wang, X., Li, H., Chen, X., Ye, B., Liu, F., Lan, J., Ye, X., Mei, J., Tang, Q., [New animal hosts of rabies virus in mountain areas in Zhejiang province]. Zhonghua liu xing bing xue za zhi. *Zhonghua liuxingbingxue zazh*, 30 (4) (2009), 344-347.

[20] Özsoy, M., Yakıştıran, S., Özkan, E., 2000 Yılında Kuduz Aşı Merkezine Başvuran Hastaların Değerlendirilmesi, *Türk Hijyen ve Deneysel Biyoloji Dergisi*, 59 (1) (2002), 1-6.

[21] Iamamoto, K., Quadros, J., Queiroz, L., Use of aspiration method for collecting brain samples for rabies diagnosis in small wild animals, *Zoonoses and Public Health*, 58 (1)

(2011), 28-31. https://doi.org/10.1111/j.1863-2378.2009.01282.x

[22] Chomczynski, P., Sacchi, N., The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on, *Nature protocols,* 1 (2) (2006), 581-585.

[23] Heaton, P. R., Johnston, E. P., McElhinney, L. M., Cowley, R., O'Sullivan, E., Whitby, J. E., Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses, *Journal of Clinical Microbiology,* 35 (11) (1997), 2762-2766. https://doi.org/10.1128/jcm.35.11.2762-2766.1997

[24] Kumar, S., Nei, M., Dudley, J., Tamura, K., MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences, *Briefings in Bioinformatics,* 9 (4) (2008), 299-306. https://doi.org/10.1093/bib/bbn017

[25] Mukherjee, S., Goyal, S., Johnsingh, A., Leite Pitman, M. R. P., The importance of rodents in the diet of jungle cat (*Felis chaus*), caracal (*Caracal caracal*) and golden jackal (*Canis aureus*) in Sariska Tiger Reserve, Rajasthan, India, *Journal of Zoology,* 262 (4) (2004), 405-411. https://doi.org/10.1017/S0952836903004783

[26] Kilic, B., Unal, B., Semin, S., Konakci, S. K., An important public health problem: rabies suspected bites and post-exposure prophylaxis in a health district in Turkey, *International journal of infectious diseases*, 10 (3) (1997), 248-254. https://doi.org/10.1016/j.ijid.2005.05.010

[27] Winkler, W. G., Schneider, N. J., Jennings, W. L., Experimental rabies infection in wild rodents, *Journal of wildlife diseases,* 8 (1) (1972), 99-103. https://doi.org/10.7589/0090-3558-8.1.99

[28] Nel, L. H., Markotter, W., Lyssaviruses, *Critical Reviews in Microbiology*, 33 (4) (2007), 301-324. https://doi.org/10.1080/10408410701647602

[29] URL.http://www.saglik.gov.tr/TR/belge/1-15918/kuduz-korunma-ve-kontrol-yonergesi.html. (2013).

[30] Duong, V., Tarantola, A., Ong, S., Mey, C., Choeung, R., Ly, S., Bourhy, H., Dussart, P., Buchy, P., Laboratory diagnostics in dog-mediated rabies: an overview of performance and a proposed strategy for various settings, *International Journal of Infectious Diseases,* 46 (2016), 107–114. https://doi.org/10.1016/j.ijid.2016.03.016