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ORIGINAL ARTICLE

First isolation and molecular characterization of pseudorabies virus detected in Turkey

Gizem Aytogu[1] · Eda B. Toker[1] · Ozkan Yavas[2] · Berfin Kadiroglu[1] · Ozer Ates[1] · Musa Ozgur Ozyigit[2] · Kadir Yesilbag[1]

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Abstract

Background Pigs are the main host species for the pseudorabies virus. It causes fatal encephalitis in many species, including humans. This article aims to report the first clinical case of pseudorabies as well as isolation and molecular characterization of the virus from a hunting dog in Bursa province, Turkey.

Methods and results The dog shows clinical signs including pruritus and neurological signs such as stumbling and inability to stand up compatible with pseudorabies. The virus isolates were obtained from the supernatant of fresh tissue samples from the cerebellum, cornu ammonis, spleen, salivary gland, conjunctival swab, serum, and PBMC samples. The glycoprotein C region is targeted for viral DNA amplification. Pseudorabies virus genome detected both in fresh tissues and supernatants of third passage on Vero cells. The number of PCR positive samples was dramatically increased after cell culture inoculations. Genome sequencing of strain Bursa-10303, which was isolated from a non-endemic area, identified it to belong to clade A.

Conclusions This study confirms the possible presence of pseudorabies infection in the wildlife reservoirs in Turkey. Future studies may clarify the importance of the infection in Turkey region, where there is no prevalent pig production.

Keywords Dog · Molecular characterization · Pseudorabies · Virus isolation

Introduction

Pseudorabies (PR), also called Aujeszky's disease, is caused by *Suide Herpesvirus-1* (SuHV-1, or synonymously Pseudorabies virus), which is a member of *Alphaherpesvirinae* subfamily within the family *Herpesviridae* [1]. Infection is an economic concern of the swine industry. The agent is generally responsible for fatal infections and manifests as respiratory distress, nervous or genital disorders in pigs. However, high mortality rates are seen in piglets rather than swine. Like other Alphaherpesviruses, Pseudorabies virus (PrV) can develop a lifelong latent infection in neuronal tissues of pigs. Even if the affected animal survives, the virus circulates among the wild boar and domestic pig populations. Saliva, nasal discharge, and airborne particles are the sources of transmission [2].

Swine (suids) is the primary host and reservoir of PrV, but the virus also has a broad spectrum of host species, including cattle, sheep, goats, dogs, cats, chickens, rodents, rabbits, and guinea pigs [1, 3]. Moreover, the infection can be sporadically transmitted to humans and may induce encephalitis [4, 5]. However, inter-species transmission or evolution of PrV has been reported to vary between regions [2]. Dogs are assessed to be the dead-end host and infected mainly by consuming contaminated pork meat or offal as well as direct contact with infected pigs [6].

PrV infection in dogs has been reported in different parts of the world. The regions where the presence of the infection in dogs has been reported include America, Europe, and China [7–13]. This study describes the first isolation and genetic characterization of pseudorabies virus from hunting dog exhibited neurological signs in Turkey.

✉ Kadir Yesilbag
kyesilbag@uludag.edu.tr

[1] Department of Virology, Faculty of Veterinary Medicine, Bursa Uludag University, 16059 Bursa, Turkey

[2] Department of Pathology, Faculty of Veterinary Medicine, Bursa Uludag University, 16059 Bursa, Turkey

Material methods

The case and samples

Based on the information from the field veterinarian, the case was a male dog exhibiting clinical signs compatible with PrV-infection. The dog owner permitted the analysis to detect the infection and declared that the dog consumed raw pork meat and offal. The dog came from Mustafakemalpaşa district of Bursa province; Turkey was presented to the veterinarian exhibiting difficulty in breathing, fever, unilateral ocular swelling, and pruritis a few days after consuming the raw meat. A few hours after reaching the clinic, the dog started to display stumbling and inability to stand up. The whole blood and serum samples were taken while the animal was alive; however, the dog died shortly after blood sampling. Samples of cerebrum, cerebellum, cornu ammonis, lung, heart, lymph nodes, liver, spleen, duodenum, pancreas, kidney, salivary gland, conjunctival swab, and also PBMC and serum samples were collected during necropsy.

After the PrV case was detected, sampling was performed for antibody screening in hunting dogs in the region. For this purpose, serum was collected from 31 dogs actively used in pig hunting from Mustafakemalpaşa district, where the infected dog is sheltered. Sera were obtained according to the national and local ethical committee (HADYEK; 2021–05/01). Blood samples were centrifuged at 1500 rpm for 10 min for serum separation and 3000 rpm for 10 min for peripheral blood mononuclear cell (PBMC) separation. Sera were heat-inactivated at 56 °C for 30 min. Collected PBMC were re-suspended in phosphate buffer saline (PBS), then both were stored at − 20 °C. Half of the tissue samples were immersed in 10% neutral buffered formaldehyde, and the remaining part was immediately used for virological examinations without freezing. The tissue samples were homogenized in PBS and were filtrated through 0.22 µm-pore filters after centrifugation at 3000 × rpm for 10 min.

Virus isolation

The virus isolation was performed on cell lines of Vero (African green monkey) and MDBK (Madin-Darby Bovine Kidney). All cell lines used in this study were obtained from Bursa Uludağ University Faculty of Veterinary Medicine Department of Virology stock. Supernatants of tissue homogenates (0.2 ml) were inoculated into 24 well plate and incubated for 60 min at 37 ºC in an atmosphere of 5% CO_2. At the same time, PBS which was used at tissue homogenate preparation was inoculated to control wells. After incubation, DMEM without fetal calf serum was added into each well and further incubated at 37 ºC for daily examination of cytopathogenic effect (CPE) using an inverted light microscope. The virus isolation process was continued for 3 passages for all the samples to increase the viral load for subsequent characterization. Viral titers in the samples with CPE in the third passage level in the Vero cell line were calculated using the Spearman-Karber method.

Histopathological analysis

Specimens of the brain, cerebellum, lungs, kidney, liver, spleen, heart and intestine were collected, fixed in 10% buffered formalin solution, and processed routinely. The samples were embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin.

Virus neutralization assay

The virus neutralization test (VNT) was performed to analyze the antibody titers in diseased dogs, and samples from dog possibly have sufficient contact with infected wild pigs in the region. Serial two-fold dilution, starting at 1:2 was, prepared in a serum-free medium. An equal volume of virus suspension with a titer of 100 $TCID_{50}$ and diluted sera were mixed in 96-well tissue culture plates. After incubation for 120 min at 37 °C and 5% CO_2, Vero cell suspension (2×10^5 cell/ml) was added. The plate was observed under an inverted light microscope for CPE for four days post-infection.

Virus identification

The DNA extraction was carried out using a commercial kit (Machery & Nagel, Germany) before virus identification by Polymerase Chain Reaction (PCR). Amplification of a 791 bp fragment using primers gC-2U GTTTCCTGA TTCACGCCCACGC and gC-1L GAAGGGCTCACCGAA GAGGAC [14] targeting a part of the gC (UL44) were performed using Dream Taq™ Hot Start PCR Master Mix (Thermo-Fisher Sci). PCR was performed using the following conditions: denaturation step for 5 min at 95 °C, followed by 35 cycles of 50 s at 55 °C, 50 s at 60.7 °C, 50 s at 72 °C and final elongation step for 5 min at 72 °C. Sequence analysis was performed on the sample (cerebellum), showing the fast characteristic CPE appearance and strongest band on the gel. Sequence reading is performed bidirectionally to avoid possible loss of reliability. Obtained and reference sequences (Table 1) were edited

Table 1 Origin, host species and accession numbers of reference sequences used for phylogenetic analyses

Accession number	Year	Origin	Species (attitude)	Clade	Accession number	Year	Origin	Species (attitude)	Clade
JQ081290	2010	Austria	Wild boar	A	KF779462	1976	Belgium	Swine	A
JQ081292	2010	Austria	Hunting dog	A	KF779465	1988	Belgium	Swine	A
JQ081284	2005	Austria	Hunting dog	A	KP862617	2012	Italy	Dog	A
KP862619	2014	Italy	Hunting dog	A	KP780805	1993	Italy	Dog	A
KP862614	2011	Italy	Hunting dog	A	KF779458	2007	Belgium	Hunting dog	B
MN590177	2017	France	Dog	A	MN590215	2009	France	Dog	B
MN590216	2017	France	Dog	A	GQ259100	1999	France	Hunting dog	B
MF101748	2015	Argentina	Domestic dog	A	GQ259125	2004	Spain	Wild boar	B
JQ768125	2007	Italy	Dog	A	GQ259107	2000	Germany	Wild boar	B
JF767011	2010	Oklahoma	Hunting dog	A	GQ259099	1999	France	Hunting dog	B
KJ717942	–	Hungary	Wild boar	A	MN443965	2018	China	Dog	Asian
KT983811	2010	Greece	Wild boar	A	AF158090	–	China	Swine	Asian
KY398740	2011	–	Bartha-61/cell strain	A	KP098534	2012	China	Wild boar	Asian
MN443969	2018	China	Dog	A	KM061380	2012	China	Wild boar	Asian
D49435	1996	Japan	Swine	A	AF403051	1962	China	Cattle	Asian

using BioEdit software and aligned with the ClustalW method. Phylogenetic analysis was performed using the Maximum-likelihood method and the Kimura two-parameter model, with 1000 replicates for bootstrap analyses, by MEGA X.

Results

Virus isolation

The dense rounded cells, diffuse degradation, and lysis was detected after inoculations of serum, cerebellum, cornu ammonis, spleen, salivary gland, conjunctival swab and PBMC samples (Fig. 1). Virus isolation was performed as three blind passages. However, virus isolation could not be successfully performed in pancreas tissue because of the adverse effect of its structure-specific enzymes. When the isolation process in cell lines was compared, there was no difference in the characteristic CPE appearance. In the first passage, CPE was obtained in both cell lines from different samples. Although CPE-like morphological changes were detected in the first passage, CPE could not be obtained from most of the samples (except cerebellum and cornu ammonis) in repetitive passages in the MDBK cell. Of all the samples, isolates in Vero cell line were obtained from serum, cerebellum, cornu ammonis, spleen, salivary gland, conjunctival swab and PBMC samples (Table 2). However, isolation was successful in both cell lines in cerebellum and spleen samples at all passage repeats. And all control wells were morphologically in normal appearence at every passage.

Molecular and sequence analysis

When PCR was performed on original samples, only one sample (cerebellum tissue) gave a positive result. However, the specific genomic sequence was amplified in all nucleic acid extracts from the 3rd passages of the samples creating CPE in the Vero cells (n = 7) (Table 2, Fig. 2). To identify the molecular characteristics of the PrV isolate, the gC gene of the nucleotide sequence of the cerebellum sample, which was both positive from tissue sample and culture supernatant, was analyzed. In all PCR analyses, nuclease-free water, which was also added to samples, was used as a negative control, and no replicative bands were seen in any of the control repeats. The amplified gC gene was 791 bp in length. Sequences of Pseudorabies viruses, obtained from dog, domestic pig, or wild boar, were not distributed in separate branches. The dog sequences were located both in clade A and B. It was observed that most of the sequences are not separated according to their geographical distribution as well. While most Asian sequences and also cattle isolate were found to be grouped outside clade A and B in the tree, it was determined that only one dog sequence obtained from China took place in clade A. Mentioned China sequence in clade A grouped with a vaccine strain and a dog strain from France (Fig. 3). The genome sequence of isolate PrV/Dog/TR-Bursa-10303 grouped with wild boar, hunting dog, and swine sequences from Belgium and Austria. On the alignment of sequences, similar to Yamagata S-81 strain, an amino-acid deletion in the strain TR-Bursa-10303 was detected in the nucleotide positions 71–73 when compared with the Kolchis (KT983811), (KJ717942), Yamagata (D49435) reference,

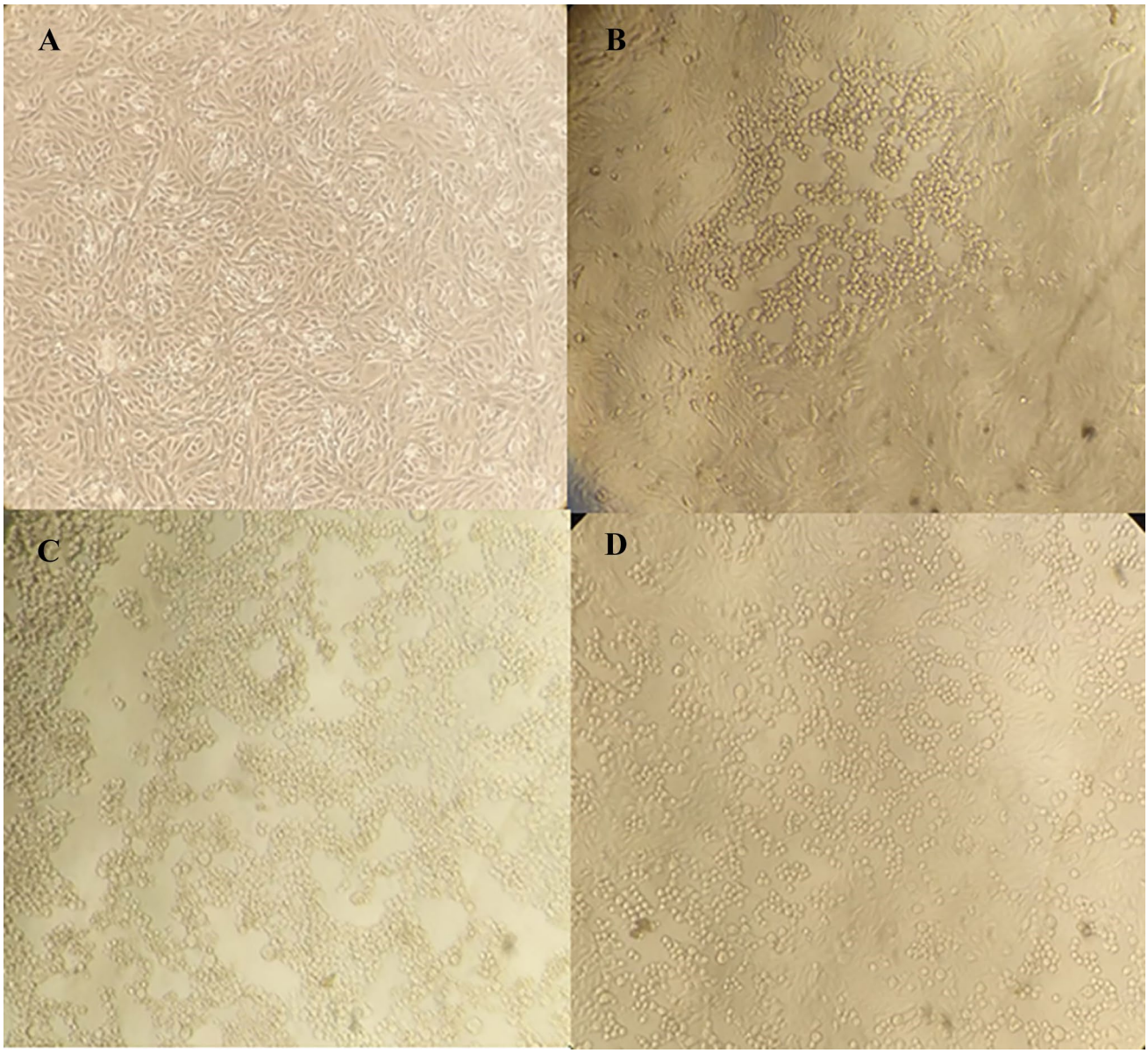

Fig. 1 Cytopathic effect caused by the isolate TR-Bursa-10303 on Vero cell line (×10). Vero monolayer (A) non infected cell control well, (B) second day of the cell line infected with PBMC sample, (C) third day of the cell line infected with cornu ammonis supernatant (D) third day of the cell line infected with conjunctival swab sample

and Bartha (KY398740) vaccine strains (Fig. 5.). Some nucleotide deletions were also detected in the amplified region. However, point mutations were found to be more common between positions 535–555. The sequence identity in the gC was 100% overlapped only with the strain BEL-55 (KF779462) the Belgian isolate but varied between 98.16 and 99.75% with aligned reference sequences (Fig. 4).

Virus neutralization

Apart from these assays, no specific antibody against the PrV virus was detected in the neutralization test with blood serum taken while the dog was alive. In addition, the presence of neutralizing antibodies was not detected in any of the blood samples randomly collected from the

Table 2 Comparison of PrV positivity results obtained by PCR and virus isolation

Sample	Virus isolation results[i] (in Vero cells)	Virus titers*	PCR results	
			Tissue sample	Cell supernatant
Serum sample	3p	$10^{5,0}$	–	+
PBMC	3p	$10^{3,5}$	–	+
Salivary gland	3p	$10^{5,25}$	–	+
Conjunctival swab	1p	$10^{5,75}$	–	+
Cerebrum	–	*NP*	–	*NP*
Cerebellum	1p	$10^{5,0}$	+	+
Cornu ammonis	3p	$10^{5,75}$	–	+
Lung	–	*NP*	–	*NP*
Heart	–	*NP*	–	*NP*
Lymph nodes	–	*NP*	–	*NP*
Liver	–	–	–	*NP*
Spleen	1p	$10^{5,0}$	–	+
Duodenum	–	–	–	*NP*
Pancreas	*NP*	*NP*	–	*NP*
Kidney	–	*NP*	–	*NP*

*Presented as $TCID_{50}$ 10^{x}/ 0,1 ml at 3rd passage, [i]the passage number which the first CPE is detected, *NP* the corresponding analysis is "not performed" in this sample, – not detected

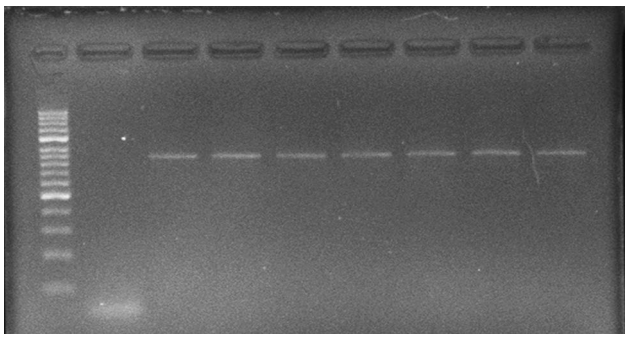

Fig. 2 Polymerase chain reaction (PCR) amplification of gC region using thirth passage of cell culture isolation. Line 1: Negative control (DNase, RNase free water); Line 2: Serum sample, Line 3: PBMC, Line 4: Salivary gland, Line 5: Conjunctival swab, Line 6: Cerebellum, Line 7: Cornu ammonis, Line 8: Spleen (expected amplicon size 791 bp)

hunting dogs in the area where the infection was detected. However, virus replication was detected in all the virus control wells.

Gross lesions and histopathology

At necropsy, diffuse bleeding areas were observed in the brain, cerebellum, lung, and intestine. Diffuse hemorrhages were noticed in the lung, kidney, spleen, liver, and intestines in histopathological examinations, particularly in the duodenum. In brain tissues, intranuclear, irregular inclusion bodies in glial cells were noticed. There was Gitter cells infiltration around diffuse demyelination area in white matter. Interalveolar thickening by mononuclear cells infiltration and edema formation were seen. In the liver, parenchymatous degeneration and intranuclear eosinophilic inclusion bodies were observed in hepatocytes. (Fig. 5) (for details see supplemental material).

Discussion

Due to its excessive economic impact, some countries, including European Union (EU) member states, incorporated PrV in surveillance programs and obtained PrV-free status in domestic pigs [7, 15]. However, PrV present in wild boar and latent course of the virus continues to pose a risk to domestic animals. By examining the genetic similarities of the isolated strains, it has been shown that hunting dogs are also involved in this spill-over. The relationship between hunting dogs and wild boars sequences and the sequence distinction of domestic pigs was shown [15]. Besides, clustering of canine PrVs together with pigs and wild boars isolates suggests that canine PrV isolates may have originated from pigs [2].

The first reported case of PrV in Turkey was in a cow from the eastern part of the country detected by immunohistochemical staining in 2016 [16]. Although rare human cases have been reported worldwide, there are no reported human cases in Turkey. In addition, there is no reported clinical case, including virological confirmation and characterization in Turkey. However, the detected PrV isolate from a hunting dog in this study in the northwestern part of the country after a cattle case in the eastern end [16], demonstrates that there may be an endemic status of the infection in wild boars in the country. Apart from these findings, veterinarians may not need laboratory diagnosis due to the rapidly developed death that occurs within 6–48 h following the onset of clinical signs in dogs [13, 15]. They may not consider PrV a causative factor. For these reasons, there may be deficiencies in detecting the possible circulation of PrV infection.

The PrV antibody prevalence can be used to evaluate the presence of infection in a region mostly for pigs [7] but dogs as well. ELISA method is predominantly preferred in serological screenings and virus neutralization

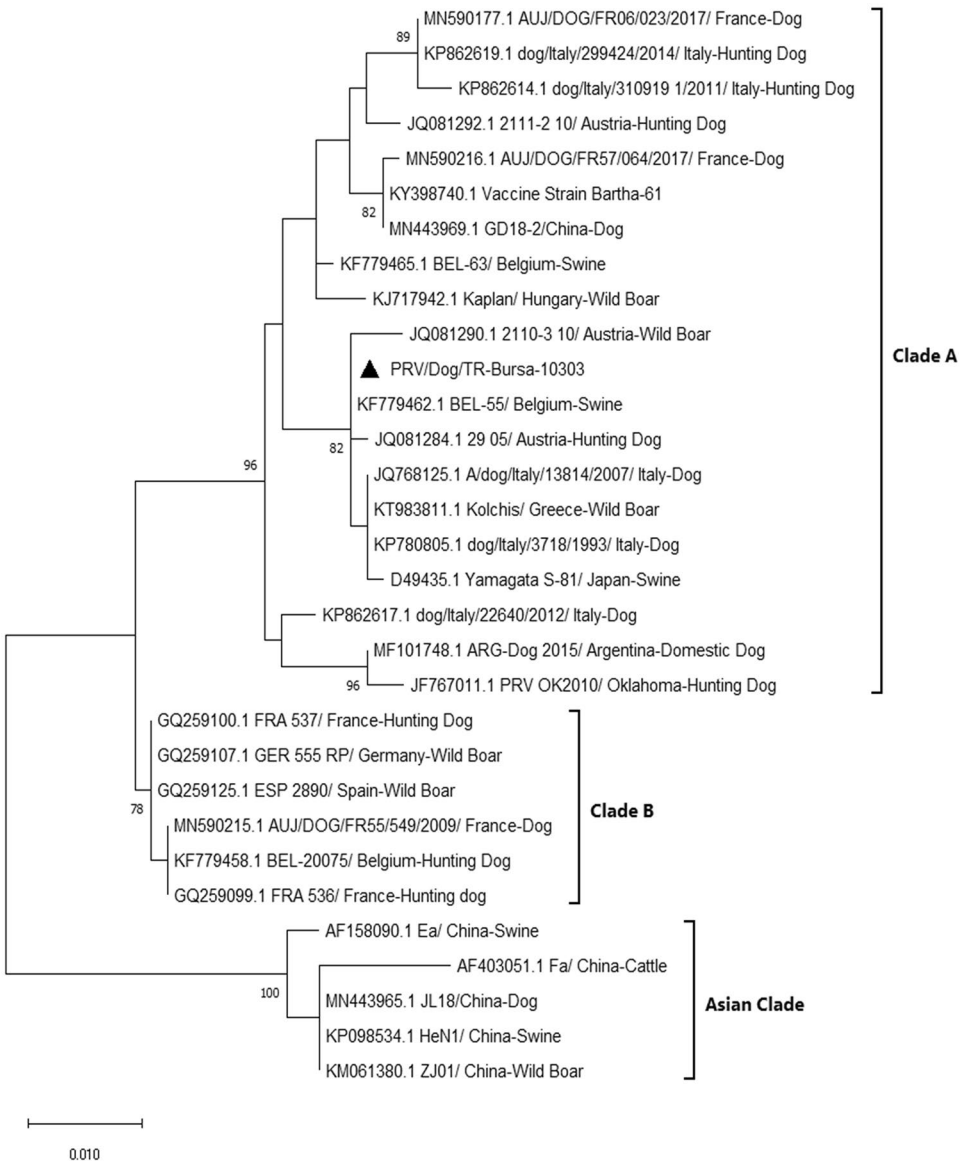

Fig. 3 Phylogenetic tree presenting the position of isolate PrV/Dog/TR-Bursa-10303 in Clade A. The target sequences are on gC genomic region of the pseudorabies virus strains. The tree was obtained using maximum likelihood method with 1000 bootstrap replicates. The Turkish dog sequence was shown as marked

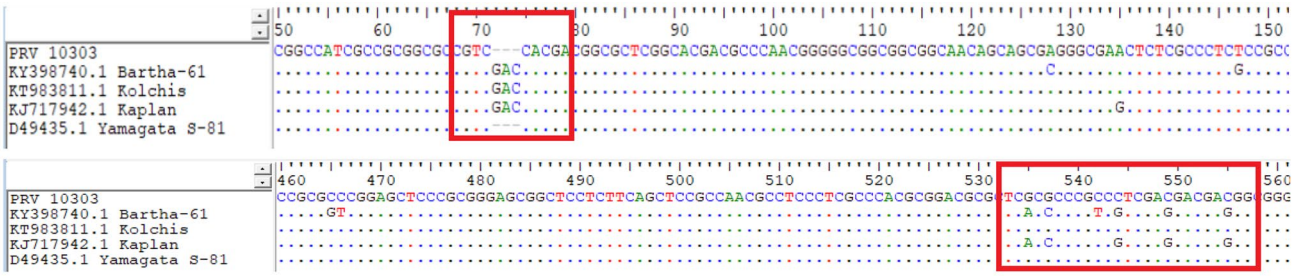

Fig. 4 Alignment of deduced nucleotide sequences of gC coding region. Alterations are indicated in boxes

assay is included only in a small proportion of studies. In China [17], the ELISA method demonstrated the presence of antibodies in 6 of 19 dog sera housed in pig farms or pet hospitals. In addition, the presence of antibodies was demonstrated by VNT in 2 of 466 samples in hunting dogs in Spain [10]. By VNT the presence of antibodies in

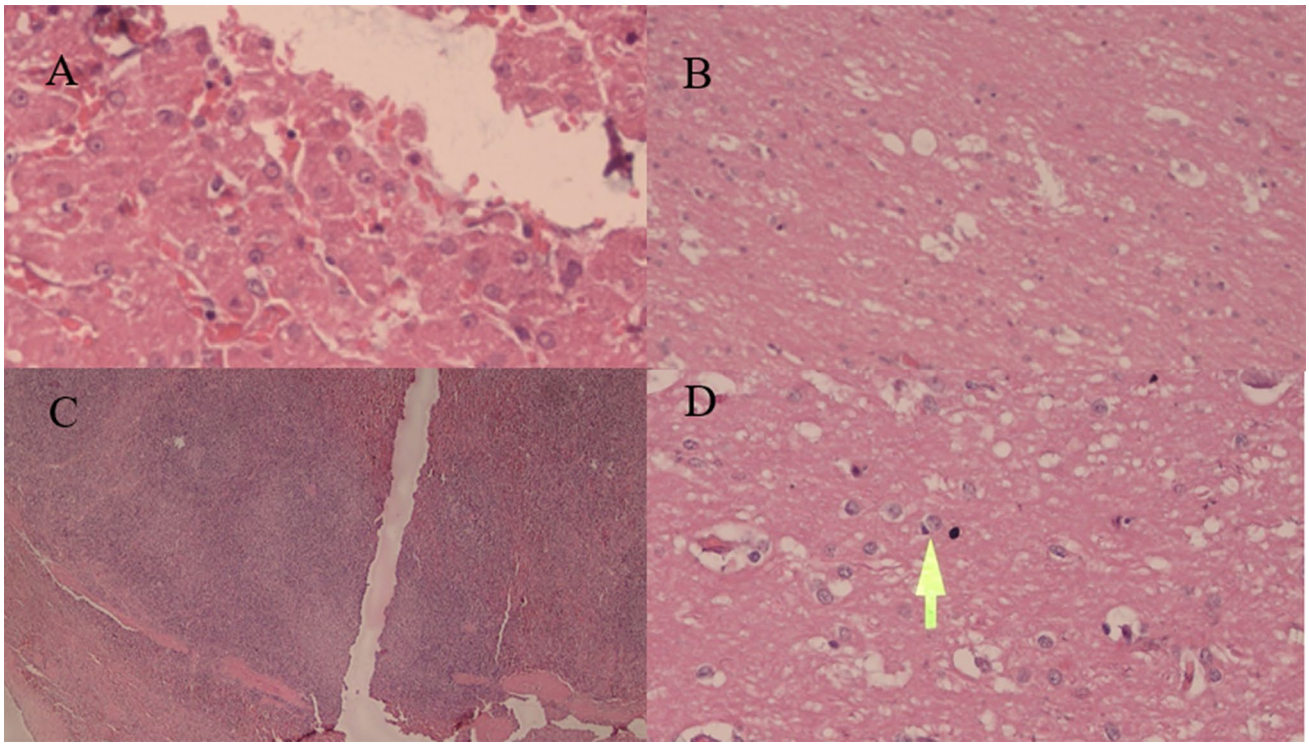

Fig. 5 H&E staining in the histopathological study of PrV infected dog tissues. (A) Extensive liver degeneration, and intranuclear eosinophilic inclusion body 40x, H-E (B) Demyelination in white matter, brain, 40x, H-E (C) Hyperplasia in white pulp, spleen 40x,H-E (D) Intranuclear inclusion body in white matter, brain

a recent outbreak in cattle in Italy has also been reported [18]. However, the dog sera tested in this study was found to be antibody negative. For PrV, the incubation period in dogs is usually 2–9 days long [19]. The period between the dog's ingestion of pork meat and its death in this study is not more than a week. Seronegative detection in the virus neutralization test of the examined dog is thought to be due to the incubation time, which may be insufficient for developing a detectable antibody response. At the same time, no antibodies were detected in the sera obtained from dogs that are actively used for pig hunting in the region. In this case, a definite conclusion could not be reached about the prevalence of the disease in the region. A survey of wild boars may help to improve understanding of the epidemiology of PrV in Turkey.

PrV can infect various types of cell cultures. In addition to the Vero cell line, which is frequently used, studies have also been carried out on the MDBK cell line [20, 21]. Wang et al. revealed no significant difference between the one-step growth kinetics of three different PrV strains but in plaque sizes. Though stated difference in CPE in comparison of variants of PrV [22]. In this study, unexpectedly, it was determined that CPE-like morphological changes could not be detected at the following passage levels of most samples in the MDBK cells. While CPE was detected at isolations of 7 samples in the Vero cell line, it was only detected at two tissue samples in the MDBK cell line. Though obtaining only two isolates in the MDBK cell line shows that preferring the Vero cell line for isolation studies will provide more successful results.

Besides, all of the PCR tests performed on the samples directly, except the cerebellum, were negative, while all the identification tests performed on the cell supernatants with CPE were positive. This may be attributed to the low amount of viral load in other tissues. Results of this study indicate that cell culture isolation, which is considered the gold standard, still has more robust reliability. In genotyping analyses, various sequences from worldwide, including European, American, or Asian territories, are included. Due to the main route of transmission to dogs is the consumption of infected meat, wild pig and dog sequences are mainly included in the tree. The absence of grouping according to host category supports the circulation by interspecies transmission. According to the information obtained from the animal owner, dogs in that region could be given adopted voluntarily to Greece. Finding similarities of the current sequences can attribute to facilitating tracing in the future. In alignment, PrV/Dog/TR-Bursa-10303 sequence was identical with a Belgium isolate (BEL-55) for the analyzed 791 bp region of gC. Moreover, both grouped with Austria strains.

All sequences of different species were intertwined. High similarity levels of sequences obtained from such distant geographic area is still obscure.

Conclusion

While the notification of infection in domestic pigs decreases in some regions of the world, PrV reported in dogs from different parts of the world is increasing [7–10, 13, 15]. The genomic characterization of PrV from the secondary host as obtained in this study will help trace the population diversity and infection origin for areas where the disease has not yet been detected or is still a problem. Future studies are warranted to assess the prevalence of PrV infections in the country in order to take precautions for the future.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06974-x.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study was approved by the Bursa Uludag University Experimental Animals Local Ethics Committee in accordance with ethical guidelines for the Care and Use of Laboratory Animals (approval number: HADYEK; 2021–05/01).

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