First Isolation and Molecular Characterization of Pseudorabies Virus Detected in Turkey

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Abstract

Pigs are the main host species for the pseudorabies virus, while it causes fatal encephalitis in many species including human being. The objective of this article is 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. The dog showing clinical signs including pruritus and neurological signs as stumbling and inability to stand up compatible with pseudorabies. The virus isolate was obtained from supernatant of fresh tissue samples from cerebellum, cornu ammonis, spleen, salivary gland, and conjunctival swab, as well as serum, and buffy coat samples. The glycoprotein C region is targeted for viral DNA amplification. Pseudorabies virus genome was detected both in fresh tissues and supernatants of 3rd passage on Vero cells. Number of PCR positive samples were dramatically increased after having cell culture inoculations. Genome sequencing revealed that the strain (Bursa-10303) isolated in Turkey where a non-endemic area is for the virus is dropped into clade A. This study confirms the presence of psedorabies infection in the wild life reservoirs in Turkey. Future studies may clarify the importance of the infection for Turkey where there is no common pig production.

Introduction:

Pseudorabies (PR), also called Aujesky'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 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 lifelong latent infection in neuronal tissues of pigs. Even if the affected animal survives, the virus continues to circulate among the wild boar and domestic pig populations. Saliva, nasal discharge and airborne particles are the sources of transmission [2].

Swine (suids) known as primarily host and reservoir of PrV, but the virus also has a wide spectrum of host species including cattle, sheep, goat, dog, cat, chicken, rodents, rabbits and guinea pigs [1, 3]. PRV is not only an important disease for animals but also for human. Moreover the infection can be sporadically transmitted to human being 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 secondary dead-end host and mostly infected 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, Austria and China until today [7−13]. This study describes the first isolation and genetic characterization of pseudorabies virus from hunting dog exhibited neurological signs in Turkey.

Material Methods:

The case and samples

Based on the alert by the field veterinarian, the case was a male dog exhibited same clinical findings typical for PrV. The dog owner also declared that the dog consumed raw pork 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 few days after consuming the raw meat. Few hours after reaching to the clinic, 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 taking blood samples. Samples of cerebrum, cerebellum, cornu ammonis, lung, heart, lymph nodes, liver, spleen, duodenum, pancreas, kidney, salivary gland, conjunctival swab and also leukocyte and serum samples were collected at the necropsy.

After the PrV case was detected, a sampling was performed for antibody screening in hunting dogs in the region. For this purpose, blood 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 rules by 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 and collected leukocytes re-suspended in phosphate-buffer saline (PBS), then stored at -20°C. Half part of the tissue samples were immersed in %10 neutral buffered formaldehyde and the remained 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 3000x rpm for 10 min.

Virus isolation

The virus isolation was performed on Vero (African green monkey) and MDBK (Madin-Darby Bovine Kidney) cell lines. Supernatants of tissue homogenates (0.1ml) were inoculated and incubated for 60 min at 37 °C in an atmosphere of 5% CO_2. After incubation, DMEM without fetal calf serum were added into each well and further incubated at 37 °C for daily examination of cytopathogenic effect (CPE) using inverted light microscope. The virus isolation process was continued for 3 passages for all the samples in order to increase viral load for subsequent characterization. Viral titers in the samples with CPE in the third passage level in Vero cell line were calculated using the Spearman-Karber method.

Histopathological analysis

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

Virus neutralization assay

The virus neutralization test (VNT) was performed for analyzing the antibody titer in diseased dog and also in serum samples from dogs possible have efficient contact to infected wild pigs in the region. Serial two-fold dilution, starting by 1:2, prepared in serum-free medium was mixed with equal volume of virus suspension with a titer of 100 $TCID_{50}$ in 96-well tissue culture plate. After incubation for 120 min at 37 °C and 5% CO_2 Vero cell suspension ($2x10^5$ cell/ml) was added. The plate was observed under an inverted light microscope for cpe during 4 days post infection.

Virus identification

Supernatants obtained from all the tissue samples and inoculated cell cultures where cpe observed were used for virus identification by Polymerase Chain Reaction (PCR). The DNA extraction was carried out by a commercial kit (Machery & Nagel, Germany). Amplification of an 791bp fragment using primers gC-2U GTTTCCTGATTCACGCCCACGC and gC-1L GAAGGGCTCACCGAAGAGGAC [14] targeting a part of the gC (UL44) were performed using Dream Taq™ Hot Start PCR Master Mix (Thermo-Fisher Sci). PCR was performed with modifications as following conditions: denaturation step for 5 min at 95 °C, 35 cycles of 50 sec at 55 °C, 50 sec at

60.7 ºC, 50 sec at 72 ºC and final elongation step for 5 min at 72 ºC. Sequence analysis was performed on the sample (cerebellum) which both showed the fast characteristic cpe appearance and strongest band on the gel. Obtained and References sequences (Table 1.) were edited using BioEdit soft ware and aligned with the ClustalW method. Phylogenetic analyzes inferred using Maximum-likelihood method with the Kimura two-parameter model, with 1000 replicates for bootstrap analyses, using MEGA X.

Results:

Virus isolation:

The dense rounded cells, diffuse degradation and lysis was detected in cell cultures inoculated with serum, cerebellum, cornu ammonis, spleen, salivary gland, conjunctival swab and PBMC samples (Fig. 1.). Virus isolation was repeated three times to increase the virus titer. But virus isolation could not be successfully performed in pancreas tissue because 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 the 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 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.). But isolation was successful in both cell lines in cerebellum and spleen samples at all passage repeats.

Molecular and sequence analysis:

At the PCR performed on original samples a positive result is obtained in only one sample (cerebellum tissue). However, in all nucleic acid extracts from the 3rd passages of the samples creating cpe in the Vero cells (n = 7), the specific genomic sequence were amplified (Table 2.). To identify the molecular characteristics of the PrV isolate, gC gene of nucleotide sequence of cerebellum sample which was both positive from tissue sample and culture supernatant was analyzed. The amplified gC gene was 791 bp in length. According to phylogenetic analyses, dog, domestic pig or wild boar sequences were not grouped in different branches separately but they were intertwined. 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. 2.). 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 and Bartha (KY398740) vaccine strains (Fig. 4.). Some nucleotide deletions were also detected in 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) Belgian isolate but varied between 98.16–99.75% with aligned reference sequences in Fig. 3.

Virus neutralization:

Apart from these assays, no specific antibody against PrV virus was detected in the neutralization test performed 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 hunting dogs in the area where the infection was detected.

Gross Lesions and Histopathology:

At necropsy, diffuse bleeding areas in the brain, cerebellum, lung and intestine were observed. In histopathological examinations, diffuse haemorrhages were noticed in lung, kidney, spleen, liver and intestines particularly in duodenum. There was interalveolar thickening by mononuclear cells infiltration and edema formation. In liver, parenchymatous degeneration were observed. Severe degeneration of tubular epithelium in the kidney (Fig. 4) (for details see supplemental material).

Discussion:

Due to its excessive economic impact, some countries, including European Union (EU) member states, inserted PrV in surveillance programs and obtained PrV-free status in domestic pigs [15, 16]. 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. In a research [15] relationship between hunting dogs and wild boars, and also sequence distinction of domestic pigs was shown. Besides clustering of canine PrVs together with isolates of pigs and wild boars suggests that canine PrV isolates may have originated from pigs [2].

The first reported case of PrV in Turkey was in a cow from eastern part of the country detected by immunohistochemical staining in 2016 [17]. Although rare human cases have been reported around the world, there are no reported human case 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 [17], demonstrates that there may be an endemic status of the infection in wild boars in the country. Apart from this findings, veterinarians may not need laboratory diagnosis due to the rapidly developed death which occurs within 6–48 hours following the onset of clinical symptoms in dogs [13, 15] or they may not consider PrV as 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 [16] but dogs as well. ELISA method is predominantly preferred in serological screenings and virus neutralization assay is included in a small range. In China [18], the presence of antibodies in 6 of 19 dog sera housed in pig farms or pet hospitals by ELISA method was demonstrated. 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 a recent outbreak in cattle in Italy has also been reported [19]. 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 [20]. 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 the development of 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 created about the prevalence of the disease in the region. A survey of wild boars may help to better understanding of the epidemiology of PrV in Turkey.

PrV can infect various types of cell cultures. In addition to Vero cell line which is frequently used, studies have also been carried out on the MDBK cell line [21, 22]. Wang et al. revealed that there were 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[23]. In this study, unexpectedly, it was determined that cpe-like morphological changes couldn't be detected at following passage levels of most samples in the MDBK cells. While cpe was detected at isolations of 7 samples in Vero cell line, it was only detected at two tissue samples in MDBK cell line. Though when obtained isolate numbers were compared, obtaining only 2 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 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 indicates that cell culture isolation, which is considered as the gold standard, still has a stronger reliability. In genotyping analyses various sequences from all over the world 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 homogeneous distribution in phylogenetic analysis and absence of grouping according to host category supports the circulation by interspecies transmission. According to the information obtained from the animal owners during the sera sampling of the dogs in the region, it was learned that animals in that region could be given adopted voluntarily to Greece. Finding similarities of the current sequence can be attributed to facilitate the tracing in future. In alignment investigation PRV/Dog/TR-Bursa-10303 sequence was identical with a Belgium isolate (BEL-55) for the analysed 791 bp region of gC. And also both grouped with Austria strains. High similarity levels of samples from such distant geographies rather than sequence data from neighboring countries 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 [9, 10, 13, 15, 16, 24]. The genomic characterization of PrV from secondary host as obtained in this study will help to trace the population diversity and infection origin for the areas where the disease has not yet been detected or is still a problem. Future studies are warranted to assess the prevalence PrV infections in the country in order to take precautions for the future.

Declarations:

Acknowledgements

Conflict of interest

The authors declare that they have no conflicts of interest.

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Tables:

Table 1. Origin, species and accession numbers of reference sequences strains 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

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

Sample	Virus isolation results☐ (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, (☐):the passage number which the first cpe is detected, (*NP*): the corresponding analysis is "not performed" in this sample, (-): not detected.
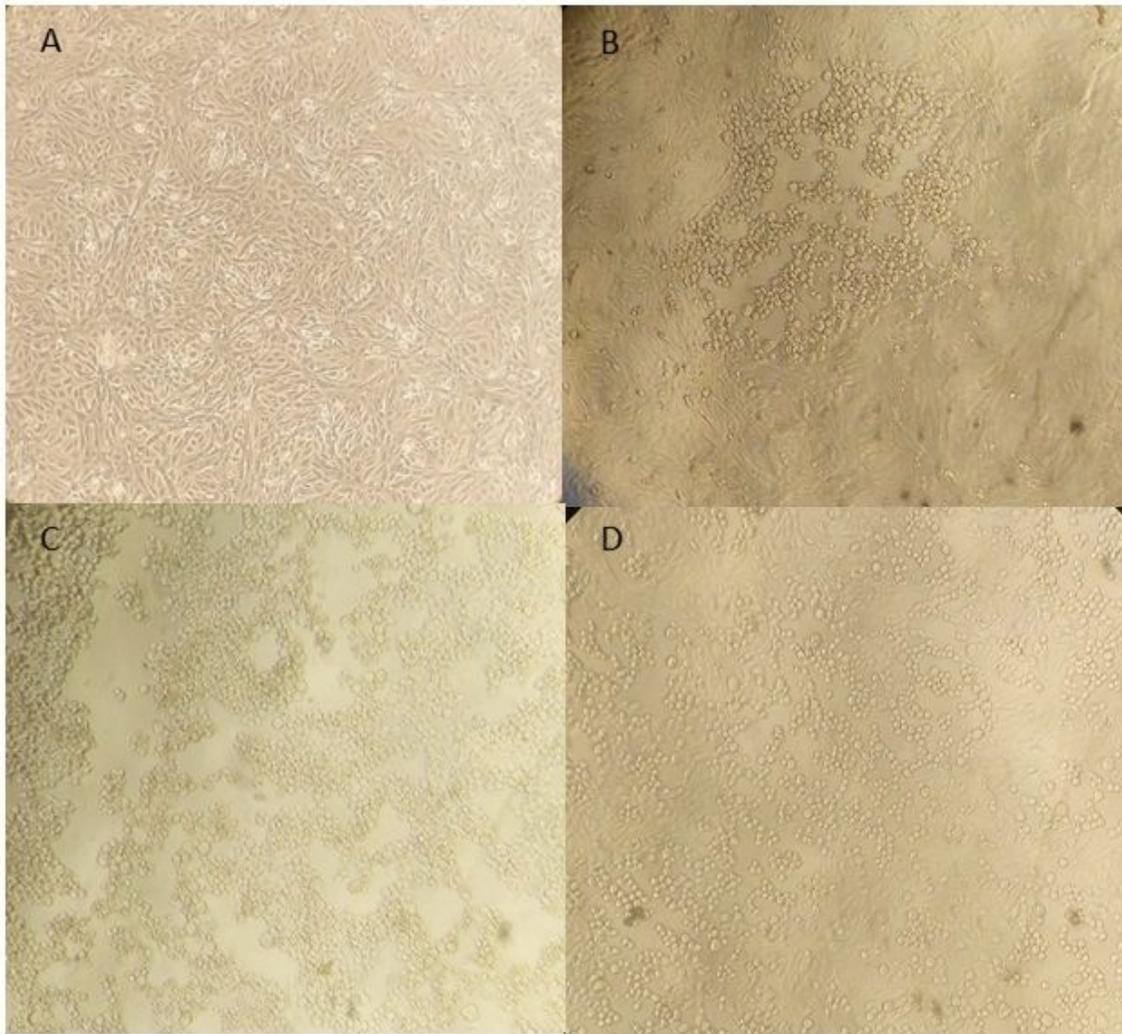
Figures

Figure 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.
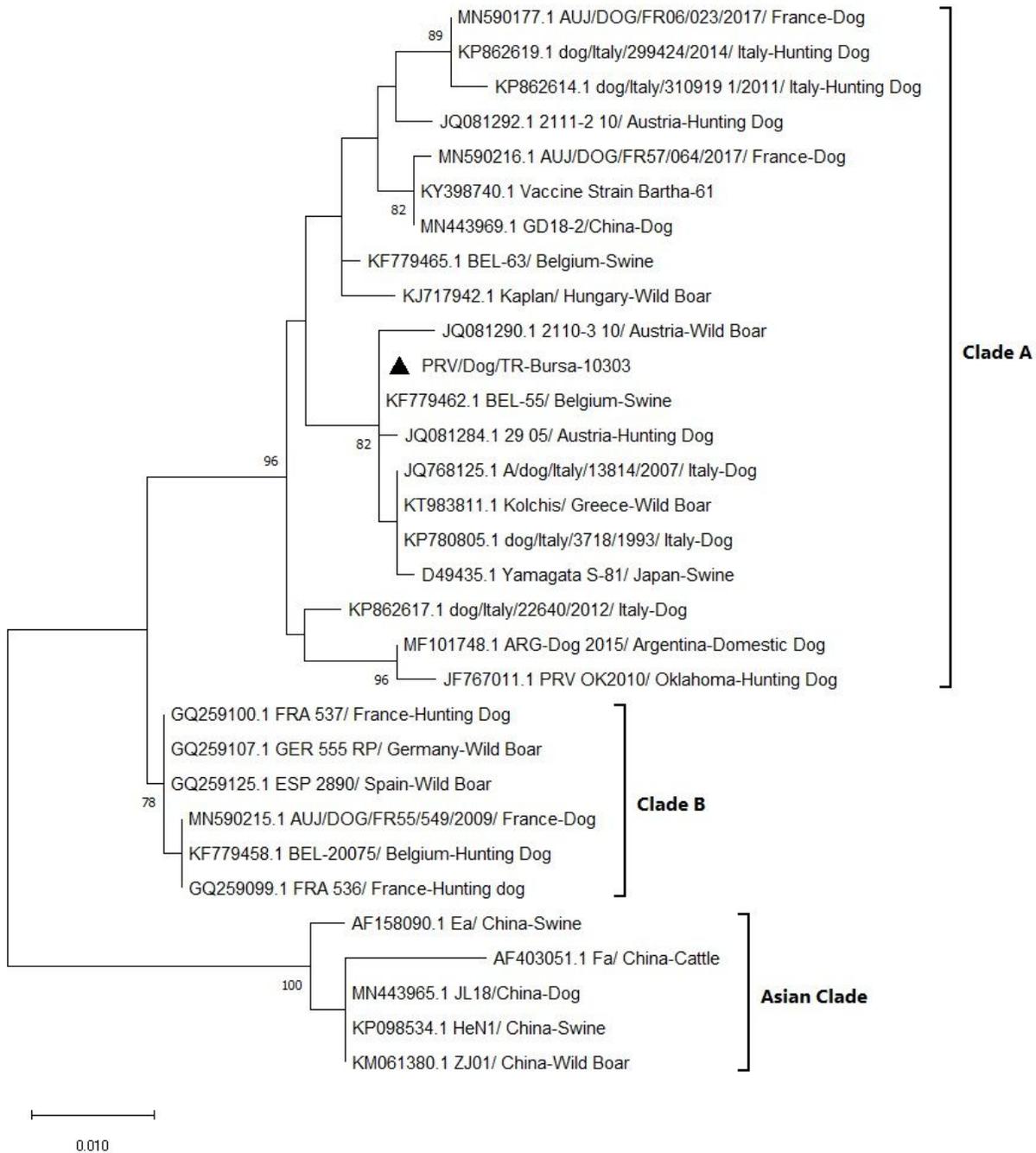
Figure 2

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.
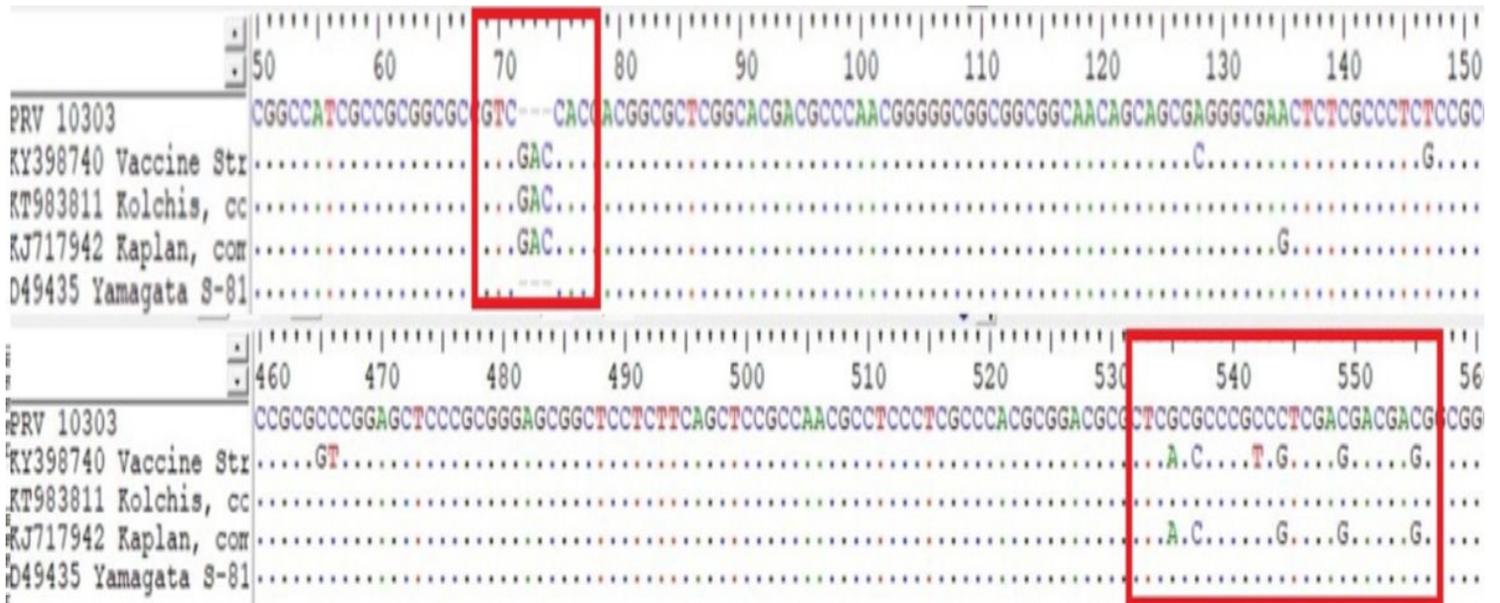
Figure 3

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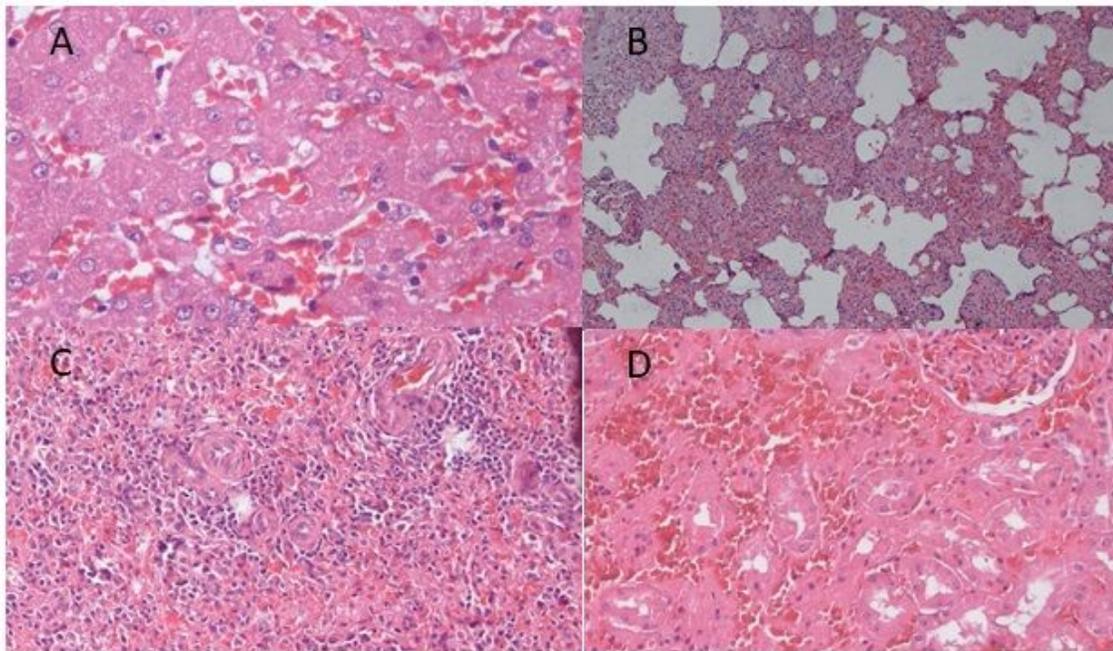

Figure 4

H&E staining in histopathological study of PrV infected dog tissues. (A) Extensive liver degeneration, 40x, H-E, (B) Thickening and hyperemia in the interstitial area of the lung,10x, H-E, (C) Hassal corpuscular in the spleen, 20x, H-E, (D) Severe bleeding and tubular degeneration in the interstitial area and glomerulus of the kidney 20x, H-E

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