Antigenic and Histopathologic Searching with Molecular Characterization and Identification of BPIV3 in Cattle with Symptoms of Respiratory System Infection

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Abstract

Parainfluenza virus is one of the viral agents causing crucial respiratory system diseases in cattle. In this study, we aimed to determine the role and genotype of BPIV3 in circulation in respiratory system diseases in cattle. For this purpose, analyses by molecular and virological ways were applied on the samples taken. During the study, a total of 570 samples as nasal swabs, conjunctival swabs and leukocyte from 190 cattle of various race, age and gender not vaccinated against the agent with symptoms of respiratory system infection were taken. Inoculums prepared from swab samples for virus isolation were planted into MDBK cell lines using adsorption method, their blind passages were performed for two times but BPIV3 could not be isolated. As a result of RT-PCR technique applied on 570 samples for molecular diagnosis, viral genome was detected in two nasal swab samples while nucleic acid could not be found in conjunctival and leukocyte samples. As a result of the sequence analysis applied on one of these samples, the agent was proved to exist in BPIV3 genotype. In addition, as a result of DIF antibody staining test applied on preparates prepared from 380 nasal and conjunctival swab samples, antigenic structures belonging to the agent were seen in 6 swab samples while virological diagnosis could not be performed in conjunctival swab samples. In two samples where viral genome presence was detected, virological diagnosis was performed by DIF test. Histopathological studies were carried out on a lung tissue sample of an animal found with molecular and virological BPIV3. At the end of these studies, some pathological findings were revealed such as septal tissue thickening indigenous to pneumonia cases and inflammatory cell infiltrations. This study has stated that in cattle with respiratory system infection in southwest regions of Turkey, PIV3 is an etiological agent and the BPIV3 genotype C of the agent is in circulation.

Introduction

The set of *Bovine Parainfluenzavirus Type 3* (*Bovine Respirovirus 3*- BPIV3) *Mononegavirales* is located in *Respirovirus* genus of *Paramyxoviridae* family *Orthoparamyxovirinae* subfamily (Maclachlan et al. 2017; Rima et al. 2019). The virus lipoprotein, which has a linear, non-segmental, negative polarity and single filament RNA genome, is surrounded by a membrane (Elenkumaran 2013). BPIV3 has 9 proteins coded by 6 genes. Among these, nucleoprotein (N), matrix protein (M), phospoprotein (P), fusion protein (F), haemagglutinin-neuroaminidase (HN) and large protein (L) are structural proteins while V, C and D proteins are non-structural ones (Ellis 2010; Spilki 2016). According to the study carried out based upon gene areas coding M and HN proteins, the agent had three different genotypes as BPIV3genotype A, BPIV3genotype B and BPIV3genotype C (Zhu et al. 2011; Elenkumaran 2013).

The agent seen as endemic all around the world causes infections in many animal species, especially cattle (Ellis 2010; Elenkumaran 2013). Immune suppressive factors such as stress, poor nutrition and poor caring conditions cause the animals to be predisposed against the disease. The virus, usually contaminated by aerosol ways, might spread in other direct or indirect ways (Sharma and Adlakha 2009; Spilki 2016). BPIV3 might cause diseases in animals of all ages; however, its prevalence is higher in animals of 2-8 months old (Elenkumaran 2013). Compared to other periods, the virus mostly proceeds

subclinically causing infections more frequently during autumn and winter months. When it is coinfected with other upper respiratory tract infection pathogens, clinical symptoms such as dyspnoea, cough, high fever and nasal and conjunctival flow appear (Maclachlan et al. 2017).

In diagnosing BPIV3, molecular techniques such as RT-PCR are frequently used as well as serological tests such as ELISA, immunofluorescence, haemagglutinin inhibition and virus neutralization. Besides, virus isolation method plays an important role in diagnosing the virus (Ellis 2010; Spilki 2016).

The aim of this study was to detect BPIV3 in molecular ways in nasal and conjunctival swab and leukocyte samples taken from animals showing respiratory system clinical findings and not vaccinated against the agent and in antigenic ways in conjunctival and nasal swab samples taken from the same animals. In addition, the strain detected by sequence analysis and drawing the phylogenetic tree was targeted to be proved to have a genetic similarity with other strains.

Material And Method

Preparing the animals and samples to be used in the study

In this research, a total of 570 samples as nasal swab (NS), conjunctival swab (CS) and leukocyte taken from 190 cattle of different race, age and gender showing respiratory system infection clinical findings and not vaccinated against the agent were used. (Table 1) Blood samples taken from *Vena Jugularis* of the animals were brought into the diagnosis lab in 5 ml tubes with EDTA, and nasal and conjunctival samples in 1.5 ml Dulbecco's Modified Eagle Medium (DMEM) added by 500 IU/ml Penicillin and 20 mg/ml Streptomycin according to cold chain procedure.

Leukocyte layer was made to disjoint by centrifuging at 1200 rpm +4**°C for 10 minutes. This layer was taken into 2 ml tubes containing 1 ml PBS by the help of a sterile Pasteur pipette. 250 microlitre (**µl) of the leukocyte washed and centrifuged at 2000 rpm +4**°C for 10 minutes was taken and put into 2 ml tubes containing no RNase-DNase. Then** 750µl total RNA isolation commercial solution was added onto it and it was kept under -80**°C until it was used in the molecular study. Having been brought into the lab, NS and CS samples were compared by vortex. Later, they were centrifuged at** 3000 rpm +4**°C for 20 minutes and 250 µl was taken from the supernatant and put into 2 ml tubes containing no RNase-Dnase in order to search the presence of viral nucleic acid. 750 µl of total RNA isolation commercial solution was added onto this sample and it was kept under –80°C until nucleic acid extraction after it was kept in room temperature for 5 minutes. The remaining supernatant was taken into 2 ml tubes and kept under –80°C to be used in cell culture studies. Also, direct immunofluorescence test was applied on preparates prepared from NS and CS samples and virological scanning was performed. Samples brought into diagnosis lab were compared by vortex. Then, they were** centrifuged at 1200 rpm +4**°C for 10 minutes and the pellet was collected. The obtained pellet was diluted with 50 µl DMEM and the preparate was prepared.** For fixation of the cells, preparates dried in room temperature were treated with acetone cooled under **-20°C** for 15 minutes.

A preparation was prepared for histopathological examination from the tissue materials after the necropsy of the animal, which died one week after the sample was taken and BPIV3 was detected molecularly and antigenically.

Cell Line and Virus Isolation

For virus isolation, Madin-Darby Bovine Kidney (MDBK) cell line and microplates with 24 compartments were used. During preparation of MDBK cell culture, 10% foetal calf serum (FDS) and 1% DMEM containing penicillin/streptomycin was used. 1 ml of cell culture suspension and DMEM was added into each compartment of the plates. The cells were incubated in a stove (Nüve, Turkey) with 5% CO_2 environment at **37** °C and were used in planting of the samples when they covered 80% of plate base.

Swab samples kept under **-80**°C in a deep freezer were thawed out swiftly in a 37°C water bath and their inoculums were prepared by passing them through 0.22 µm filters. 400 *µl was inoculated into each compartment of the microplates and was taken into* 5% CO_2 stove at **37**°C. After incubated for one hour here, the content was removed and 2 ml nonserum DMEM was added on, and then reincubated with the same values. The plate was examined for 4-10 days with an invert microscope so as to detect cytopathogen effects that could appear in cells. Blind passage was performed using supernatants obtained from microplates.

RNA Extraction, RT-PCR, Sequence

RNA extraction was performed with all the samples taken. For this purpose, together with commercial RNA isolation (Wizbiosolutions, WizolReagent, Korea) solution, chemicals such as chloroform (Sigma, USA), isopropyl alcohol (Sigma, USA) and ethanol (Sigma, USA) and cooled centrifuge (Nüve, Turkey) for high speed centrifuge during extraction were used. The process was carried out by the producing company as prescribed. In order to detect BPIV3 in molecular way, specific primers were synthesized into gene area that codes M protein at a length of 328 basepair (bp) (Mforw:AGTGATCTAGATGATGATCCA Mrev: GTTATTGATCCAATTGCTGT) (Maidana et al. 2012). All samples were treated with RT-PCR technique. Commercial kit (Geneall®HyperScript™one-step RT PCR master mix, Korea) was used for one-step RT-PCR method. *5 µl* RNA template was added onto *RNAse freewater* 2,5 *µl, BPIV3 M forw 1 µl, BPIV3 M rev 1,5 µl, master mix 10 µl (dNTPs (10 mM), $MgCl_2$ (2,5 mM), Hyperscript RTase,* AmpONE HS-Taq DNA polymerase*) and the total reaction volume was brought up to* 20 *µl. Firstly, a cDNA synthesis was achieved at* 55 *°C for 30 minutes. Following this process, viral genome amplification was performed in 35 cycles as 1 cycle 95°C* 15 min. predenaturation and 94 *°C 1 min. denaturation*, 50 *°C 1 min.* annealing and 72 *°C 1 min.* extension stages. In order to monitor amplicons, 1.2% agarose gel containing 0.07 *µl*/ml SafeView Classic (Abm, Canada) was used. Sanger sequence method was used in positive samples (Letgen, Turkey). Sequence data was aligned using Mega X program and maximum likelihood analysis was performed with 1000 boots trap replication. The reference sequences used during the regulation of phylogenetic tree were taken from GenBank.

Antigenic Detection using Direct Immunofluorescence Test

DIF test was applied on all conjunctival and nasal swab samples. The cells fixed onto a slide were stained with polyclonal antiserum conjugate (VMRD, USA) marked with a fluorescent stain (Fluoresceinisothiocyanate-FITCH) so as to reveal antigen presence. Having been incubated at 37°C for 30 minutes in a humid environment, it was evaluated under a fluorescent microscope (Olympos Co. Japan).

Histopathology

A lung tissue sample taken from an animal which died after sampling and underwent necropsy was used for histopathologic examination. The tissue samples detected by 10% formaldehyde solution were blocked onto paraffin by alcohol and xylol. They were taken onto slides after 5 micron thick serial sections were taken. Preparates were stained with haematoxylin and eosin and examined under a microscope (Olympos Co. Japan). Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan) computer program was used for morphometric analysis.

Results

Virus Isolation

Nasal and conjunctival swab samples taken from 380 animals used in the study were planted into MDBK cell cultures for BPIV3 isolation. The samples had blind passage process for two times and no cytopathological effect was found in cells at the end of microscopical examinations.

Molecular Research and Sequence Analysis

In the study carried out using RT-PCR technique for nasal swab, conjunctival swab and leukocyte samples taken from 190 animals used in the study, viral nucleic presence was found in only two nasal swab samples. (sample no: 163 and 175) BPIV3 viral nucleic acid presence was detected (Table 2). BPIV3 nucleic could not be detected in conjunctival swab and leukocyte samples. Nasal swab samplings with positive results were performed in December-February, when respiratory system diseases are frequently seen and the animals were believed to have acute infections due to symptoms ongoing for 3-7 days such as abdominal respiration, fever over 39 °C, mucopurulent nasal flow and cough. Using Mega X program, as a result of the phylogenetic analysis of the sample numbered as 163NS, whose BPIV3 agent was found by RT-PCR technique using primers indigenous to the gene area coding M protein, it was believed to have been located within BPIV3 genotype C. In addition, the agent was recorded in GenBank with MT949524 access number. On the other hand, no data could be obtained as a result of the sequence analysis of the sample numbered as 175NS, which we detected as positive. The reason for this was thought to be the fact that the extracted viral RNA had a fragile structure and was degraded during safekeeping or transportation. The sequences similarity of BPIV3 based on M gene amplicons (328 bp) has been given in Appendix 1

Antigenic Results

An antigenic research was carried out by direct immunofluorescence staining method in 380 nasal and conjunctival swab samples taken from 190 animals. During the test, BPIV3 antigen was found in 6 out of 190 nasal swab samples BPIV3 could not be detected in conjunctival swab samples (Table 2).

Histopathological Results

Number 163 animal, whose nasal swab sample showed BPIV3 at the end of molecular and antigenic RT-PCR and DIF test, died in the following days and following its necropsy, an histopathological examination was performed on lung tissue samples. In addition, the lung of a healthy animal in clinical care was used as control. No macroscopic or microscopic pathologic finding was seen in this healthy lung while samplings were performed macroscopically on areas with lesions in the lung infected with BPIV3 and severe hyperaemia in veins and partly necrotic areas drew attention during the histopathological examination. Increases in ligaments epithelial cell proliferations, thickness in septal tissue and inflammatory cell infiltrations were among frequently observed findings were observed.

Since the infected areas of the lungs were removed in formaldehyde following the necropsy, antigenic or molecular diagnosis applications from the tissue could not be performed.

Discussion

Respiratory system disease complex of cattle (BRDC) is one of the commonly observed multifactorial health problems around the world. In its ethiology, it contains many pathogen factors such as viral ones like BPIV3, BHV-1, BCoV, BRSV, BAV, BVDV and bacterial ones like *Pasteurella multicidae, Mannheimia haemolytica, Histophilus somni* (Griffin et al. 2010; Headley et al. 2018). One of the most important viral factors providing a basis for this disease complex, BPIV3, usually occurs during autumn and winter months and mostly proceeds subclinically. Depending on the decrease in animal welfare based on infection, weight loss, decrease in quality of carcase, prophylaxis, increase in expenses of veterinarian services, decrease in fertility and fatal bronco pneumonias caused by joining of other pathogens to the infection cause breeders to have extensional economic losses (Ellis 2010). Serological and epidemiological researches performed on livestock and dairy animals show that BPIV3 is endemic all over the world (Tiwari et al. 2016). Besides, a seropositivity at the rate of 18-94.6% was detected in serological researches in Turkey (Alkan et al. 2000; Yıldırım et al. 2009; Kale et al. 2013; Gür 2018).

In this study, molecular diagnosis of BPIV3 was aimed to be revealed by RT-PCR technique in nasal swab, conjunctival swab and leukocyte samples of 190 non-vaccinated animals of different race, gender and ages showing respiratory system infection clinical findings and antigenic diagnosis of BPIV3 in nasal and conjunctival swab samples of the same animals by DIF method. In order to see the effects of the virus on tissue damage, a histopathological examination was carried out in lung tissue samples following the necropsy of an animal diagnosed with BPIV3 by antigenic and molecular methods.

Virus isolation could not be performed in our cell culture planting process. Similar to our study, in their study, Noori et al. (2014) performed an agent isolation from cell culture passages in only one out of 25

samples detected as positive in DIF and PCR analysis. The reason of this was thought that the agent had a fragile structure and could be isolated from a single sample since it could hardly adapt to cell lines. These results show the hardships of cell culture studies in diagnosing BPIV3 infection.

In many countries of the world, there are studies on molecular detection of BPIV3 (Zhu et al. 2011; O'Neill et al. 2015; Moore et al. 2015; Veljovic et al. 2016; Kishimoto et al. 2017; Headley et al. 2018; Kamdi et al. 2020). In these studies, the agent prevalence varied between 0% and 21.6%. Similar to these studies, in those in Turkey, Hacıoğlu (2011) obtained a positivity at the rate of 1.41% (1/71) in nasal swab samples by RT-PCR technique, Timurkan et al. (2019) 1.94% (3/155) in nasal swab and lung tissue samples and Toker and Yesilbağ (2021) 0.51% (1/193) in nasal swab and lung tissue samples. In addition, in their study on teat tissue samples by qRT-PCR technique, Çomaklı et al. (2019) detected BPIV3 viral genome at a rate of 21.67% (26/120).

This difference in prevalence values was believed to differ depending on the specifity (different primers) and sensitivity (qPCR) of the applied molecular diagnosis technique, the viral load carried by the material used (nasal swab, lung tissue sample, trachea sample), whether the animals being seropositive or in their convalescence period and the fragility of the agent.

163 NS strains obtained in this study were detected to be in a close genetic relation with some isolates in China, Turkey, Japan, the USA and South Korea and were located in BPIV3 genotype C. Besides, when this strain was compared to the isolates obtained in other studies in Turkey, Albayrak et al. (2019) found that it was 99.68% similar to the isolate (MH357343) they found in their study in Samsun and Timurkan et al. (2019) 97.99% similar to the one they found in their study in Erzurum. In accordance with this information, in our study, we supported the fact that BPIV3 genotype C was in circulation in cattle populations around Turkey. This study is the first one on antigenic and molecular detection and genetic examination of BPIV3 in south Turkey.

The strain we obtained in our study showed similarities to the isolates (HQ530153, KU198929, KT071671) found by Zhu et al. (2011) and Wen et al. (2012) in China at a rate of 99.2%, to the ones (LC000638, LC040886) obtained by Konishi et al. (2014) in Japan at rates of 96.62% and 97.31% respectively, to the virus (JX969001) isolated by Oem et al. (2013) in South Korea at a rate of 97.31% and to the strains (KJ647285, KJ647287, KJ647289) isolated by Neill et al. (2015) in the USA at rates of 97.65%, 98% and 80.45% respectively. On the other hand, it had a 79.22% genetic similarity with the strain (KP757872) isolated by Sobhy et al. (2017) in Egypt. The genetic similarity of the strain obtained in our study to SF-4 was accepted as the reference strain (AF178655) is 82.12% Bailley (2000).

During the studies performed on the antigenic detection of BPIV3 by DIF test, the prevalence varied between 9.8% and 41.5% (Alkan et al. 2000; Gençay and Akça 2004; Delgado et al. 2005; Çeribaşı et al. 2012; Çeribaşı et al. 2014; Maidana et al. 2012; Saeed et al. 2016; Kamdi et al. 2020). In this study, the positivity rate was found lower than those in the other studies. This result was believed to have occurred due to conscious breeding in sampling area, improved veterinarian services, common vaccination applications and better struggling with infections. In preventing BPIV3 infection, mucosal immunity is in

the foreground compared to systemic immunity and while mucosal immunity plays a role in preventing the infection and decreasing the dispersion, systemic immunity allows an occurring infection to proceed more slightly (Elenkumaran 2013; Maclachlan et al. 2017). In accordance with this information, the fact that the sampled animals had strong mucosal immunity, they were seropositive or they had maternal antibodies against the agent could be considered as other biological agents causing low prevalence rate.

In our study, due to the fact that immune response was not sufficient, the diagnosis of the agent could be performed in animals whose nasal swab samples were taken to be detected in terms of BPIV3 in molecular and antigenic ways. Although clinical symptoms were seen, in animals whose BPIV3 viral genome could not be detected, a respiratory system infection caused by other viral, bacterial or parasite pathogens or a clinical picture dependent on secondary infections occurring after BPIV3 infection was believed to have formed. In addition, in sampled animals whose clinical symptoms subsided, the reason why the agent could not be detected was thought to be the fact that the disease did not contain the agent as it was in convalescence period or the animals were in convalescence period since 1-2 weeks passed after they showed severe symptoms. On the other hand, detection of the agent could not be performed on conjunctival swab samples as it showed affinity against respiratory tract cells where NANA receptor molecules such as BPIV3 pneumocytes, bronchial cells and trachea epitheliums were abundant (Maclachlan et al. 2017). In addition, because the agent did not have viremia period or it was too short, it was considered natural not to be able to detect the virus in leukocyte samples.

Conclusion

In our study, during lab diagnosis of BPIV3 infection, DIF test showed a higher positivity compared to RT-PCR. In this respect, it was concluded that more samples and more studies were needed to be carried out so as to determine and compare the specifity and sensitivity of these two methods in diagnosing BPIV3. In virological diagnosis of BPIV3, during the acute period of the disease, nasal swab samples, taken properly and brought into diagnosis lab swiftly, might be used in smear ways using a suitable transport fluid under cold chain. In addition, in virological and molecular diagnosis studies of the agent using nasal swabs, it was considered better to use samples taken from animals showing respiratory system infection symptoms and to convey samplings during acute period of the disease.

Declarations

Availability of data and material All data and material are available.

Code availability Not applicable for that section

Author's contribution YY and AK conceived and designed research. AK and YY conducted experiments. YY and AK contributed new reagents or analytical tools. YY and AK analyzed data. YY and AK wrote the manuscript. All authors read and approved the manuscript.

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Ethics Approval The sampling was conducted according to permission from local ethical committee for animal experiments (MAKU-HADYEK 318/2017).

Consent to Participate Consent of participation in this study was obtained.

Consent of Publication All authors agree to publish this manuscript.

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this article.

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Tables

Table 1. General information about sampled animals

Data of Sampled Animals	
Number of Animal Sampled (*n)*	190
Age	
< 1 age	137
> 1 age	53
Gender	
Female(♀)	92
Male(♂)	98
Races	
Montofon (Brown Swiss)	40
Simmental	78
Holstein	72

Table 2. Comparison of positivity rates according to test results.

Samples	Number of Samples (*n*)	Number of Positive Samples (%)	
		RT-PCR	DIF
Leukocyte	190	%0 (0/190)	-*
Nasal swab	190	%1,05 (2/190)	%3.15 (6/190)
Conjunctival swab	190	%0 (0/190)	%0 (0/190)

*** No Test Available**

Figures

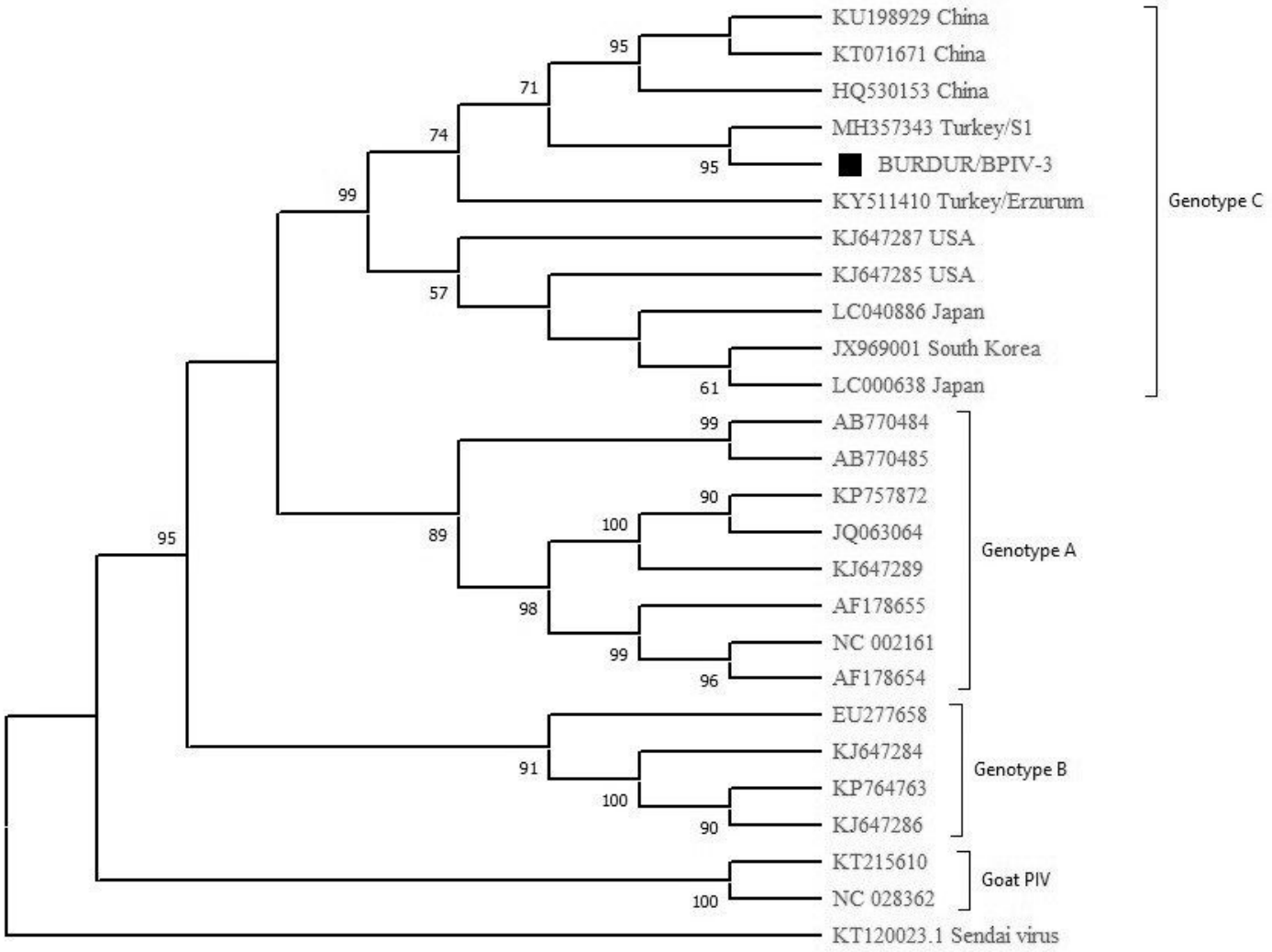

Figure 1

Phylogenetic tree generated based on the nucleotide sequence alignment of the BPIV3 matrix (M) gene region of the virus obtained in the study (BURDUR/BPIV-3/ Feb.2019) and viral strains from Genbank

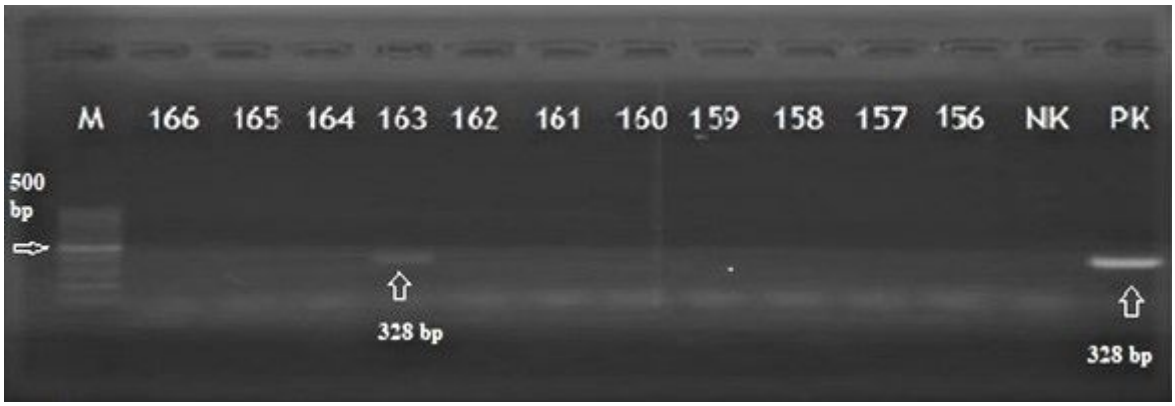

Figure 2

Gel electrophoresis image of BPIV3 RT-PCR products, 156-166 field nasal swab samples, 163 positive nasal swab samples.

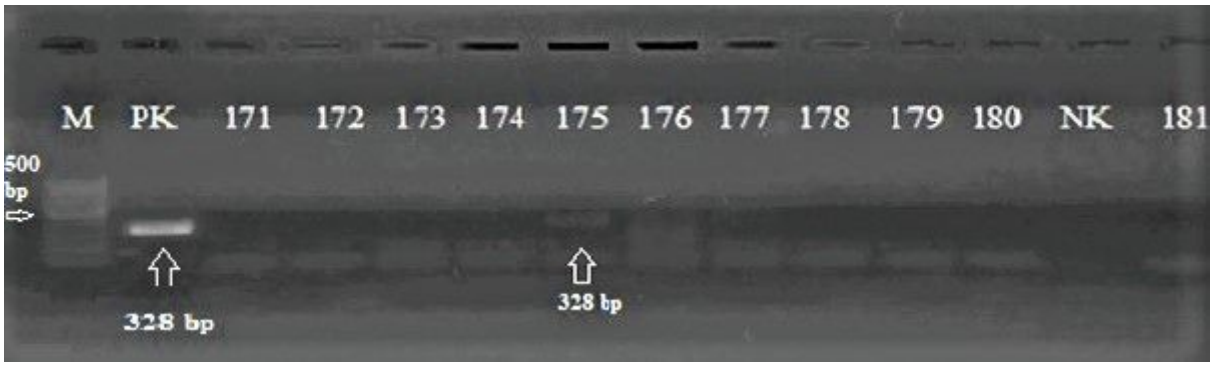

Figure 3

Gel electrophoresis image of BPIV3 RT-PCR products, 156-166 field nasal swab samples, 175 positive nasal swab samples.

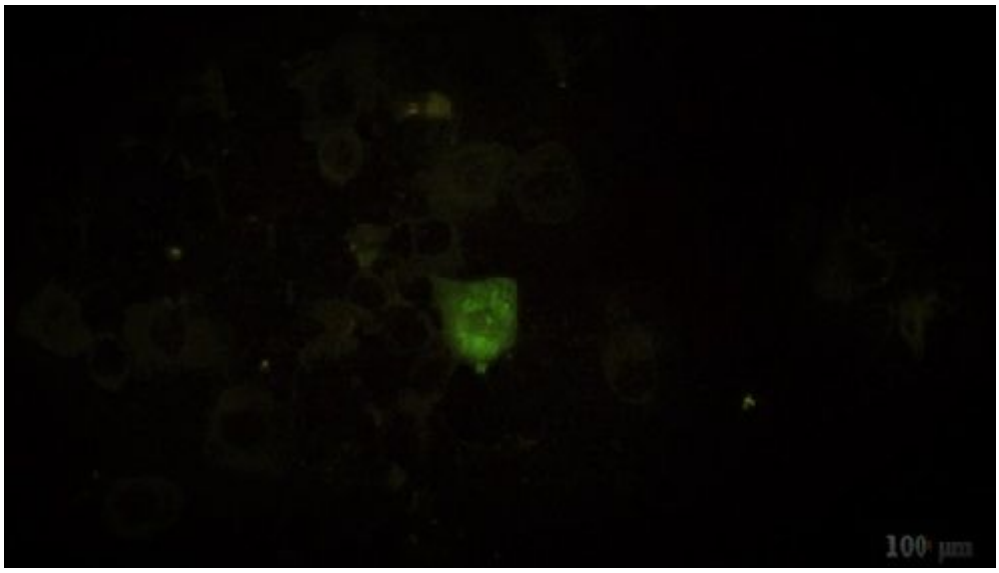

Figure 4

DIF Positive nasal swab sample (sample number 163)

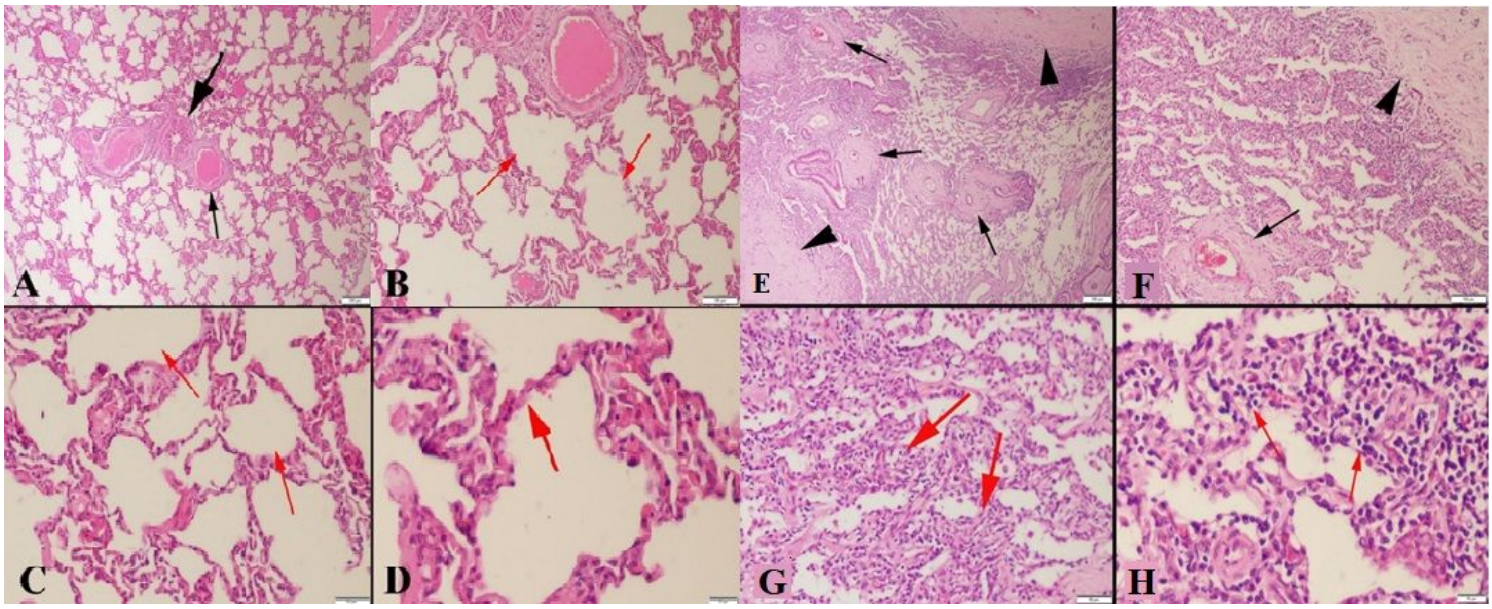

Figure 5

The histological view of a normal looking lung (A), lung veins by small scale magnification (black thin cursor) and black (thick cursor) normal microscopic view of a bronchiole, Bar= 200µm. (B), view of alveoles (red thin cursors), Bar= 100µm. (C), close view of alveoles (red thin cursors), Bar= 50µm. (D), view of alveole septal tissue (red thick cursor), Bar= 20µm, Hemotoxylin Eosine (HE). Histopathological view of a lung with pneumonia (E), increase in veining (black thin cursors) and common ligament growth (black cursor heads), Bar= 200µm. (F), close view of veining and ligament increase, Bar= 100µm. (G), septal tissue thickening (red thick cursors), Bar= 50µm. (H), inflammatory cell infiltration (red thin cursors), Bar= 20µm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Appendix.docx