



# Pathological and Molecular Investigation of Porcine Kobuvirus (PKV) from Indian Pigs

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## ABSTRACT

**Background:** The porcine kobuvirus (PKV) is a single-stranded, positive-sense, RNA virus of the family Picornaviridae which composed of small, non-enveloped viruses. The PKV is suspected to cause large outbreaks of diarrhoea and dehydration along with vomition in suckling piglets with a high morbidity rate. Despite of its economical importance very few studies are available on the pathology of PKV. The present study was conducted with the aim to investigate PKV in naturally infected Indian pigs.

**Methods:** A total of 41 intestinal content and tissue samples from dead pigs were collected from post-mortem facility of, ICAR-IVRI during April, 2019 to July 2020. All the carcasses were subjected to detailed necropsy examination and appropriate tissue samples were collected for histopathological examination and molecular investigation. The amplification of 3D region of PKV was carried out via RT-PCR using the specific primers. The Genetic characterization of Indian isolate of the PKV was done on the basis of viral 3D gene of PKV.

**Result:** A total of six out of 41 necropsied piglets were found positive for the PKV. The diarrhoea and dehydration were observed as the main clinical signs. The thickening and clouding of brain meninges, congestion of brain, pneumonia along with catarrhal and haemorrhagic enteritis was observed as common gross lesions. Microscopic lesions observed in brain were mild to moderate perivascular cuffing, congestion of meningeal and cerebral vessels, focal gliosis and neuronophagia. Mild to severe interstitial pneumonia and emphysema was noticed in lungs. Intestinal mucosa revealed severe inflammatory changes along with sloughing of villi. The genetic characterization revealed maximum identity of 91-93% with Indian isolate of PKV MH394282 and Chinese strain of PKV FJ459905. This study reported the first pathological investigation of PKV from Indian pigs and described the molecular findings in detail. Further explorative surveillance along with epidemiological studies are highly warranted to find out the real impact of the PKV on Indian pigs.

**Key words:** Genetic characterization, Indian pigs, Pathology, Porcine kobuvirus, PKV.

## INTRODUCTION

The Kobuvirus is a single-stranded, positive-sense, RNA virus of the family Picornaviridae which composed of small, non-enveloped viruses (Reuter *et al.*, 2009; ICTV, 2019). The genus Kobuvirus is consist of six species viz. Aichivirus A, Aichivirus B, Aichivirus C, Aichivirus D, Aichivirus E and Aichivirus F, infecting a variety of hosts like pigs, sheep, goats, cattle, ferrets, bats, dogs, cats and humans (Reuter *et al.*, 2011; Khamrin *et al.*, 2014 and Lu *et al.*, 2018). The Aichivirus C is the only Kobuvirus species reported to infect pigs, previously known as "porcine kobuvirus (PKV)" (Khamrin *et al.*, 2014). The PKV was first identified in Hungary from faecal samples of pig in 2008 followed by China (Yu *et al.*, 2009; Reuter *et al.*, 2010). There after, PKV was subsequently reported from Asia, Africa, Europe and United states (Khamrin *et al.*, 2009; Barry *et al.*, 2011; Verma *et al.*, 2013; Amimo *et al.*, 2014 and Zhou *et al.*, 2016). The virus has been detected from both diarrhoeic (Khamrin *et al.*, 2009; Park *et al.*, 2010; Cromeans *et al.*, 2014; Van Dung *et al.*, 2016 and Almeida *et al.*, 2018) and non-diarrhoeic pigs (Dufkova *et al.*, 2013; Goecke *et al.*, 2017 and Jackova *et al.*, 2017). Prevalence of PKV in domestic pigs was reported from 13-99% (CFSPH, 2015), which decreases as age advances (Shan *et al.*, 2011; Chen *et al.*, 2013). Furthermore, PKV is suspected to cause large outbreaks of

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diarrhoea and dehydration along with vomition in suckling piglets with a morbidity rate of 80-100% and mortality of 50-90% in China (Cao *et al.*, 2012). The large outbreaks of diarrhoea with highly significant mortality rate may contribute to great economic losses. The pathological studies of PKV in piglets have not been conducted in India and unavailability of a vaccine for protection of piglets from PKV together raises the need for further studies.

The present study has been conducted for pathological and molecular characterization of PKV infection in Indian

pigs. The pathological characterization of natural PKV infection in Indian pigs is likely being attempted for the very first time.

## MATERIALS AND METHODS

### Sample collection

A total of 41 intestinal content and tissue samples (brain, tonsil, lung, liver, spleen, heart and intestine) from dead pigs (Table 1) were collected from post-mortem facility of Division of Pathology, ICAR- Indian Veterinary Research Institute (ICAR- IVRI) during April, 2019 to July 2020. The clinical history of all the presented carcasses was recorded. Thereafter, the carcasses were subjected to detailed systematic necropsy examination and gross abnormalities were recorded carefully. Appropriate representative tissue samples were collected in 10% neutral buffered formalin and stored at -80°C for histopathological examination and molecular investigation respectively.

### Histopathological examination

For histopathological processing, the thin pieces of formalin fixed tissue samples were washed overnight under running tap water followed by dehydration through ascending grades of alcohol. After dehydration, clearing was done with acetone and benzene followed by embedding in paraffin wax blocks by automatic tissue processor. The 4-5 µm thick paraffin sections were trimmed by microtome and stained with routine haematoxylin and eosin stain using standard protocol (Bancroft and Gamble, 2008). The stained sections were examined microscopically and the histopathological lesions were recorded carefully and photographed digitally (Olympus BX41, USA).

### Molecular examination

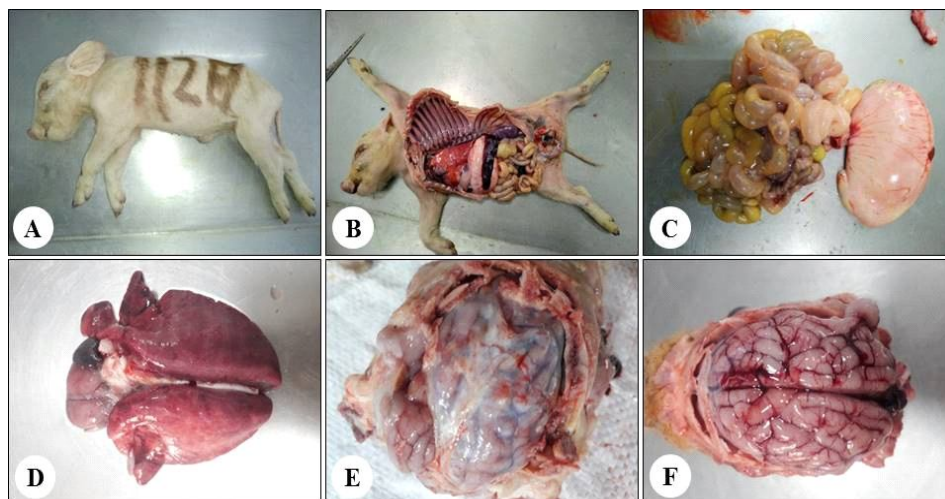
Total RNA from all the intestinal content and tissue samples was extracted as per manufacture's protocol from all PKV suspected samples using commercial TRIzol® Reagent

(Thermo Fisher Scientific, USA). All extracted RNA samples were subjected for quantification by NanoVue plus (Thermo Fisher Scientific, USA) and the purity of RNA was also checked by A260/230 and A260/280 ratio. The cDNA synthesis was performed as described earlier by Ray *et al.* (2018) and stored at -20°C for further use. The amplification of 3D region of PKV was carried out *via* RT-PCR using the primer forward: 5'-TGGAC GACCAGCTCTTCCTTAAACAC-3' and reverse: 5'-AGTGCAAGTGCAAGTCTGGGTTGC AGCCAACA-3' (Yu *et al.* 2009). The samples from all dead animals were also screened for porcine teschovirus (PTV) and porcine sapelovirus (PSV) using specific primers targeting 5' UTR gene.

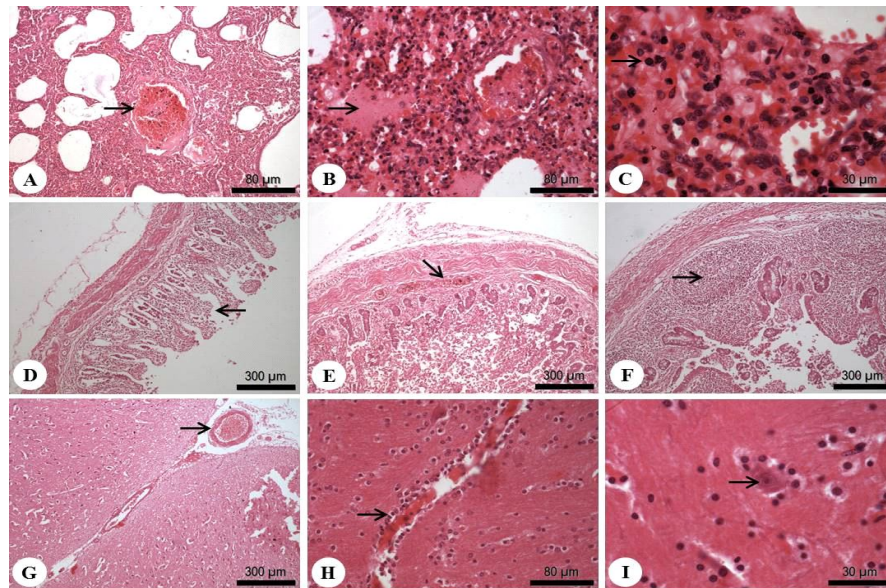
PCR reaction (12 µL) was carried out in 0.2 ml PCR tubes containing 6.0 µL of DreamTaq™ Green PCR Master Mix (2X), 0.5 µL of forward primer (10 pmol/µL), 0.5 µL of reverse primer (10 pmol/µL), 2.0 µL of cDNA (100 ng/µL) and 3.0 µL of nuclease-free water by using a thermocycler (S1000™ Thermal Cycler, BIO-RAD Laboratories India). The PCR was carried out with an initial denaturation of 94°C for 4 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing) and 72°C for 50 seconds (extension), and a final extension at 72°C for 7 minutes. The visualization of amplified PCR products was done by agarose gel (1.5% w/v) electrophoresis after staining with 0.5 µg/mL ethidium bromide at 90 V for 1 h under UV transilluminator (Geldoc, USA).

### Phylogenetic analysis

The amplified PCR products were further confirmed by commercial DNA sequencing facility (Genotypic Technology Pvt. Ltd., Bangalore, India) *via* employing Sanger sequencing method using specific forward and reverse primers for the target gene used in the study. The 3D gene based phylogenetic analysis was performed using sequence data received in form of coloured electropherograms and text files to establish the genotypes of the sequenced PKV



**Fig 1:** Gross lesions of PKV affected animals; A) Severely dehydrated and weak piglet; B) Congested lungs and intestines filled with diarrhoeic content C) Congested, haemorrhagic and distended intestine filled with yellowish diarrhoeic content; D) Non collapsible lungs with pneumonic changes; E) Clouding and thickening of brain meninges; F) Congestion of cerebral vessels.



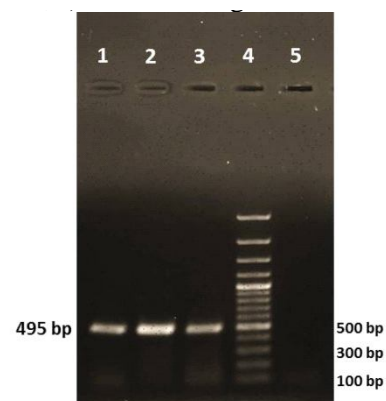
**Fig 2:** Microscopic lesions of PKV affected animals; Lungs: **A)** Severe interstitial pneumonia with capillary engorgement (arrow), H and E, 400X; **B)** Severe mononuclear cell infiltration in interalveolar septa with accumulation of protein rich edema fluid (arrow), H and E, 400X; **C)** Thickening of inter alveolar septa due to haemorrhages and severe infiltration of mononuclear cells, H and E, 1000X. Intestine: **D)** Jejunum of intestine showing sloughing of villi (arrow), H and E, 100X; **E)** Jejunum showing congestion of vessels (arrow) and sloughing of villi, H and E, 100X; **F)** Peyer's patches of Ileum showing lymphoid depletion (arrow) and mononuclear cell infiltration, H and E, 100X. Brain: **G)** Severe congestion of meningeal vessels (arrow), H and E, 100X; **H)** Severe perivascular infiltration of mononuclear cells (arrow), H and E, 400X; **I)** Neuronal degeneration and satellitosis (arrow), H and E, 1000X.

strains. The sequences of various isolates corresponding to other genotypes from different countries were retrieved from Gen Bank and used as input sequences for multiple sequence alignment. The sequence alignment was carried out using ClustalW programme of MEGA v.6 software followed by construction of a phylogenetic tree (Tamura *et al.*, 2013).

## RESULTS AND DISCUSSION

In the present study a total of six out of 41 samples were found positive for PKV whereas all the PKV positive samples were found negative for PTV and PSV. The history of all PCR positive PKV cases revealed diarrhoea as the common clinical sign as discussed by Cao *et al.* (2012). Among PKV positive piglets the consistent lesions noticed were clouding and thickening of brain meninges, congestion of brain, severe to moderate degree of pneumonia along with congestion of lungs, presence of frothy exudate in trachea, congestion of mesenteric lymph nodes and thickening of intestinal mucosa along with catarrhal enteritis (Fig 1). Variable degree of consolidation and emphysema was observed in the different lobes of the lungs. Microscopic lesions in brain mainly include congestion of cerebral and meningeal vessels, infiltration of mononuclear cells, neuronophagia, mild to severe perivascular cuffing and gliosis. Mild to severe interstitial pneumonia along with congestion and haemorrhages was observed in the lungs. Moreover, infiltration of mononuclear cells in the interalveolar septa and accumulation of protein rich fluid was also observed. Intestines revealed congestion of mucosal and

submucosal vessels, sloughing of villous epithelium along with infiltration of mononuclear cells in lamina propria. Lymphoid depletion in the Peyer's patches was also observed in the ileum along with congestion of vessels (Fig 2). However, the above lesions were found associated with PKV positive cases with the history of diarrhoea and the possibility of involvement of other porcine enteric picornaviruses (PTV and PSV) has also been ruled out but presence of other pathogens and their role in the development of pathology cannot be ignored. Therefore, the above lesions may only be strongly considered as the



**Fig 3:** Ethidium bromide stained 1.5% agarose gel showing amplification of 495 bp for targeted 3D region of PKV: Lane 1-3: Positive sample, Lane 4: Marker (100bp) and Lane 5: Negative test control.



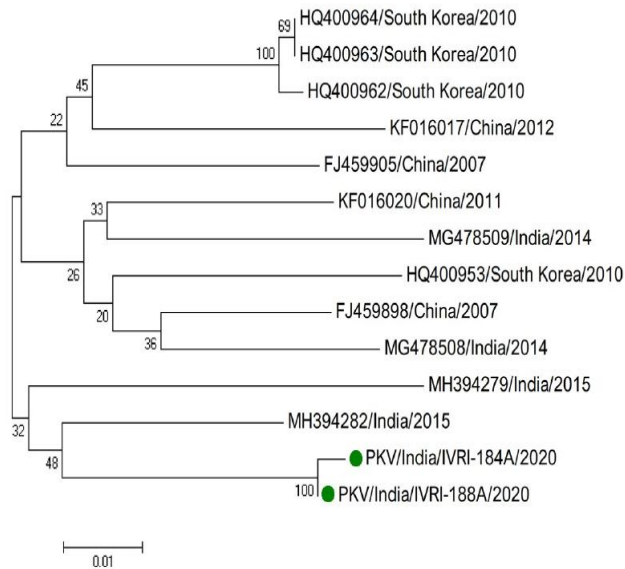
speculative lesions associated with PKV infection. Further studies with inoculation of experimental animals and natural hosts are highly warranted to ascertain the PKV specific lesions.

In RT-PCR specific amplicons of 495 bp were obtained from PKV positive samples (Fig. 3). Among six PKV positive cases, 2 were 6 day old, 2 were 10 day old, 1 was 11 days old and 1 piglet was 20 days of age suggesting the prevalence of the virus in piglets of less than 1 month of age (Table 1). The 3D regions of 12 PKV sequences were retrieved from NCBI and used for the construction of phylogenetic tree along with the Indian isolates detected in the present study. The Indian isolates of the present study

were named as PKV/India/IVRI-184A/2020 (Accession no. MW018696) and PKV/India/IVRI-188A/2020 (Accession no. MT946377). Phylogenetic analysis of the 3D region revealed that Indian isolate of this study is closely related to other Indian isolates MH394282/India/2015 and MH394279/India/2015. Indian isolates PKV/India/IVRI-188A/2020 and PKV/India/IVRI-184A/2020 were clustered together in a single node with a bootstrap value of 100. On Blast analysis of 3D gene maximum identity of 91-93% with query coverage of 74%-94% with Indian isolate MH394282/PKV/India/IVRI-187/2015 and Chinese strain FJ459905/swine/2007/CHN isolate Z324 was observed (Fig 4).

**Table 1:** Details of animals included in the study and results of RT-PCR.

Necropsy No.	Breed (CB = Crossbred)	Age (D = Day, M = Month)	Sex	PCR Result
52 A/19	CB	1 M	F	Negative
80 A/19	CB	10 D	M	Negative
81 A/19	Non-descript	3 D	M	Negative
82 A/19	Non-descript	3 D	F	Negative
99 A/19	CB	1 M	M	Negative
107 A/19	CB	1 M	M	Negative
108 A/19	CB	25 D	F	Negative
120 A/19	CB	1 M	M	Negative
127 A/19	Landrace	1.5 M	F	Negative
135 A/19	CB	1.5 M	F	Negative
136 A/19	CB	1 M	F	Negative
145 A/19	CB	37 D	M	Negative
146 A/19	Non-descript	42 D	F	Negative
148 A/19	CB	45 D	M	Negative
153 A/19	CB	1.5 M	M	Negative
160 A/19	CB	1.5 M	M	Negative
166 A/19	CB	20 D	F	<b>Positive</b>
167 A/19	CB	29 D	F	Negative
179 A/19	CB	1 M	M	Negative
181 A/19	CB	1.5 M	M	Negative
182 A/19	CB	1.5 M	M	Negative
183 A/19	CB	1 M	F	Negative
184 A/19	CB	1 M	F	Negative
185 A/19	CB	1.5 M	M	Negative
192 A/19	CB	1 M	F	Negative
199 A/19	CB	39 D	M	Negative
200 A/19	CB	40 D	F	Negative
320 A/19	CB	6 D	F	Negative
341 A/19	CB	13 D	M	Negative
345 A/19	CB	9 D	M	Negative
183 A/20	CB	6 D	M	<b>Positive</b>
184 A/20	CB	6 D	F	<b>Positive</b>
186 A/20	CB	9 D	M	Negative
188 A/20	CB	10 D	M	<b>Positive</b>
189 A/20	CB	11 D	F	Negative
191 A/20	CB	13 D	F	Negative
202 A/20	CB	16 D	F	Negative
209 A/20	CB	10 D	F	<b>Positive</b>
218 A/20	CB	11 D	F	<b>Positive</b>
231 A/20	CB	33 D	M	Negative
239 A/20	CB	56 D	F	Negative



**Fig 4:** Phylogenetic tree of the Indian isolates of PKV (PKV/India/IVRI-184A/2020 and PKV/India/IVRI-188A/2020) with reference sequences based on 3D region of PKV genome using the Neighbor-Joining method.

## CONCLUSION AND FUTURE PROSPECTS

The present pathological and molecular investigation of PKV in naturally infected piglets concluded that PKV primarily infected intestine, brain and lungs. Moreover, mild to severe encephalitis, interstitial pneumonia and severe catarrhal and haemorrhagic enteritis was observed as major pathological findings. Although very limited information on pathology of PKV is available it is hoped that this study will add to the understanding of PKV infection in pigs. Moreover, further studies in suitable animal model will definitely provide better insights of the pathology and pathogenesis of the PKV.

## CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

## ACKNOWLEDGEMENT

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## REFERENCES

- Almeida, P.R., Lorenzetti, E., Cruz, R.S., Watanabe, T.T., Zlotowski, P., Alfieri, A.A. and Driemeier, D. (2018). Diarrhea caused by rotavirus A, B, and C in suckling piglets from southern Brazil: molecular detection and histologic and immunohistochemical characterization. *Journal of Veterinary Diagnostic Investigation*. 30: 370-376.
- Amimo, J.O., Okoth, E., Junga, J.O., Ogara, W.O., Njahira, M.N., Wang, Q., Vlasova, A.N., Saif, L.J. and Djikeng, A. (2014). Molecular detection and genetic characterization of kobuviruses and astroviruses in asymptomatic local pigs in East Africa. *Archives of Virology*. 159: 1313-1319.
- Bancroft, J.D. and Gamble, M. (2008). *Theory and Practice of Histopathological Techniques*. 6<sup>th</sup> ed. Churchill Livingstone, Elsevier, Philadelphia. p 657.
- Barry, A.F., Ribeiro, J., Alfieri, A.F., van der Poel, W.H. and Alfieri, A.A. (2011). First detection of kobuvirus in farm animals in Brazil and the Netherlands. *Infection, Genetics and Evolution*. 11: 1811-1814.
- Cao, W., Zheng, H., Zhang, K., Jin, Y., Lv, L., Yang, F. and Liu, X. (2012). Complete genome sequence of the porcine kobuvirus variant CH/HNXX-4/2012. *Journal of Virology*. 86(21): 11947.
- CFSPH. (2015). Porcine kobuvirus ([http://www.cfsph.iastate.edu/pdf/shic-factsheet-porcine-kobuvirus#:~:text=Porcine%20kobuvirus%20\(PKoV\)%20is%20a%20suspected%20cause%20of%20diarrhea%20in,in%20the%20clinical%20signs%20observed](http://www.cfsph.iastate.edu/pdf/shic-factsheet-porcine-kobuvirus#:~:text=Porcine%20kobuvirus%20(PKoV)%20is%20a%20suspected%20cause%20of%20diarrhea%20in,in%20the%20clinical%20signs%20observed)). (Accessed on 14/04/2021).
- Chen, L., Zhu, L., Zhou, Y.C., Xu, Z.W., Guo, W.Z. and Yang, W.Y. (2013). Molecular and phylogenetic analysis of the porcine kobuvirus VP1 region using infected pigs from Sichuan Province, China. *Virology Journal*. 10: 281.
- Cromeans, T., Park, G.W., Costantini, V., Lee, D., Wang, Q., Farkas, T., Lee, A. and Vinje, J. (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology*. 80: 5743-5751.
- Dufkova, L., Scigalkova, I., Moutelikova, R., Malenovska, H. and Prodelalova, J. (2013). Genetic diversity of porcine sapoviruses, kobuviruses and astroviruses in asymptomatic pigs: an emerging new sapovirus GIII genotype. *Archives of Virology*. 158: 549-558.
- Goecke, N.B., Hjulsgaard, C.K., Kongsted, H., Boye, M., Rasmussen, S., Granberg, F., Fischer, T.K., Midgley, S.E., Rasmussen, L.D., Angen, O., Nielsen, J.P., Jorsal, S.E. and Larsen, L.E. (2017). No evidence of enteric viral involvement in the new neonatal porcine diarrhoea syndrome in Danish pigs. *BMC Veterinary Research*. 13: 315.
- ICTV (International Committee on Taxonomy of Viruses). (2019). Virus Taxonomy (<https://talk.ictvonline.org/taxonomy/>). (Accessed on 14/04/2021).
- Jackova, A., Sliz, I., Mandelik, R., Salamunova, S., Novotny, J., Kolesarova, M., Vlasakova, M. and Vilcek, S. (2017). Porcine kobuvirus 1 in healthy and diarrheic pigs: genetic detection and characterization of virus and co-infection with rotavirus A. *Infection, Genetics and Evolution*. 49: 73-77.
- Khamrin, P., Maneekarn, N., Kongkaew, A., Kongkaew, S., Okitsu, S. and Ushijima, H. (2009). Porcine kobuvirus in piglets, Thailand. *Emerging Infectious Diseases*. 15: 2075-2076.
- Khamrin, P., Maneekarn, N., Okitsu, S. and Ushijima, H. (2014). Epidemiology of human and animal kobuviruses. *Virus Disease*. 25: 195-200.
- Lu, G., Zhang, X., Luo, J., Sun, Y., Xu, H., Huang, J., Ou, J. and Li, S. (2018). First report and genetic characterization of feline kobuvirus in diarrhoeic cats in China. *Transboundary and Emerging Diseases*. 65: 1357-1363.
- Park, S.J., Kim, H.K., Moon, H.J., Song, D.S., Rho, S.M., Han, J.Y. and Park, B.K. (2010). Molecular detection of porcine kobuviruses in pigs in Korea and their association with diarrhea. *Archives of Virology*. 155(11): 1803-1811.

- Ray, P.K., Desingu, P.A., Kumari, S., John, J.K., Sethi, M., Sharma, G.K., Pattnaik, B., Singh, R.K. and Saikumar, G. (2018). Porcine sapelovirus among diarrhoeic piglets in India. *Transboundary and Emerging Diseases*. 65(1): 261-263.
- Reuter, G., Kecskémeti, S. and Pankovics, P. (2010). Evolution of porcine kobuvirus infection, Hungary. *Emerging Infectious Diseases*. 16(4): 696-698.
- Reuter, G., Boldizsar, A. and Pankovics, P. (2009). Complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus, a member of a new species in the genus Kobuvirus, family Picornaviridae. *Archives of Virology*. 154: 101-108.
- Reuter, G., Boros, A. and Pankovics, P. (2011). Kobuviruses – a comprehensive review. *Reviews in Medical Virology*. 21: 32-41.
- Shan, T., Li, L., Simmonds, P., Wang, C., Moeser, A. and Delwart, E. (2011). The fecal virome of pigs on a high density farm. *Journal of Virology*. 85(22): 11697-11708.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*. 30(12): 2725-2729.
- Van Dung, N., Anh, P.H., Van Cuong, N., Hoa, N.T., Carrique-Mas, J., Hien, V.B., Sharp, C., Rabaa, M., Berto, A., Campbell, J., Baker, S., Farrar, J., Woolhouse, M.E., Bryant, J.E. and Simmonds, P. (2016). Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam. *The Journal of General Virology*. 97: 378-388.
- Verma, H., Mor, S.K., Abdel Glil, M.Y. and Goyal, S.M. (2013). Identification and molecular characterization of porcine kobuvirus in US swine. *Virus Genes*. 46(3): 551-553.
- Yu, J.M., Jin, M., Zhang, Q., Li, H.Y., Li, D.D., Xu, Z.Q., Li, J.S., Cui, S.X., Yang, S.H., Liu, N. and Duan, Z.J. (2009). Candidate porcine Kobuvirus, China. *Emerging Infectious Disease*. 15(5): 823-825.
- Zhou, W., Ullman, K., Chowdry, V., Reining, M., Benyeda, Z., Baule, C., Juremalm, M., Wallgren, P., Schwarz, L., Zhou, E., Pedrero, S.P., Hennig-Pauka, I., Segales, J. and Liu, L. (2016). Molecular investigations on the prevalence and viral load of enteric viruses in pigs from five European countries. *Veterinary Microbiology*. 182: 75-81