SHORT COMMUNICATION

Immunocytochemistry assay in BHK-21 cell line infected with Porcine *Sapelovirus*

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Abstract The present study describes an immunocytochemistry (ICC) assay with self-raised hyperimmune sera and a Baby Hamster Kidney-21 (BHK-21) cell line infected with Porcine Sapelovirus (PSV). Sapelovirus/IVRI/SPF-c-6/2015 strain Indian PSV was isolated from the porcine IBRS-2 cell line and investigated for growth on non-porcine cell lines. After two passages, PSV was successfully grown in BHK-21 and produced the same cytopathic effects as in IBRS-2 such as shrinking of cytoplasm, rounding of cells and detachment of cells from the surface of flask within 24 h. For raising of hyperimmune sera, PSV was grown in IBRS-2 cell line up to the required

volume and purified by ultracentrifugation. With self-raised hyperimmune sera in laboratory rats, ICC was performed in BHK-21 cells infected with PSV. Positive signals consisted of large granular aggregates of virus in the cytoplasm near the nucleus, suggesting that PSV can infect cell lines other than those of porcine origin.

Keywords Porcine Sapelovirus · Immunocytochemistry · BHK-21 · IBRS-2

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Porcine sapelovirus (PSV) is a member of newly classified genus *Sapelovirus*, belongs to family Picornaviridae which currently consists of 29 genera divided into 50 species (ICTV Virus Taxonomy 2015). *Sapelovirus* genus comprised three species, Porcine Sapelovirus (Fauquet et al. 2005), Simian Sapelovirus (Oberste et al. 2003) and Avian Sapelovirus (Tseng and Tsai 2007). PSV was previously named Porcine Enterovirus 8 (PEV-8) and classified as Porcine Enterovirus A (PEV A) (Krumbholz et al. 2002). PSV is non-enveloped, spherical, about 30 nm in diameter, the genome is a linear, non-segmented, single-stranded positive sense RNA with length of 7.5–8.3 kb nucleotides (Lan et al. 2011; Schock et al. 2014). PSV was identified as a causative agent of acute diarrhoea, respiratory distress, and polioencephalomyelitis in an outbreak of gastroenteritis in pigs of swine farm in Shanghai, China in 2009 (Lan

et al. 2011) and cause of encephalomyelitis outbreak in 2008–2009 in United Kingdom (Schock et al. 2014). PSV has been frequently isolated from faeces of healthy piglets (Buitrago et al. 2010; Chen et al. 2012; Kim et al. 2016; Ray et al. 2018; Bai et al. 2018; Kumari et al. 2019b; Sunaga et al. 2019).

Diagnosis of Sapelovirus can be done by isolation of the virus in cell lines like IBRS-2, and BHK21 (Knowles et al. 1979). PSV causes unique type II cytopathic effects (CPE) in porcine kidney cells (Knowles et al. 1979; Honda et al. 1990). Affected cells of cell line with PSV show marked pyknotic nuclei and shrunken cytoplasm with irregular protrusions in stained preparation. Cells first became granular, gradually degenerated and sloughed off the glass. They rarely form aggregates (Kadoi et al. 1970). Routine laboratory diagnosis of PSV infection depends mainly on virus isolation and characterization, but this technique is less effective and time-consuming for initial detection. Several molecular methods for detecting PSV have been developed such as reverse transcription PCR (Palmquist et al. 2002), nested reverse transcription PCR (Zell et al. 2000) and real-time quantitative PCR (Krumbholz et al. 2003). Real-time PCR detection (Chen et al. 2014) and real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) detecting Porcine Sapelovirus (Wang et al. 2014) has been developed and validated. Immunohistochemistry has been successfully used to demonstrate PSV antigen in brain and spinal cord sections of pig in an outbreak of encephalomyelitis in United Kingdom (Schock et al. 2014). In addition, Sapelovirus A was also detected in CNS with severe polioencephalomyelitis in pig by in situ hybridization in the USA (Arruda et al. 2017). However, only few experimental studies of PSV have been reported (Lan et al. 2011; Kim et al. 2016; Kumari et al. 2019a).

In the past, India has no report of PSV infection in wild as well as in domestic pig up to 2014. Recently, in India the virus has been isolated/detected from faeces of healthy pigs as well as those affected with diarrhoea, respiratory signs, encephalitis, skin lesions and fertility disorders during their research work on porcine picornavirus (Ray et al. 2018; Kumari et al. 2018b). The objective of this study was to develop immunocytochemistry (ICC) assay for PSV detection in BHK-21 cell line using self-raised hyper-immune sera in laboratory rats.

In the current study, we used Sapelovirus/IVRI/SPF-c-6/2015 strain (Strain C-6) Indian isolate of PSV which was obtained from Swine Disease Laboratory, Division of Veterinary Pathology, IVRI, Izatnagar. We increased the volume and concentration of PSV by repeated infection of IBRS-2 cell line (Details, Supplementary file 1). We attempted to grow the PSV in different cell lines. We found PSV is producing same cytopathic effect in BHK-21 cell line as in IBRS-2 cell line (Supplementary file 1). This virus produced cytopathic effects characteristic of CPE group II in IBRS-2 and BHK-21 cell lines which could be confirmed by RT-PCR and real time PCR (Kumari et al. 2018a) methods.

We detected PSV antigens in cell line using the indirect immunoperoxidase procedure (Schock et al. 2014) with some modification. The glass coverslips were treated with 100% methanol for 10 min and then placed in small culture plates. The coverslips were inoculated with 500 µl of inoculum containing BHK-21 cells of 18th passage suspension by putting over the coverslips without spillage. 1 ml of culture media was then added and the cells were uniformly distributed by 1 ml pipette. The plates were then incubated for 48 h at 37 °C in 5% CO₂ incubator. Culture plates with coverslip containing non infected BHK-21 cells of 18th passage suspension were also maintained for virus control. Cover slip monolayers of BHK-21 cell line were inoculated with 600 µl of the high titre PSV suspension and incubated at 37 °C in a 5% CO₂ incubator. After incubation, cover slips were harvested before detachment of BHK-21 cells because this virus causes complete detachment of monolayers within 24 h. Therefore coverslips were harvested after 9 h, 12 h, 16 h, 20 h and 22 h in order to avoid full detachment. Both infected and virus control coverslips were fixed with 1:1 acetone and methanol solution for 10 min at room temperature (RT). The coverslips were drained thoroughly and dried at room temperature (RT). Fixed preparations were stored at -20 °C in a sealed bag before staining.

The immune-labelling procedure is briefly outlined as follows. The coverslips (infected and virus control) were brought to RT, mounted onto glass slides and rinsed once in PBS for 5 min. Subsequently 100 µl of 3% H₂O₂ was applied for quenching of endogenous peroxidase activity and the slides were incubated for 20 min at RT followed by a rinse with PBS from a wash bottle and washed twice in PBS for 3 min each

with continuous slow stirring. The slides were allowed to drain and 100 µl of 5% rabbit serum in PBS was used as a blocking agent. After pouring the blocking serum, slides were incubated for 30 min at RT. After incubation, excess serum was discarded and rat anti-PSV serum (polyclonal, 1:100) was applied to the coverslip and the slides were incubated overnight in a humidified chamber at 4 °C. Infected BHK-21 monolayers with Primary Antibody control and incubated with pre-immune serum of rat (collected at 0 day of hyperimmune sera raising protocol as a control) (Supplementary Fig. 1). After incubation, the slides containing coverslips (infected, control and infected pre-immune sera) were rinsed from wash bottle containing PBS and washed twice in PBS for 3 min each with continuous slow stirring. The slides were allowed to drain, excess fluid was shaken off and 100 µl of rabbit anti-rat IgG horseradish peroxidase (HRPO) (Sigma) conjugate (1:1000) diluted in PBS was applied and the slides were further incubated for 60 min in a humidified chamber at RT. After incubation, the slides were rinsed thrice in PBS for 5 min each. The slides were allowed to drain, excess fluid was shaken off and AEC (3-Amino-9-ethylcarbazole) substrate was dissolved in recommended volume of deionized water to make working solution immediately before use. After carefully wiping around the sections, sufficient amount of substrate solution was added to cover the moist coverslip monolayers and incubated for 3 min. The reaction was terminated by washing with distilled water and then counterstained with Meyer's haematoxylin for 1 min and blueing was done with 0.2% ammonia water solution and rinsed in tap water. AEC stained sections were mounted with Crystal Mount aqueous mounting medium (Sigma-Aldrich, USA) and kept for drying. Then slides were examined under light microscope. Anti-Rat IgG (whole molecule) peroxidase conjugated antibody produced in rabbit (product no. A5957, Sigma Aldrich, USA) was used as secondary antibody for ICC.

In the current study, PSV infected IBRS-2 cell line showed round, refractile, detached floating cells with pyknotic nuclei, after 18 h post-infection (PI) (Fig. 1) and BHK-21 infected cell line also showed CPE with pyknotic nuclei, rounding and detachment of cells after 22 h (Fig. 2). Infected monolayers of BHK-21 cell line at different time intervals (18 h, 20 h and 22 h) were subjected to ICC by using primary

Fig. 1 PSV infected IBRS-2 cell line: PSV infected cell line showing round, refractile, detached floating cells, enlarged with pyknotic nuclei, after 18 h PI. bar = 20 µm. (In inset control normal IBRS-2 cell line)

Fig. 2 PSV infected BHK-21 cell line: PSV Infected cell line showing pyknotic nuclei, rounding and detachment of cells after 22 h (CPE group II) at PI. bar = 20 µm. (In inset control normal BHK-21 cell line)

antibody (anti-PSV hyperimmune sera in rats). On microscopic examination, large granular aggregates of virus particles were seen in the cytoplasm of cells near the nucleus showing cytoplasmic positive red signals (Figs. 3, 4) and without applied primary antibody used as antibody control (Supplementary Fig. 2).

In United Kingdom, immunohistochemistry has been successfully used to demonstrate PSV antigen in brain and spinal cord sections of pig in an outbreak (Schock et al. 2014). CPE were characterized by

Fig. 3 Immunocytochemistry of BHK-21 cell line infected with PSV: PSV infected cell line showing cytoplasmic positive red signals (AEC substrate) after 18 h of PI, bar = 20 μ m, at 400X (inset showing virus aggregation in the cytoplasm at 1000X) Mayer's haematoxylin used as a counter stain

Fig. 4 Immunocytochemistry of BHK-21 cell line infected with PSV: PSV infected cell line showing cytoplasmic positive red signals (AEC substrate) after 22 h of PI, bar = 20 μ m, at 400X (inset showing positive signal rounding and detachment of cell after 22 h) Mayer's haematoxylin used as a counter stain

shrunken cytoplasm of cells, rounding and detachment and floating of cells from surface of flask within 24 h. In the present study, PSV isolate was grown in IBRS-2 and BHK-21 cell lines. Virus multiplied rapidly in cell lines, showed CPE characteristic of CPE group II viruses within 24 h, which helped in differentiating this virus from other viruses of *Picornaviridae*. Diagnosis of CPE group II viruses (PSV) was done

by isolation of the virus in cell lines such as IBRS-2 and BHK-21 (Knowles et al. 1979). PEV-8 (PSV) caused unique type II CPE in porcine kidney cells (Knowles et al. 1979; Honda et al. 1990). Bai et al. (2018) reported that Vero E6, Hep G2/C3a and PGMKC non porcine origin cells are also susceptible to PSV infection. In CPE group II, affected cells of cell line show marked pyknotic nuclei and shrunken cytoplasm with irregular protrusions in stained preparations. Cells first became granular, gradually degenerated and sloughed off from the surface of culture dish. They rarely formed aggregates (Kadoi et al. 1970). The present study concluded that, PSV is able to infect cell lines other than those of porcine origin. The application of immunocytochemistry is used to demonstrate the multiplication of virus in the cell cytoplasm besides the other molecular methods such as PCR and realtime PCR. Antibody raised against PSV cultured in BHK21 cell line would be useful for diagnostic and research applications.

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Compliance with ethical standards

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

Ethical statement All the experimental procedures on animals were carried out according to the recommendations and approval of the Institute Animal Ethics Committee (IAEC) as per the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

References

- Arruda PH, Arruda BL, Schwartz KJ, Vannucci F, Resende T, Rovira A, Sundberg P, Niefeld J, Hause BM (2017) Detection of a novel sapelovirus in central nervous tissue of pigs with polioencephalomyelitis in the USA. *Transbound Emerg Dis* 64:311–315
- Bai H, Liu J, Fang L, Kataoka M, Takeda N, Wakita T, Li TC (2018) Characterization of porcine sapelovirus Isolated from Japanese swine with PLC/PRF/5 cells. *Transbound Emerg Dis* 65:727–734
- Buitrago D, Cano-Gómez C, Agüero M, Fernandez-Pacheco P, Gómez-Tejedor C, Jiménez-Clavero MA (2010) A survey

- of porcine picornaviruses and adenoviruses in fecal samples in Spain. *J Vet Diagn Invest* 22:763–766
- Chen JW, Chen F, Zhou Q, Li W, Song Y, Pan Y, Zhang X, Xue C, Bi Y, Cao Y (2012) Complete genome sequence of a novel porcine sapelovirus strain YC2011 isolated from piglets with diarrhea. *J Virol* 86:10898–10898
- Chen J, Chen F, Zhou Q, Li W, Chen Y, Song Y, Zhang X, Xue C, Bi Y, Cao Y (2014) Development of a minor groove binder assay for real-time PCR detection of porcine Sapelovirus. *J Virol Methods* 198:69–74
- Fauquet CM, Mayo M, Maniloff J, Desselberger U, Ball LA (2005) Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses: Access Online via Elsevier. eISBN: 9780080575483
- Honda E, Kimata A, Hattori I, Kumagai T, Tsuda T, Tokui T (1990) A serological comparison of 4 Japanese isolates of porcine enteroviruses with the international reference strains. *Nippon Juigaku Zasshi* 52:49–54
- ICTV (2015) International Committee on Taxonomy of Viruses. <http://ictvonline.org/virusTaxonomy.asp>. Accessed 11 Oct 2016
- Kadoi K, Kobori S, Morimoto T (1970) Studies on swine enteroviruses. Japanese 6th serotype and relationship between heat susceptibility and cytopathic effects. *Jpn J Microbiol* 14:111–121
- Kim DS, Son KY, Koo KM, Kim JY, Alfajaro MM, Park JG, Hosmillo M, Soliman M, Baek YB, Cho EH, Lee JH, Kang MI, Goodfellow I, Cho KO (2016) Porcine sapelovirus uses 2,3-linked sialic acid on GD1a ganglioside as a receptor. *J Virol* 90:4067–4077
- Knowles NJ, Buckley LS, Pereira HG (1979) Classification of porcine enteroviruses by antigenic analysis and cytopathic effects in tissue culture: description of 3 new serotypes. *Arch Virol* 62:201–208
- Krumbholz A, Dauber M, Henke A, Birch-Hirschfeld E, Knowles NJ, Stelzner A, Zell R (2002) Sequencing of porcine enterovirus groups II and III reveals unique features of both virus groups. *J Virol* 76:5813–5821
- Krumbholz A, Wurm R, Scheck O, Birch-Hirschfeld E, Egerer R, Henke A, Wutzler P, Zell R (2003) Detection of porcine teschoviruses and enteroviruses by LightCycler real-time PCR. *J Virol Methods* 113:51–63
- Kumari S, Ray PK, Singh R, Desingu PA, Sharma GT, Saikumar G (2018a) Development of a Taqman-based real-time PCR assay for detection of porcine sapelovirus infection in pigs. *Anim Biotechnol*. <https://doi.org/10.1080/10495398.2018.1549561>
- Kumari S, Singh R, Saikumar G (2018b) Epidemiological study of porcine sapelovirus infection in pigs at Bareilly area of Uttar Pradesh, India. *Biol Rhythm Res* 10:15. <https://doi.org/10.1080/09291016.2018.1557838>
- Kumari S, Ray PK, Singh R, Saikumar G (2019a) Pathogenicity of porcine sapelovirus infection in mice. *Indian J Anim Sci* 89:123–127
- Kumari S, Ray PK, Singh R, Desingu PA, Varshney R, Saikumar G (2019b) Pathological and molecular investigation of porcine *Sapelovirus* infection in naturally affected Indian pigs. *Microbial Pathog* 127:320–325
- Lan DL, Ji WH, Yang SX, Cui L, Yang Z, Yuan C, Hua X (2011) Isolation and characterization of the first Chinese porcine sapelovirus strain. *Arch Virol* 156:1567–1574
- Oberste MS, Maher K, Pallansch MA (2003) Genomic evidence that simian virus 2 and six other simian picornaviruses represent a new genus in Picornaviridae. *Virology* 314:283–293
- Palmquist JM, Munir S, Taku A, Kapur V, Goyal SM (2002) Detection of porcine teschovirus and enterovirus type II by reverse transcription-polymerase chain reaction. *J Vet Diagn Invest* 14:476–480
- Ray PK, Desingu PA, Kumari S, John JK, Sethi M, Sharma GK, Pattnaik B, Singh RK, Saikumar G (2018) Porcine sapelovirus among diarrhoeic piglets in India. *Transbound Emerg Dis* 65:261–263
- Schock A, Gurrall R, Fuller H, Foyle L, Dauber M, Martelli F, Scholes S, Roberts L, Steinbach F, Dastjerdi A (2014) Investigation into an outbreak of encephalomyelitis caused by a neuroinvasive porcine sapelovirus in the United Kingdom. *Vet Microbiol* 172:381–389
- Sunaga F, Masuda T, Ito M, Akagami M, Naoi Y, Sano K, Katayama Y, Omatsu T, Oba M, Sakaguchi S, Furuya T (2019) Complete genomic analysis and molecular characterization of Japanese porcine sapeloviruses. *Virus Genes* 55(2):198–208
- Tseng CH, Tsai HJ (2007) Sequence analysis of a duck picornavirus isolate indicates that it together with porcine enterovirus type 8 and simian picornavirus type 2 should be assigned to a new picornavirus genus. *Virus Res* 129:104–114
- Wang C, Yu D, Cui L, Hua X, Yuan C, Sun H, Liu Y (2014) Rapid and real-time detection of Porcine Sapelovirus by reverse transcription loop-mediated isothermal amplification assay. *J Virol Methods* 203:5–8
- Zell R, Krumbholz A, Henke A, Birch-Hirschfeld E, Stelzner A, Doherty M, Wurm R (2000) Detection of porcine enteroviruses by nRT-PCR: differentiation of CPE groups I-III with specific primer sets. *J Virol Methods* 88:205–218

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