SHORT COMMUNICATION

Identification of swinepox virus from natural outbreaks in pig population of Assam

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Abstract Outbreaks of swinepox [caused by a swinepox virus (SWPV)] in pigs were investigated in 3 districts of Assam, a north eastern state of India. Diagnosis of the disease was carried out employing both standard virological as well as molecular methods. Three representative isolates from different places were selected for inoculation into confluent monolayers of Porcine Kidney-15 (PK-15) cell line. The cytopathic effects were characterized by cell rounding, nuclear vacuolation, cell fusion, granulation of cells and finally detachment from third blind passage onwards. The three genes viz., *SPV18–20* and *P42* of SWPV was targeted for confirmation of the virus. Swinepox virus was successfully adapted to the PK-15 cell line from seventh passage onwards. The isolated viruses were characterized by sequencing and phylogenetic analysis of *P42* gene (extracellular envelope protein), a homologue of vaccinia virus F13L gene. In India, studies on swine pox are very limited. This is the first report on successful isolation of swinepox virus from north eastern region of India. Assam and the other north-eastern states of India being a hub for pig husbandry, isolation of swinepox virus will

help in developing and formulating control strategies against the disease.

Keywords Swinepox · Diagnosis · Swinepox virus · Isolation · Molecular characterization · *SPV18–20* gene · *P42* gene

Swinepox is an acute, often mild, infectious disease characterized by typical pox lesions in the skin and affects only pigs [7]. The causative agent for swinepox is swinepox virus (SWPV), the sole member of the genus *suipoxvirus*, belonging to the sub family *chordopoxvirinae* under family *Poxviridae* [20]. It is an enveloped virus with typical poxviral brick-shaped morphology and a large linear double-stranded DNA genome with a cytoplasmic site of replication [13]. The 146-kbp swinepox virus genome consists of a central coding region bounded by identical 3.7-kbp inverted terminal repeats and contains 150 putative genes. The swinepox virus contains three open reading frames namely, *SPV018*, *SPV019* and *SPV020* which are unique for SWPV [1].

Although worldwide in distribution, the SWPV is highly host specific and pigs are the only host species of SWPV in nature [4]. Virus transmission is usually associated with poor hygienic conditions and occurs by direct or indirect contact. The pig louse (*Haematopinus suis*) and domestic flies (*Musca domestica*) has been reported to be the mechanical vectors for SWPV transmission [17]. The mortality is generally low; the morbidity in affected herds may reach up to 100%. Piglets up to 4 months of age are most frequently affected and present severe lesions than adult animals [3]. Swinepox is a typical pox disease, with the development of the lesions progressing through the classic stages of macule, papule, vesicle and pustule or

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crust formation [16]. Clinical course of the disease is characterized by skin lesions usually along the ventral aspect of the abdomen, inside the legs and in inguinal areas [9]. The disease can be diagnosed by observing the typical clinical signs and lesions. However, isolation and electron microscopy has been used as gold standard for identification of the virus [18]. Over the recent years, few molecular detection methods have been developed for diagnosis of swinepox [14, 15]. Molecular based identification and detection of viral diseases are said to be fast, accurate, sensitive and more reliable compared to the traditional laboratory procedures and presently it is recognized as “gold standard” for many viral diseases [8].

Swinepox was reported from India for the first time in 1987 by Manickam and Mohan [12] after which sporadic cases have been reported from time to time [10, 19]. The incidence and prevalence of swinepox virus has recently been reported from the north eastern part of the country [5]. However, research on swinepox virus has not so far been carried out in greater detail as compared to other poxviruses from India. Swinepox is prevalent in most parts of Assam, but its outbreak often goes unreported as the disease itself is self-limiting and primarily occurs in rural areas with poor veterinary infrastructure. Pig rearing being the most common and economical enterprise among the small and marginal farmers of the state, extensive studies are required to characterize and understand the epidemiology of the disease.

In this study, outbreaks of swinepox in pig population of three districts of Assam, viz. Kamrup, Karbi-Anglong and Lakhimpur during 2015–2017 were investigated. The affected animals were mostly crossbred (Hampshire X Local) animals of either sex, in the age group of 6 months to 1 year. Clinical samples in the form of skin scabs were collected using sterilized forceps from naturally infected pigs showing symptoms of swinepox from different places of these three districts. Samples were processed to prepare a 10% suspension in phosphate buffered saline (pH 7.2) and was used for SWPV detection and isolation. The swinepox reference virus (AVA-121, P-9) was received from Veterinary Type culture Collection (VTCC), ICAR-National Research Centre on Equines, Hisar, Haryana was used as a positive control throughout the experiment.

Presence of the SWPV in the scab samples were screened by PCR amplification of swinepox specific genes (SPV18–20 gene) using previously published primer sets SPV18–20F (5'-CGCTACTACTACAACGTTC-3') and SPV18–20R (5'ATGCACCTTAAAAATGAAGT-3') [19]. For this, genomic DNA was extracted from processed scab materials by using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. The PCR amplification was carried out in 50 µl reaction mixture containing 5 µl of 10× buffer, 2.5 mM of MgCl₂, 10 mM

dNTP, 10 pico mole of each forward and reverse primer, 2.5 IU of Dynazyme (Thermo Scientific) along with 3 µl of template DNA. The contents were mixed thoroughly and spun briefly for a few seconds and transferred to a thermal cycler (Applied Biosystems). The amplification parameters were set as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 50 s, 50 °C for 50 s, and 72 °C for 1 min, and a final extension phase of 72 °C for 10 min. The DNA of purified reference swinepox virus was used as positive control. The PCR products were visualized on 1.5% agarose gel.

Samples found to be positive for presence of swinepox virus in PCR were processed for isolation using PK-15 cell line. A 24 h confluent PK-15 cell monolayer (25 cc flask) was inoculated with 1 ml of scab/tissue preparation following standard method [19]. The supernatant was allowed to adsorb for 1 h at 37 °C. After 1 h, the supernatant was discarded and the monolayer was washed with prewarmed calcium magnesium free phosphate buffer saline (Ca²⁺, Mg²⁺ free PBS). The monolayer was covered with 5 ml of maintenance media (Minimum Essential Medium Eagle with Earle's Salt) containing antibiotics and 5% Fetal Calf Serum (Sigma Aldrich). The cells were incubated for 5 days at 37 °C and observed daily under microscope for the appearance of CPE. Swinepox virus specific CPE if observed in the inoculated flasks were marked as positive which was further confirmed by PCR amplification of SWPV specific genes (SPV18–20 gene and P42 gene) using previously published primers [11, 19]. The PCR products were visualized on 1.5% agarose gel. The DNA of purified reference swinepox virus was used as positive control for all the PCR reactions. Molecular characterization of the isolated viruses were done by sequencing and phylogenetic analysis of P42 gene (extracellular envelope protein), a homologue of vaccinia virus (VV) F13L gene, following standard method. The full length P42 gene of SWPV was amplified by using specific primers (SPV1FP: ATAGCATCGTTTTGTTGTAATCTT and SPV1RP: CGTATTAAACTAACGAACGCATG) as reported elsewhere [11], purified and cloned to pGEMT vector. Positive clones were selected and sequenced commercially at Eurofins Ltd, Bangaluru.

In the present study, the pigs suspected to be affected with SWPV had high rise in temperature (up to 103.2 ± 0.74 °F), small circular pustular growth over the entire body (Fig. 1a) which later coalesced to form large pustular lesions along with secondary bacterial infection. Various workers have reported similar clinical findings in swinepox affected animals [5, 6, 10, 19]. Animals showing these clinical signs were diagnosed as SWPV infections and scab samples were collected from different districts/outbreaks (Lakhimpur, Kamrup and Karbi Anglong) of

Fig. 1 **a** Affected animals showing typical pox lesions on the skin in different parts of the body, **b** healthy PK-15 cell monolayer mock infected; **c** cytopathic effects shown by isolated virus in PK-15 cells at 6dpi, $\times 400$

Assam for laboratory confirmation as well as isolation and molecular characterization of the virus.

The scabs collected from affected animals were subjected for amplification of SWPV specific SPV18–20 gene in PCR. On agarose gel electrophoresis, clear expected band sizes of 555 bp (data not shown) could be observed indicating presence of SWPV in the samples. Various methodologies have been developed and applied for confirmatory diagnosis of SWPV infection in the laboratory by different workers. Molecular based diagnostics have played a major role in confirmation of various etiological agents of different diseases by detecting the nucleic acid of the agent in the clinical materials [21]. For SWPV diagnosis, PCRs targeting different genes have been developed. These include the SPV18–20 gene [19], viral late transcription factor-3 (VLTF-3) gene [14] and P42 gene [11] are common. The SPV18–20 gene is stated to be unique to SWPV and can be efficiently used for detection and differentiation of SWPV from other related poxviruses [19].

A total of three different pooled samples from different outbreaks/places of Assam, viz., Lakhimpur ($n = 1$), Kamrup ($n = 1$) and Karbi Anglong ($n = 1$) were inoculated to confluent monolayers of PK-15 cell line maintained in Eagle's minimum essential medium (EMEM) containing 10% foetal calf serum (FCS). For maintenance medium a reduced (5%) FCS was used. Samples treated with antibiotic–antimycotic solution when incubated for an hour, completely eliminated bacteria and fungi present in the suspension. The virus recovered from the scab samples was used to infect PK-15 cells but no cytopathic effect (CPE) was observed even up to three blind passages. Virus induced cytopathic changes were observed from the fourth passage onwards and was initiated by day 4 post inoculation (dpi) and progressed to 80% by day 6. Cytopathic effects were characterized by rounding, ballooning, increased granularity, vacuolation, degeneration and finally detachment of the monolayer by day 6 (Fig. 1b, c). Virus adaptation was further confirmed by PCR amplification of

SWPV specific genes, viz., *SPV18–20* and *P42* gene which yielded expected product size of 555 and 880 bp respectively, from 7th passage onwards (data not shown). All three inoculated sample showed typical CPE as well as positivity in PCR, indicating that all three samples were adapted to PK-15 cell line and hence successfully isolated in the laboratory.

Virus isolation in cell culture is regarded as the “gold standard” method for detection of poxviruses. In the present study, isolation of swinepox virus from the clinical samples in PK-15 was attempted. Typical SWPV induced CPE was observed in PK-15 cell line as reported previously [19]. These include rounding, increased granularity, vacuolation, degeneration and finally detachment of the monolayer by day 6 (Fig. 1b, c). To adapt the SWPV from clinical samples to PK-15 cells, 3–4 blind passages were necessary which may sometimes be more [9]. From India, there is only one report on successful isolation of SWPV [19]. Earlier reports have clearly mentioned about the CPE shown by SWPV in PK-15 cells. The present findings are also revealed that PK-15 cells may be a good choice for isolation of SWPV in laboratory. PCR technique has been used as a faster and more effective method of SWPV detection and confirmation. Laboratory adaptation as well as isolation of SWPV was further confirmed by PCR amplification of SWPV specific genes in the cell culture harvests. Amplification using *SPV18–20* and *P42* genes of SWPV was targeted in this study for confirmation of adaptation of the SWPV to the cell line. Also, *SPV18–20* is a unique open reading frame present in the genome of SWPV with no known function [1, 19]. The *VLTF-3* gene and *P42* gene which encodes a viral late transcription factor and the envelope protein of SWPV respectively have been frequently used for identification and characterization of SWPV worldwide [11, 14]. *P42* is a highly immunogenic envelope protein encoded in the genome of the swinepox virus, a homologue of vaccinia virus envelope protein antigen P37 (F13L gene product) [2]. On agarose

Fig. 2 Phylogenetic tree constructed based on *P42* gene of SWPV. Red cone represents the reference SWPV (AVA121) from India, blue rectangles represents the viruses isolated in the present study

gel electrophoresis, both the genes targeted for amplification, yielded an expected product sizes of 555 bp (SPV18–20 gene) and 880 bp (*P42* gene) in case of positive sample, confirming that the SWPV has been successfully adapted and isolated in the present study.

The amplified products of *P42* (envelope protein) genes of all three isolates were successfully cloned to pGEMT vector and positive clones were sequenced commercially. The envelope protein gene sequences of other related poxviruses like sheepox, goatpox, buffalopox, orf, camelpox and fowlpoxviruses were retrieved from the GenBank database and included for comparison with *P42* gene sequences of swinepox virus. On sequence analysis, it was revealed that SWPV isolates from pig population of Assam (SWPV 01_KA_Assam, SWPV 02_KM_Assam and SWPV 03_LKM_Assam) showed a high degree (97–100%) of sequence identity among themselves. All three isolates shared maximum identity (99–100%) with the previously reported SWPV isolate from India (AVA121) as well as from other countries. The swinepox viruses are clustered as a separate clade from other poxviruses in the phylogenetic tree (Fig. 2). Similar observations on molecular characterization of SWPVs have been reported by other workers (12, 15, 16, 17). Hence the

findings of the present study are in conformity with the reports of the earlier workers that the swinepox virus reported from different parts of the world shares a high degree of similarity in terms of its nucleotide level as well as at amino acid level (16, 21).

In India, studies on swine pox are very limited. This is the first report on successful isolation of swinepox virus from north eastern region (NER) of India. Assam and the other north-eastern states of India being a hub for pig husbandry, isolation of SWPV will help in developing and formulating control strategies against the disease. Isolation of more virus isolates will give a better picture of epidemiology of the disease. Further studies may be carried out to develop a good vaccine candidate for controlling the disease.

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