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Detection of Fowlpox virus carrying distinct genome segments of Reticuloendotheliosis virus

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ABSTRACT

Fowlpox virus (FWPV), the type species of the genus *Avipoxvirus* family *Poxviridae*, is a large double-stranded DNA virus that causes fowlpox in chickens and turkeys. Notably, sequences of the avian retrovirus reticuloendotheliosis virus (REV) are frequently found integrated into the genome of FWPV. While some FWPV strains carry remnants of the REV long terminal repeats (LTRs), other strains have been shown to contain insertions of nearly the full-length REV provirus in their genome. In the present study we detected heterogeneous FWPV populations carrying the REV LTR or the near full-length REV provirus genome in a Merriam's wild turkey (*Meleagris gallopavo merriami*). The bird presented papules distributed throughout the non-feathered areas of the head. Avipoxvirus-like virions were observed in the lesions by transmission electron microscopy and the presence of FWPV was confirmed by DNA sequencing. Metagenomic sequencing performed on nucleic acid extracted from the skin lesions revealed two FWPV genome populations carrying either a 197-nt remnant of the REV LTR or a 7939-nt long fragment corresponding to the full-length REV provirus. Notably, PCR amplification using primers targeting FWPV sequences flanking the REV insertion site, confirmed the natural occurrence of the heterogeneous FWPV genome populations in one additional clinical sample from another turkey affected by fowlpox. Additionally, sequencing of a historical FWPV isolate obtained from chickens in the US in 2000 also revealed the presence of the two FWPV-REV genome populations. Results here demonstrate distinct FWPV populations containing variable segments of REV genome integrated into their genome. These distinct genome populations are likely a result of homologous recombination events that take place during FWPV replication.

1. Introduction

Avipox viruses (APVs) comprise a broad group of poxviruses that are known to infect multiple avian species, including domestic poultry and wild birds (Bolte et al., 1999). APVs are enveloped, double-stranded DNA viruses with genome lengths ranging between 260 to 365 kpb and are classified within the genus *Avipoxvirus* of the family *Poxviridae* (Knipe and Howley, 2013). Several types of APVs have been described, with each type/strain usually named after the species of bird from which it was first isolated (Bolte et al., 1999; ICTV, 2015). Fowlpox virus (FWPV) is the type member of the genus *Avipoxvirus* and causes fowlpox in domestic poultry (chickens and turkeys) and wild turkeys (Tripathy et al., 2000; Weli and Tryland, 2011).

FWPV infections can lead to significant economic losses to the poultry industry (Weli and Tryland, 2011). The virus is spread through breaks in the skin, by contaminated water and food, or by biting insects. Another potential source of infection is aerosolized virus, which may be important for FWPV transmission in commercial settings (Tripathy et al., 2000; van Riper C III, 2007). FWPV infections in poultry can cause two common forms of disease, historically defined as cutaneous-

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or diphtheritic fowlpox (Tripathy et al., 2000; van Riper, 2007). Recently, a systemic form of fowlpox was described in chickens (Akanbi et al., 2016). Cutaneous fowlpox is the most common clinical presentation of FWPV infection and is characterized by epithelial hyperplasia affecting mainly non-feathered regions of the body, including the wattle, comb, and eyelids. The diphtheritic form of disease presents as proliferative necrosis of the mucous membranes of the respiratory and/or digestive tracts (Tripathy et al., 2000; van Riper, 2007). Diphtheritic fowlpox is usually more severe, resulting in higher mortality rates than the cutaneous disease. Although significant mortality rates have been observed during outbreaks of cutaneous fowlpox, mortality is often associated with secondary bacterial infections (van Riper, 2007). Outbreaks of both disease forms have been described in chickens, as well as in domestic and wild turkeys (Tripathy et al., 2000; van Riper, 2007; Weli and Tryland, 2011).

FWPV vaccines consist of live attenuated strains of FWPV or of other antigenically related APV strains such as pigeonpox virus (PGPV) (Singh et al., 2000; Wang et al., 2006). Interestingly, in recent years, many vaccine and field strains of FWPV have been shown to contain genome segments of the avian retrovirus reticuloendotheliosis virus (REV) integrated in their genome. Notably, these insertions can vary from only a few nt, corresponding to the long terminal repeats (LTRs) of the REV genome, to the near full-length REV provirus which is ~7.5 kb in length (Fadly et al., 1996; García et al., 2003; Singh et al., 2003; Weli and Tryland, 2011). Integration of REV into the FWPV genome could occur during co-infections of a host with FWPV and REV. Loss of the provirus seems to be a natural event that presumably occurs by homologous recombination between the REV LTRs during FWPV replication or by retroviral excision from the FWPV genome (Ball, 1987; Hertig et al., 1997; Niewiadomska and Gifford, 2013). Thus, it has been postulated that infected birds could potentially carry FWPV with either the REV LTR or the full REV genome (Ball, 1987; Hertig et al., 1997; Niewiadomska and Gifford, 2013). To date, however, this possibility has not been confirmed. Here we detected heterogeneous FWPV genome populations carrying the REV LTR or the full-length provirus in a Merriam's wild turkey (*Meleagris gallopavo merriami*) using metagenomics sequencing. The occurrence of heterogeneous FWPV populations seems frequent as analysis of one additional sample obtained from a wild turkey affected with fowlpox, and of a historical FWPV isolate (FWPV-MN00) obtained from chickens, also revealed the presence of at least two FWPV-REV genome populations.

2. Materials and methods

2.1. Case history

A juvenile male Merriam's wild turkey (*Meleagris gallopavo merriami*) was harvested by a hunter in Bon Homme County, South Dakota on January 10, 2015. The turkey presented multiple skin lesions covering the non-feathered areas of the head and was submitted to the SD Animal Disease Research and Diagnostic Laboratory (ADRDL) (under ID SD15-670) for diagnostic investigation. Routine pathological examination was conducted, and skin samples were collected and processed for transmission electron microscopy, and virological and molecular diagnostic investigation.

2.2. Transmission electron microscopy (TEM)

One cubic millimeter of skin tissue was submitted for TEM at the University of Minnesota Veterinary Diagnostic Laboratory (VDL). Briefly, thin sections (60–70 nm) were obtained and collected on a 200 mesh copper grid (Electron Microscopy Sciences) using a perfect loop. Grids were stained with 5% uranyl acetate for 20 min and with Satos' lead citrate for 6 min. Tissue sections were observed under a JEOL 1200 EX II transmission electron microscope (JEOL LTD, Tokyo, Japan). Images were obtained using a Veleta 2 K × 2 K camera with iTEM software (Olympus SIS, Munster, Germany). Negative staining was performed in homogenized skin biopsies. A 5 µl aliquot of the resuspended sample was placed on parafilm and placed in coated copper grids. Grids were stained with 1% phosphotungstic acid (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were visualized under the JEOL 1200 EX II TEM, and images obtained as described above.

2.3. Nucleic acid extraction

Nucleic acid was extracted from skin lesions from turkey SD15-670, and from one additional turkey presenting skin lesions in the head that was received a few months later at the SD ADRDL (ID SD15-157). DNA was also extracted from two additional FWPV strains/isolates. The first, FWPV-MN/00, was isolated from chickens affected by a severe form of fowlpox in the State of Minnesota, US, in 2000. The second is the vaccine strain FWPV-Cutter. Nucleic acid was extracted using the MagMAX viral RNA/DNA isolation kit (Life Technologies) following the manufacturer's instructions.

2.4. Metagenomics sequencing and sequence analysis

Next-generation sequencing was used to obtain the complete genome sequence of the FWPV strain associated with the infection in the Merriam's wild turkey (SD15-670) and the isolate FWPV-MN00. DNA isolated from skin lesions was used for library preparation using the Nextera XT DNA library kit (Illumina) according to the manufacturer's protocol. The DNA library was quantitated using Qubit dsDNA assay kit (Life Technologies) and the high sensitivity DNA analysis kit for Bioanalyzer 2100 (Agilent Technologies). Four nanomolar (nM) of library DNA were loaded into a MiSeq Nano Flow Cell (300 cycles, Illumina) and sequenced with the Illumina MiSeq sequencing platform (Illumina).

The FWPV genome sequences were assembled with Ray (Boisvert et al., 2012), and the terminal repeats were resolved using Celera (Myers, 2000) and Cap3 (Huang and Madan, 1999) software's. Variant base calling was performed with FermiKit (Li, 2015) and the final genome consensus sequences were mapped and visualized with Consed (Gordon et al., 1998). Open reading frames (ORFs) were inferred and annotated based on the reference FWPV strain, GeneBank accession no. NC_002188 (Afonso et al., 2000) using the genome annotation transfer utility (GATU) (Tcherepanov et al., 2006). Similarity searches were conducted for each putative ORFs, and ORFs were numbered in order from left to right of the viral genome.

2.5. Polymerase chain reaction

PCR amplification was used to confirm the presence of FWPV in skin lesions of the Merriam's turkey. A set of primers targeting a conserved region of APV DNA polymerase gene (Avi-DNAPol-Fw1-5′-GTCTGTATCCAAATGTATGCATC-3′ and Avi-DNAPol-Rv1-5′-CTATAGTAGTACACGTCTTTGC) was designed using the primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). PCR amplifications were performed in 50 µl reactions containing 25 µl of the Q5 hot start high-fidelity 2X master mix (New England Biolabs), 2.5 µl of each primer (final concentration of 0.5 µM), 19 µl nuclease-free water (Thermo-Fisher Scientific), and 1 µl of purified nucleic acid as template. The PCR conditions consisted of initial denaturation at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s. A final extension step was performed at 72 °C for 2 min. Test sample consisted of nucleic acid from skin lesions of the Merriam's turkey. Positive- (FWPV MN-00 and Cutter) and negative controls (no template) were included in all amplifications. PCR amplicons were analyzed by 1% agarose gel electrophoresis.

Additionally, PCR amplification was used to confirm the presence of REV sequences into the FWPV genome. Sets of primers targeting FWPV

sequences flanking the REV integration site (FWPV-REV-Fw-5′-CAACA ATGATACGTCTCTTCCTG-3′ and FWPV-REV-Rv-5′-GTTGTACCGAACT ACGACGA-3′). PCR conditions were performed with slight modifications to those described above. The extension step was performed at 72 °C for 4 min. Test samples consisted of nucleic acid extracted from skin lesions of both Merriam turkeys (SD15-670 and SD15-157), and FWPV strains Cutter and MN00. PCR amplicons were analyzed by 1% agarose gel electrophoresis.

2.6. Cells and viruses

The chicken embryo fibroblast cell line DF-1 was obtained from the American Type Culture Collection (ATCC CRL-12203). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 2 mm L-glutamine and containing 100 IU/mL penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin. Cell cultures were maintained at 39 °C with 5% CO_2. The FWPV strain Cutter was kindly provided by Dr. Amy McNeil (Department of Pathobiology, University of Illinois at Urbana-Champaign). The field FWPV isolate MN00 was obtained from chickens during a fowlpox outbreak in Minnesota, in 2000, and was kindly provided by Dr. Deoki Tripathy (Department of Pathobiology, University of Illinois at Urbana-Champaign). FWPV MN00 was isolated in embryonated chicken eggs and the first viral passage in the chorioallantoic membrane was used in the present study.

FWPV SD15-670 was isolated from skin lesions of the Merriam's turkey in DF-1 cells. Approximately 0.5 mg of skin lesion tissue was minced with a sterile scalpel blade and grinded using a mortar and pestle. Tissue homogenates were resuspended in 5 ml of DMEM (10% w/v) containing 5X the concentration of antibiotics used for cell culture (see above) and clarified by centrifugation at 10,000 rpm for 10 min. The clarified supernatant was filtered (0.45 µm filter) and 200 µl were inoculated in 70–80% confluent DF-1 cell monolayers cultured in 6-well plates. After 1 h adsorption 2 ml replacement media were added to each well. Mock-inoculated cells were used as controls. Inoculated cell cultures were incubated at 39 °C with 5% CO_2 and monitored daily for FWPV cytopathic effect (CPE). A second blind passage was performed at 96 h post-inoculation. Passage 2 FWPV SD15-670 was used were indicated in the present study.

3. Results

3.1. Clinicopathological findings

Gross lesions characterized by yellowish papules were observed on non-feathered areas of the head. Papules ranged from 2 to 10 mm in diameter and coalescent lesions forming large proliferative skin lesions were observed (Fig. 1A). Reddish-brown crusts covering the surface of a few lesions were also observed. No lesions were present in the oral cavity nor in the esophageal mucosa. Histological changes in the epidermis consisted of swelling and proliferation of the stratified squamous epithelium (Fig. 1B). Most epithelial cells presented edema with abundant pale eosinophilic cytoplasm containing one or more variably sized eosinophilic inclusion bodies (Fig. 1B, bottom panel). Additionally, extracellular eosinophilic accumulations were found scattered throughout the epidermis. Subjacent to the epidermal lesions, the dermis was thickened and presented edema, fibroplasia, and multifocal perivascular accumulations of pleocellular inflammatory cells.

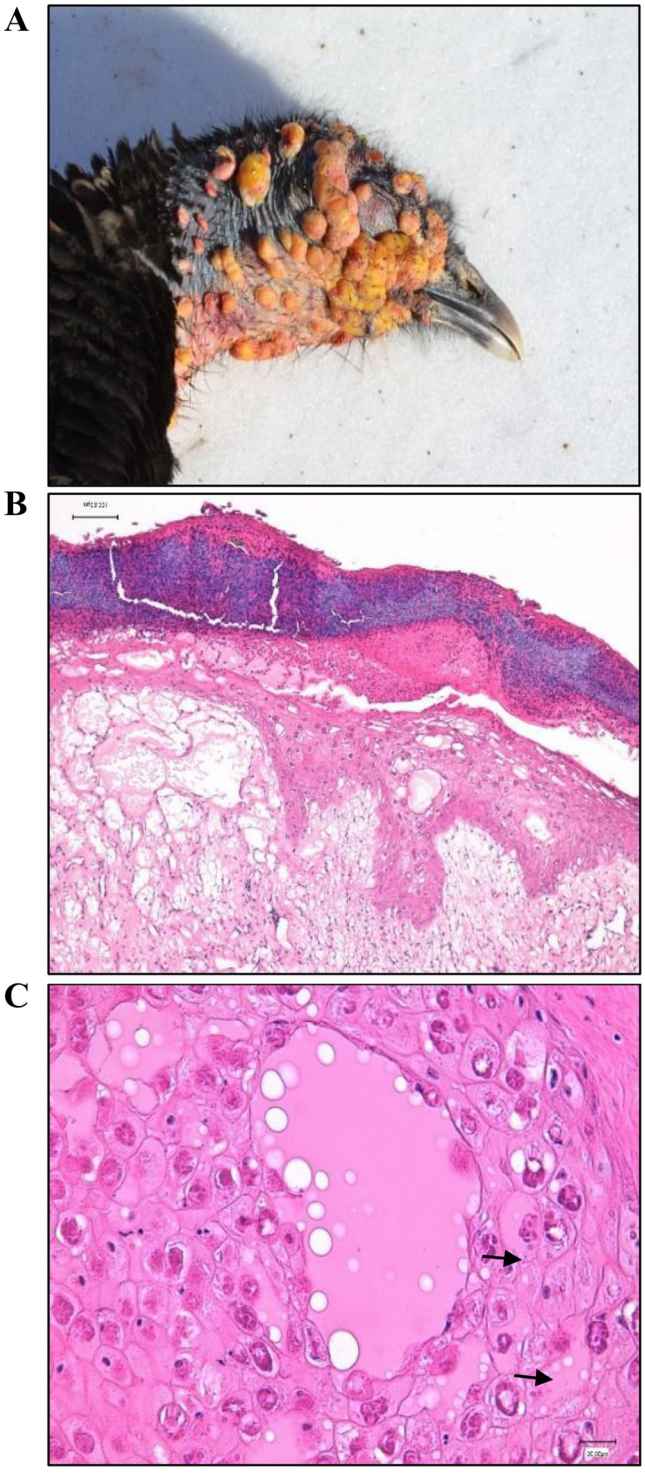

Fig. 1. Clinicopathological findings. **(A)** Gross skin lesions characterized by yellowish papules (2–10 mm in diameter) affecting non-feathered areas of the head. **(B and C)** Hematoxylin and eosin staining of skin sections demonstrating multifocal proliferative dermatitis **(B)** with epidermal cytoplasmic eosinophilic inclusion bodies indicated by arrow heads **(C).**

3.2. Detection of FWPV in skin lesions

Analysis of skin sections by TEM revealed the presence of large inclusion bodies in epithelial cells of the stratum spinosum (Fig. 2A). These viral inclusions contained numerous mature pox-like virions (Fig. 2A.2). Negative staining of skin homogenates confirmed the presence of virions with characteristic poxvirus morphology (Fig. 2C). No other viral particles were visualized on the TEM or negative stain.

The presence of an FWPV in the skin lesions of the Merriam's wild turkey was confirmed by PCR (Fig. 2D). PCR amplification using primers specific for a conserved region of APVs DNA polymerase resulted in an amplicon of ~0.45 kb (Fig. 2D). A similar product was amplified from positive controls FWPV strain Cutter and FWPV MN00. These results confirm the presence of an FWPV in skin lesions of a Merriam's

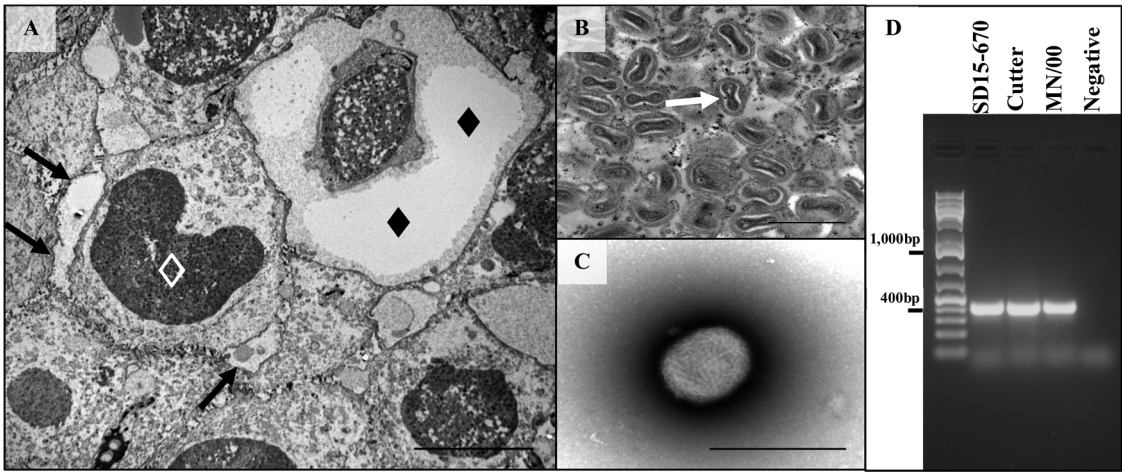

Fig. 2. Detection of avipoxvirus in the skin of a Merriam's wild turkey. **(A)** Epithelial cells of the stratum spinosum presenting larges poxvirus inclusions (Bollinger body) indicated by open white diamond. Note intercellular separation of cell of the stratum spinosum indicative of edema (arrows). One cell present lyses of organelles and distention of the cytoplasm (dark diamonds). Bar represents 10 µm. **(B)** Magnification of the viral inclusion containing numerous mature virions (arrow), displaying multiple layers and concavities of the core. Bar represents 0.5 µm. **(C)** Negative stain preparation of a poxvirus particle measuring 321–401 nm recovered from skin lesion. Bar represents 0.5 µm. **(D)** Polymerase chain reaction demonstrating the presence of avipoxvirus DNA in skin lesions of the wild turkey. DNA band shown in the image corresponds to a conserved 418-bp region of the P4b APV DNA polymerase gene. M: 1 kb DNA ladder; SD15: DNA from skin sample of the affected turkey; FWPV-Cutter: positive control DNA from FWPV strain Cutter; FWPV-MN/00: positive control DNA from FWPV strain MN/2000; No template negative control.

wild turkey.

3.3. Evidence for two FWPV genome populations in the skin of the Merriam's wild turkey

Metagenomics sequencing performed on skin samples from the affected turkey revealed two FWPV genome populations, one containing a small 197 nt insertion (FWPV SD15-670.1) corresponding to the REV LTR region and the other containing a 7939 nt insertion corresponding to the near full-length REV provirus (FWPV SD15-670.2) (Fig. 3A). The REV integration occurred at position 231,703 of the FWPV SD15-670 genome (Fig. 3A). The FWPV sequences detected in the sample shared

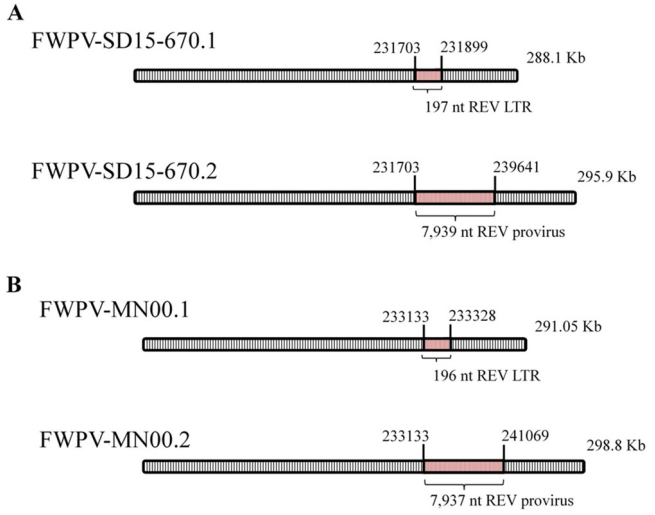

Fig. 3. Genomic features of fowlpoxvirus populations detected in the skin of the Merriam's wild turkey and the strain FWPV-MN00. **(A)** Schematic representation of the genome of FWPV-SD15-670.1 containing a short 197-nt insertion corresponding to the REV genome LTR or of FWPV-SD15-670.2 carrying a 7936 bp insertion corresponding to the full length REV provirus. **(B)** Schematic representation of the genome of FWPV-MN00.1 containing a short 196-nt insertion corresponding to the REV genome LTR or of FWPV-MN002 carrying a 7937 bp insertion corresponding to the near full length REV provirus.

~99% nt identity with other FWPV sequences available on GenBank (Fowlpox Challenge Virus - AF198100.1). Blast search with the REV full-length sequence revealed 100% nt identity with the REV strain 104,865 (KJ756349.1). Sequences of FWPV SD15-670.1 and FWPV SD15-670.2 were deposited on GenBank under accession numbers MH719203 and MH734528, respectively.

To assess whether the distinct FWPV populations were also present in other virus isolates, we performed NGS on a historical FWPV isolate obtained from chickens from Minnesota during an outbreak of fowlpox in 2000 (FWPV MN00). Sequencing of the isolate FWPV MN00 was performed on the first viral passage in the chorioallantoic membrane of embryonated chicken eggs. Sequence analysis of the FWPV MN00 revealed the presence of two FWPV-REV populations, similar to those detected in the pox lesions of the Merriam's turkey SD15-670 described above (Fig. 4A and B). The integration of REV occurred at position 233,133 of FWPV MN00 genome (Fig. 4A). Complete genome sequences of FWPV MN00.1 and FWPV MN00.2 were deposited on GenBank under accession numbers MH709125 and MH709124, respectively.

The presence of two distinct FWPV-REV populations in the skin lesion of SD15-670 and in the FWPV isolate MN00 were confirmed by PCR amplifications using primers specific for FWPV sequences flanking the REV insertion sites (Fig. 4). When nucleic acid was extracted from FWPV MN00 or from the skin of turkey SD15-670, two PCR amplicons were detected (Fig. 4). A large ~9 kb fragment containing the full-length REV genome and a small ~0.2 kb fragment were detected in both MN00 and SD15-670 samples. Notably, the same fragments (~9 and ~0.2 kb) were amplified from a skin lesion sample from another wild turkey collected in SD (SD15-157) (Fig. 4; lane 4). When the flanking primers were used to amplify nucleic acid extracted from the FWPV vaccine strain Cutter a ~0.5 kb amplicon was detected. Interestingly, a similar fragment was also evident in SD15-670 and SD15-157 samples. Together, these results confirm the presence of at least two FWPV-REV populations in the FWPV MN00, SD15-670 and SD15-157 samples. Amplification of a third DNA fragment in SD15-670 and SD15-157 samples, that is similar in size to the ~0.5 kb fragment amplified from the vaccine FWPV strain Cutter, further suggest that additional FWPV-REV viral populations may be present in FWPV infected tissues.

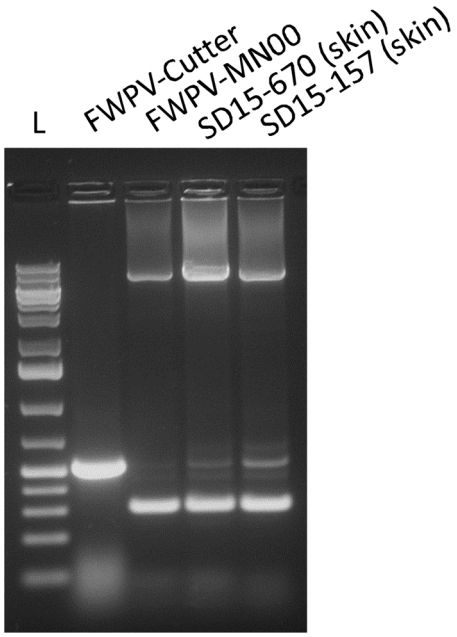

Fig. 4. Polymerase chain reaction (PCR) confirming the presence of distinct FWPV genome populations in the skin of a Merriam's wild turkey. PCR amplification using FWPV specific primers targeting sequences flanking the REV integration site. Lane 1: DNA ladder (GeneRuler 1 kb plus DNA ladder); lane 2: FWPV strain Cutter DNA; lane 3: FWPV strain MN00 DNA; lane 4: lesion skin DNA SD15-670; lane 5: lesion skin DNA SD15-157. The large ~9 kB DNA band corresponds to the fragment containing the full length REV provirus while the smaller DNA band ~0.2 kb corresponds to the fragment containing the short REV LTR remnant.

3.4. Passage of FWPV SD15-670 in cell culture reveals instability of the integrated REV provirus sequences

The stability of the REV provirus integration into the FWPV genome was investigated during virus replication *in vitro*. For this, FWPV SD15-670 was isolated in DF-1 cells (Fig. 5A and B) and passage 2 was screened by PCR using primers targeting regions flanking the REV integration site (Fig. 5C). As shown in Fig. 5C, the 9- and the 0.2-kb DNA fragments corresponding to the full-length REV provirus or the short LTR fragments, respectively, were detected in nucleic acid samples extracted directly from the skin of the SD15-670 turkey and from FWPV MN00 (passage 1). Similarly, the 0.2 kb REV LTR fragment was also detected in passage 2 FWPV SD15-670 (passage 2 in DF-1 cells); however, the 9 kb fragment comprising the near full-length REV provirus was not detected in FWPV SD15-670 passages in DF-1 cells (Fig. 5C). Notably, a smaller ~5.5 kb fragment was detected in passage FWPV SD15-670 (Fig. 5C). These results indicate the instability of REV provirus integrated in the FWPV genome and further suggest that REV proviral sequences may be lost during FWPV replication in cell culture.

4. Discussion

Here we describe the detection of two FWPV genome populations carrying the LTR or the near full-length REV provirus in a Merriam's wild turkey. Detection of these two viral populations in two clinical samples from fowlpox affected turkeys and in a historical FWPV isolate obtained from chickens demonstrate that this may be a frequent event in FWPV infected birds. Interestingly, when FWPV SD15-670 was isolated and amplified in chicken fibroblast DF-1 cell lines, the full REV provirus was lost after only two passages, indicating the instability of the REV sequences inserted into the FWPV genome.

The wild turkeys presented typical gross and histopathological lesions of avipoxvirus infection (Weli and Tryland, 2011), including papules covering the non-feathered areas of the head and swelling and proliferation of the stratified squamous epithelium. The presence of avipoxvirus virions in the skin lesions was confirmed by transmission electron microscopy (TEM) and PCR amplification. It is important to note that analysis of affected skin lesions by TEM did not reveal the presence of REV virions in affected tissues, despite the detection of REV sequences. The lack of REV particles within the affected skin, combined with metagenomics data, confirmed that the REV sequences detected in the skin lesions were integrated into the FWPV SD15-670 genome.

Previously, it has been suggested that different FWPV genome populations, carrying either the REV LTR or the full-length REV genome, could co-exist in infected cells (García et al., 2003; Singh et al., 2003). This hypothesis was based on observations that full-length REV provirus integration into the FWPV genome was unstable due, in part, to the presence of the REV LTR at each end of the inserted REV genome (Ball, 1987; Hertig et al., 1997; Niewiadomska and Gifford, 2013). Loss of the REV provirus seems to be a natural event that presumably occurs by homologous recombination between the REV LTRs during FWPV replication or by retroviral excision from the FWPV genome (Ball, 1987; Hertig et al., 1997; Niewiadomska and Gifford, 2013). Metagenomics and PCR amplification results presented here confirm this hypothesis, demonstrating that this phenomenon occurs both *in vivo* and *in vitro* in FWPV infected cells. These results demonstrate that both FWPV populations with the REV LTR or the near full-length REV genome co-exist and may be present in naturally infected birds.

Notably, after isolation of FWPV SD15-670 in DF-1 cells, a shift in the viral population, with loss of the near full-length REV provirus, was observed when PCR amplification was performed with primers targeting sequences flanking the REV insertion site. This was observed after two passages of FWPV SD15-670 in DF-1 cell cultures. Interestingly, instead of the 9 kb PCR amplicon containing the full-length REV provirus, a smaller 5.5 kb product was amplified from p.2 FWPV SD15-670. These results suggest that loss of the near full-length REV genome during passage of FWPV in cell culture seems to be common and would explain the absence of the REV provirus in FWPV vaccine strains which are likely plaque purified and mostly contain only remnants of the REV LTR region (Singh et al., 2000). The loss of the near full-length provirus genome during passage in cell culture was demonstrated in a previous study using a recombinant FWPV-REV virus expressing green fluorescent protein (Singh et al., 2003). In contrast to the vaccine strains, field FWPV isolates have been frequently reported with integration of the full-length REV provirus (Mzula et al., 2014).

While it has been demonstrated that REV is non-essential for FWPV replication *in vitro*, it was suggested that REV genes may be critical for FWPV (Singh et al., 2000). Insertion of REV in FWPV seems to be a rather new event in the evolutionary history of these viruses (Niewiadomska and Gifford, 2013) and virulent FWPV isolates without REV provirus insertion are still circulating (Laidlaw and Skinner, 2004; Mzula et al., 2014), suggesting that the insertion of REV into the FWPV genome may benefit REV. The fact that REV is also found in other viruses, including *gallid herpesvirus 2* (GHV-2), corroborate with this hypothesis (Su et al., 2013). Association of REV with FWPV may provide a more effective means of transmission and spread of the virus among susceptible hosts, for example. These possibilities, however, still need to be demonstrated experimentally in susceptible birds. It is important to note that clinical signs induced by REV, including immunosuppression, anemia, and neoplasia may increase the severity of fowlpox (Niewiadomska and Gifford, 2013; Singh et al., 2000).

Interestingly, the current study shows natural occurrence of distinct FWPV populations carrying the LTR or the full-length REV provirus genome in wild birds. While it is possible that these wild turkeys were infected through contact with domestic poultry, the possibility that FWPV is endemic in this wild bird population cannot be formally excluded. The presence/circulation of wild type FWPV in wild turkeys may have significant implications on this important wild bird species (South Dakota Department of Game Fish and Parks, 2017; van Riper,

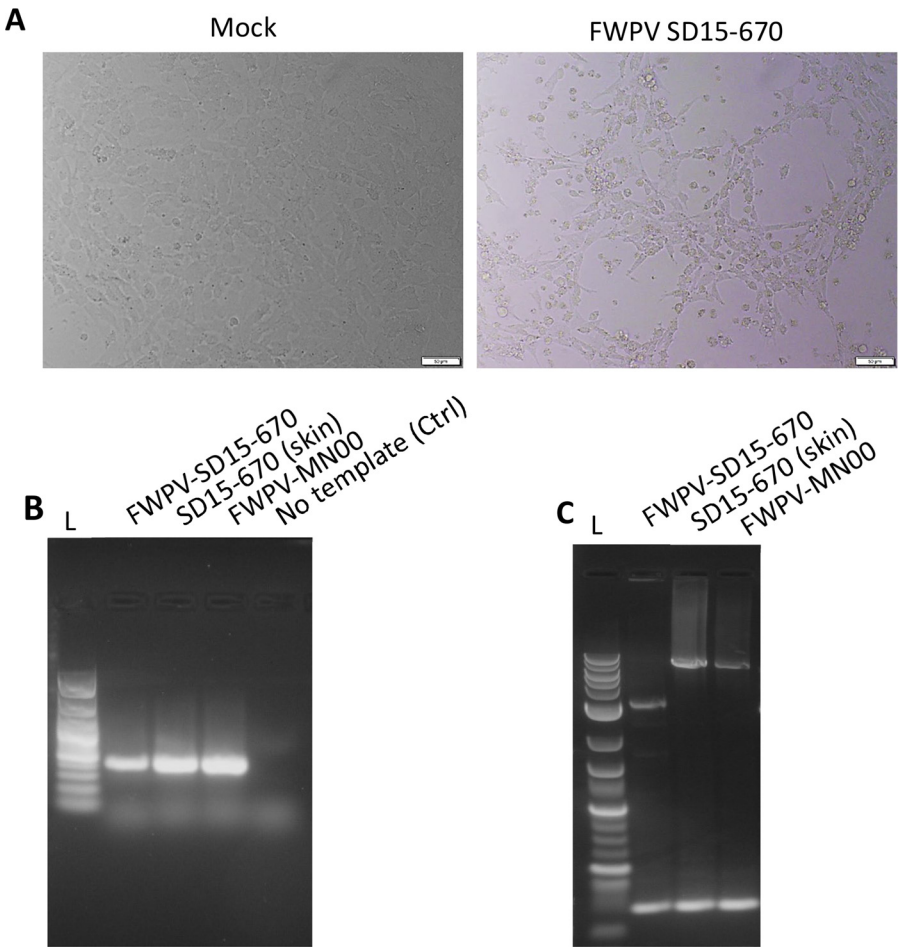

Fig. 5. Evidence of instability of REV provirus in FWPV genome. **(A)** Virus isolation in DF-1 cells. Mock infected control cells (left) and FWPV SD15-670-infected cells showing typical FWPV cytopathic effect (right) at 72 h post-infection on passage 2. **(B)** PCR amplification using avipoxvirus DNA polymerase specific primers. Lane 1: DNA ladder (GeneRuler 1 kb plus DNA ladder); lane 2: FWPV isolate SD15-670; lane 3: lesion skin DNA SD15-670; lane 4: FWPV strain MN00 DNA; lane 5: no template control. **(C)** PCR amplification using FWPV specific primers targeting sequences flanking the REV integration site. Lane 1: DNA ladder (GeneRuler 1 kb plus DNA ladder); FWPV isolate SD15-670; lane 3: lesion skin DNA SD15-670; lane 4: FWPV strain MN00 DNA The large ~9 kB DNA band corresponds to the fragment containing the full length REV provirus while the smaller DNA band ~0.2 kb corresponds to the fragment containing the short REV LTR remnant.

2007). Monitoring and surveillance for FWPV in wild turkeys may be warranted to ensure a healthy wild turkey population.

While the precise nature of the relationship between FWPV and REV is not yet completely understood, our study shows that this association is common in the field. The co-existence of distinct FWPV-REV populations indicates an unstable integration of REV into the FWPV genome. Nevertheless, detection of these distinct populations in naturally infected birds and in one virus isolate obtained several years apart suggests both viruses likely benefit from the association.

Acknowledgments

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