NOTE

Avian Pathology

Molecular characterization of avipoxviruses circulating in Windhoek district, Namibia 2021

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ABSTRACT. Samples from eleven birds (chicken, dove and peacock) with symptoms of fowlpox, caused by the avipoxvirus (APV), were collected in seven different areas of the Windhoek district, Namibia between April and October 2021. A fragment of the 4b core protein and the DNA polymerase gene of APV were amplified by PCR from the DNA of the samples and sequenced. Phylogenetic analysis revealed that the viruses present in the chickens all belonged to clade A1 while the viruses in the doves and peacock were from subclade A3.1. This is the first report of subclade A3.1 avipoxvirus in peacock. In addition, all of the samples obtained from chickens were shown by PCR to be positive for the integration of reticuloendotheliosis virus while those from the doves and peacocks were negative. This study is the first characterization of avipoxvirus in Namibia and provides additional information on the presence of avipoxvirus in southern Africa.

KEYWORDS: avipoxvirus, clade A1, clade A3.1, Fowlpox, Namibia

Avipoxviruses (APV) are large, enveloped DNA viruses that belong to the genus *Avipoxvirus* in the *Chordopoxvirinae* subfamily of the family *Poxviridae* [6]. They cause Fowlpox (FP) disease in a large number of wild and domestic bird species and can result in significant economic losses in domestic poultry as a result of a drop in egg production, reduced growth, blindness and increased mortality [6].

Phylogenetic analysis of the APV genus has largely relied on segments of the genes encoding the 4b core protein and the DNA polymerase gene which are highly conserved among all poxviruses [3, 7]. Using these loci it has been seen that the majority of the strains characterized to date cluster into three major clades A, B and C with clades A and B being subdivided further into A1, A2, A3, A3.1, A4, B1 and B2 [7, 9, 12]. Two additional clades D and E contain just one and three isolates, respectively [2, 13, 19].

The avian retrovirus, reticuloendotheliosis virus (REV) is known to partially or completely integrate into the genome of some APV viruses and it is believed that the pathogenicity of the APV is enhanced by the presence of the integrated REV [8, 10, 16, 20]. Reports from commercial flocks suggested that APV vaccine contamination with REV caused tumours in vaccinated birds [5] while, more recently, an acute outbreak of FP in China which resulted in 100% mortality of commercial chickens was caused by an APV with integrated partial REV sequences [22].

Currently in Africa, APVs have only been genetically characterised in a few countries. These include Egypt [chicken (subclade A1), pigeon (subclade A4), and turkey (subclade A2)], Nigeria [chicken (subclade A1)], Mozambique [chicken (subclade A1 and clade E), peacock (subclade A1), quail (subclade A2) and turkey (subclade A2)], South Africa [dove (subclade A3), flamingo (subclade A3), penguin (subclade A2) and pigeon (subclade A2 and A3)] and Tanzania [chicken (subclade A1)] [1, 4, 14–17]. As there was no information available on APVs in Namibia this study was undertaken to characterize viral DNA obtained from FP outbreaks in the country.

Between April and October 2021, scab samples from seven separate FPV outbreaks around the Windhoek district (Namibia) were collected and sent to the Central Veterinary Laboratory by private veterinarians (Table 1). The samples taken from the same species on the same day at the same location were from the same flock. The virus appears to have initially affected a flock of 300 broiler chickens and then backyard chickens, doves and one peacock. The clinical signs in all the chickens investigated were

Table 1. Description of samples collected and analysed in this study

Sample.	Date	Host	District	Location
A	01.04.2021	Broiler chicken	Windhoek	Dordabis
B	01.04.2021	Broiler chicken	Windhoek	Dordabis
C	06.04.2021	Backyard chicken	Windhoek	Katutura
D	06.04.2021	Backyard chicken	Windhoek	Katutura
E	12.04.2021	Backyard chicken	Windhoek	Groot aub
F	12.04.2021	Backyard chicken	Windhoek	Groot aub
G	09.09.2021	Dove	Windhoek	Klein Kuppe
H	09.09.2021	Dove	Windhoek	Klein Kuppe
I	09.09.2021	Dove	Windhoek	Klein Kuppe
J	09.09.2021	Dove	Windhoek	Klein Kuppe
K	13.10.2021	Peacock	Windhoek	Klein Kuppe

reported as listlessness and loss of appetite without associated respiratory distress. Cutaneous lesions were characterized by the presence of nodules and scabs of different sizes found on wattles, comb and eye lids. No lesions were recorded on feet and legs. The doves presented with proliferative, nodular, crusted lesions and thick scabs around the beak, eyelids and upper neck region. Associated clinical signs included mild emaciation and severe conjunctivitis; while the peacock presented with bilateral, nodular, mucocutaenous lesions that extended intraoral. Hard, caseous material was found inside the lesions at *post-mortem* examination. Associated clinical signs included severe emaciation and secondary parasitic infection.

Upon arrival at the Central Veterinary Laboratory, the samples were immediately processed. DNA was extracted from the scabs using a Maxwell®16 Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA) with an elution volume of 50 µl following the manufacturer's instructions. A fragment of the 4b core protein gene and the DNA polymerase gene were amplified using a Polymerase Chain Reaction Kit (Taq PCR Master Mix Kit, Qiagen, Hilden, Germany). For the 4b core protein gene fragment primers: FP-FW (5′- CAG CAG GTG CTA AAC AAC AA-3′); FP-RW (5′-CGG TAG CTT AAC GCC GAA TA-3′) which produces an amplicon of 578 bp, were used as previously described [7] while for the DNA polymerase gene the following primers: PoPrl (5′-CGC CGC ATC ATC TAC TTA TC-3′) and PoPr2 (5′-CCA CAC AGC GCC ATT CAT TA-3′), which produces an amplicon of 682 bp, were used [11]. Both the reactions were performed with an initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 sec, 55°C for 35 sec and 72°C for 45 sec, and then a final extension at 72°C for 7 min. In order to determine whether REV gene sequences had been integrated into the genomes of the FPVs, the following primer pairs were used to amplify a partial REV env gene and the REV env-FPV open reading frame 203, respectively: REV-env Fw1 (5′-ACC ACT CTC GAC TCA AGA AA-3′) and REV-env Rv1 (5′-CCA CAC ACA AAT ACA TGA CCC-3′) which produces an amplicon of 1,089 bp and REV env-FWPV 203 Fw1 (5′-GAA ATC TTA CGA GGC TAT GTC-3′) and REV env-FWPV 203 Rv1 (5′-TTC AAC CAC CAG GCT ACA TAA AGG-3′) which produces an amplicon of 1,437 bp [22].

All the amplified fragments were visualised on 2% agarose gels. Positive PCR amplicons for the 4b core protein and DNA polymerase gene fragments were purified using a QIAquick PCR Purification Kit (Qiagen) and were sent to LGC Genomics (Berlin, Germany) for sequencing. All sequences generated were deposited in GenBank under accession numbers MZ706997 to MZ707008. The Staden Package (http://staden.sourceforge.net/) was used to assemble the generated sequences. Multiple sequence alignment was performed using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) with default settings, incorporating all the sequences generated here combined with a selection of representative sequences available in GenBank. Phylogenetic trees were estimated using the maximum-likelihood (ML) method available in MEGA 6 [21], employing the Tamura-Nei model of nucleotide substitution and 1,000 bootstrap replications.

All of the samples tested were positive for the APV 4b core protein gene and DNA polymerase gene, while only the APV DNA obtained from the chickens tested positive for REV genome integration. ML analysis using both the 4b core protein (Fig. 1) and DNA polymerase gene fragments (Fig. 2) revealed that the sequences from the viruses from the chicken samples were identical to each other and grouped in clade A1. The sequences of the viruses from the doves and peacock were also identical to each other and belonged to subclade A3.1 according to the classification of Offerman *et al.* [18]. Avipoxviruses belonging to subclade A3.1 have been identified in columbiforms and bustards (Great and Houbara) in China, Spain, South Africa, the United Arab Emirates and the United Kingdom. To the best of our knowledge this is the first report of subclade A3.1 in peacocks. Interestingly, no clade A2 or clade E APVs, which have been identified in South Africa and Mozambique were identified in this study but this may be due to the low number of samples collected [13, 14, 18]. Therefore, whether this study is a true reflection of the circulation of APV in Namibia will require further investigation and larger sample numbers.

Regarding the data on REV genome integration, it would appear from the literature to date that only clade A1 avipoxviruses have been shown to be positive for REV genome integration. For example, clade A1 avipoxviruses described in Australia, China and the US were all REV positive while turkeys and quail infected with clade A2 avipoxviruses in Mozambique were negative for REV genome integration [10, 14, 20, 22]. Something similar has been observed here as only the clade A1 avipoxviruses were REV positive. Nevertheless, whether REV integration is only associated with clade A1 avipoxviruses requires further investigation of larger numbers of diverse samples.
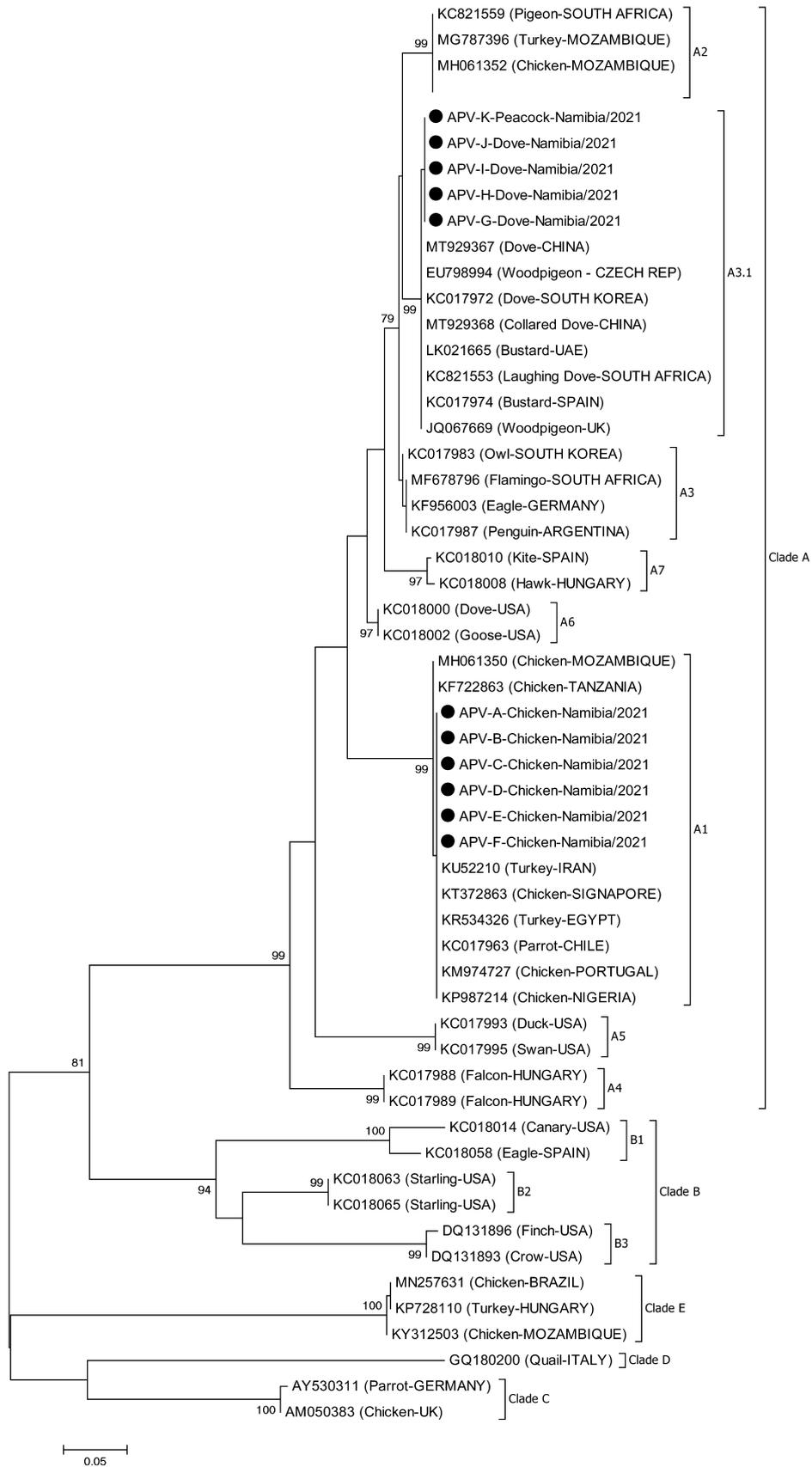
Fig. 1. Maximum-likelihood (ML) analysis using the MEGA6 software of a partial nucleotide sequence (368 bp) of the 4b core protein gene from the six samples investigated (filled circles). The numbers indicate the bootstrap values calculated from 1,000 bootstrap replicates. The scale bar represents nucleotide substitutions per site. The different clades and sub-clades are indicated as described by Gyuranecz *et al.* [7].
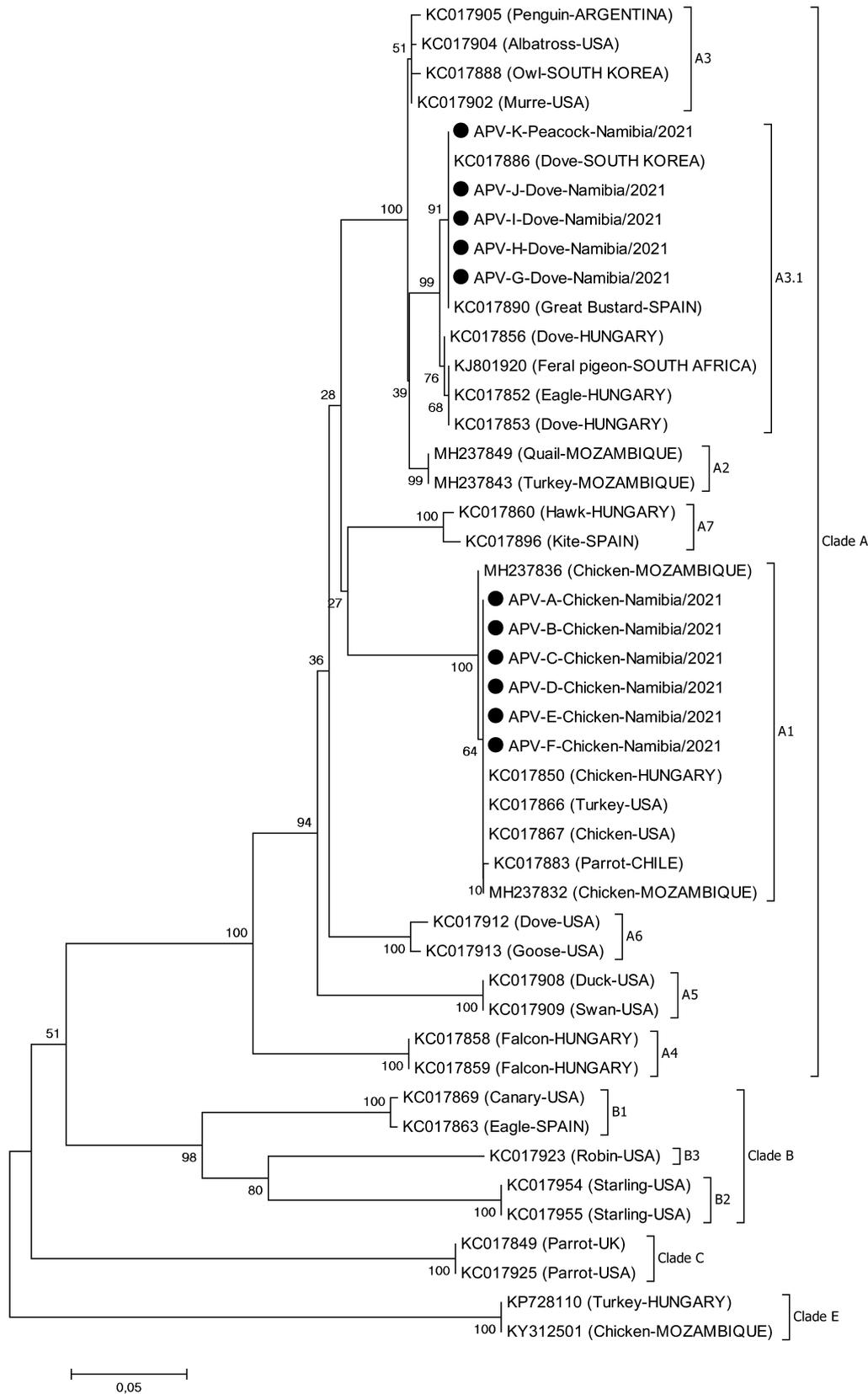
Fig. 2. Maximum-likelihood (ML) analysis using the MEGA6 software of a partial nucleotide sequence of the DNA polymerase gene (654 bp) from the six samples investigated (filled circles). The numbers indicate the bootstrap values calculated from 1,000 bootstrap replicates. The scale bar represents nucleotide substitutions per site. The different clades and sub-clades are indicated as described by Gyuranecz *et al.* [7].

Finally, none of the birds sampled in this study were vaccinated against FP even though three commercial vaccines for FP are registered and available in Namibia [i.e. Onderstepoort Fowl Pox Vaccine, AviPro AE-POX (EliLilly) and Diftosec (Boehringer Ingelheim)]. However, it appears that only large poultry companies regularly use the vaccines. Therefore, the current study should encourage veterinary authorities in Namibia to reassess vaccine and control strategies against FP in backyard and small holder farms.

CONFLICT OF INTERESTS. The authors declare that they have no conflict of interests regarding the publication of this study.

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