Circulation of A2 subclade of *Avipoxvirus* in pigeons of the Andaman and Nicobar Islands, India

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Genus *Avipoxvirus*, an important member of the family Poxviridae, has 12 species which have been recognized by the International Committee on Taxonomy of Viruses (ICTV). *Fowlpox* virus and *pigeonpox* virus are two important species that may affect other species of birds, besides chicken and fowl. Hence, accurately identifying species, clades and subclades of Avipoxvirus is vital to design and implement adequate control strategies. The present study was carried out in a pigeon colony showing symptoms of pox. A fragment of viral *Pan*-genus 4b (*P4b*) gene was amplified and sequenced. As an equivocal and prototype species, sequence information of fowlpox virus isolated from an infected bird was also generated. The generated sequence information was compared with those of previously reported strains. It was found that both fowlpox and pigeonpox viruses belonged to clade A, and there was circulation of A1 and A2 subclades in chicken and pigeon respectively. From India, one report showed the circulation of A1 and A3 subclades in Indian chickens and pigeons respectively, since the other reports were based on clinical symptoms, histopathological examination and comparison of sequence information with those available without distinct demarcation of clade and subclade of Avipoxvirus. This study describes the findings of the novel A2 subclade of Avipoxvirus and the existence of pigeonpox in the Andaman and Nicobar Islands, India.

Keywords: *Avipoxvirus*, chicken, clade and subclade, fowlpox virus, pigeon.

POX is a well-known disease for its wide host range since pox viruses may infect humans, animals, avian species as well as invertebrates. This double-stranded DNA virus with a genome size of 250–400 kb is prevalent in nearly 374 species of 23 orders, which includes domestic as well as wild birds but is not seen in kingfishers and related species due to their low species richness (contributing 1.7% of the avian species) or less contact with other species of birds[1]. Avian pox viruses belong to the genus *Avipox* under the family Poxviridae within the subfamily Chordopoxvirinae[2]. A total of 12 species have been recognized by the 'International Committee on Taxonomy of Viruses', besides a proposal of two more species[3] using two main criteria, viz. phylogenetic distance and natural host[4]. Pigeon is the natural host for pigeonpox virus, though it may affect and cause mild disease in chicken, turkey, duck and goose[5,6]. Like other avian pox viruses, pigeon pox occurs in two forms – cutaneous and diphtheritic. The former manifestation occurs from mechanical injury and through the bite of arthropod vectors. The clinical sign is characterized by proliferative nodular lesions on featherless parts of the body. The diphtheritic form occurs due to inhalation or ingestion of the virus, and there is probability of high mortality due to obstruction of the oropharynx[7,8].

There is a re-emergence of the Avipox virus in different avian species[9]. Some of these studies are based on clinical signs and histopathological examinations[10–12]. Recent studies on the Avipox virus in domestic and wild birds are based on phylogenetic signalling of *Pan* genus 4b (*P4b*) gene locus[9,13,14]. *P4b* is the most preferable molecular marker for detecting avian poxviruses, as it is highly conserved among all poxviruses[14]. To date, there is only one report on subclades of circulating Avipox virus from the Indian mainland[9]. In this study, we discuss the phylogenetic position of the pigeonpox virus from the Andaman and Nicobar Islands, which is separated from the Indian mainland by a natural geographical barrier, along with the claim of circulation of a unique clade which is yet to be reported from India.

Materials and method

Ethical permission

Ethical clearance to carry out this study was given by the Institute Animal Ethics Committee of the ICAR-Central Island Agricultural Research Institute (ICAR-CIARI), Port Blair, Andaman and Nicobar Islands, India (approval letter no. ICAR-CIARI/IAEC/2021/209 dated 13.06.2021).

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Study area and sample collection

A colony of pigeons ($n = 60$) in a residential complex of Garacharma, Port Blair, situated in South Andaman, was seen to be affected with wart-like lesions in June 2021. Observations on clinical symptoms, gross lesions, and morbidity and mortality of the birds were recorded. For confirmation and genetic characterization of the virus, nodular wart lesions were collected. The nodular lesions were excised from the infected birds and collected in separate vials containing 0.5 ml phosphate buffer saline (pH 7.2). Two randomly chosen samples from infected pigeons were collected. In addition, for characterizing the Avipox virus in chickens, biological material (nodular wart lesion, one sample) was collected from an organized poultry farm in South Andaman.

Nucleic acid extraction, amplification and sequencing of the P4b gene fragment

For the extraction of DNA, the QIAamp MinElute Virus kit (QIAGEN GmbH, Germany) was used. For amplification of the partial fragment of *P4b* gene, a primer design was adopted from Huw Lee and Hwa Lee[15], with minor modifications. The primer sequences were as follows: forward: 5′CAGCAGGRGCTAAACAACAA3′ and reverse: 5′CGGTAGCTTAACGCCGAAAA3′.

PCR amplification was carried out in a 25 µl reaction mixture containing 10× PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 1.5 U *Taq* DNA polymerase, 10 pM of each primer and 25 ng of DNA template. Cycling conditions remained the same as described earlier[14]. The PCR product was run through 1.2% w/v agarose gel and visualized in a Gel Documentation system (Labmate Asia Pvt Ltd, Chennai). PCR products were directly sent to a commercial company (Eurofins Scientific India Pvt Ltd, Bengaluru) to generate sequence information on both ends.

Bioinformatics analysis

The generated sequence information was evaluated, aligned and compared with the available sequence information using a basic local alignment search tool (BLASTn) implemented in NCBI (ncbi.nlm.nih.gov). Currently, five genetic lineages of the Avipox virus have been reported as clades A–E. Clade A is further subdivided into seven subclades (A1–A7). Accession numbers of clades and subclades of the Avipox virus used in the present study are presented in Supplementary Table 1. Nucleotide variation of Andaman isolates of pigeon pox and fowlpox with different clades and subclades of the genus *Avipox* was examined using MEGA X (ref. 16). Phylogenetic analysis based on nucleotide sequence information was performed using neighbour joining method[17] implemented in MEGA X with 1000 bootstrap replications[16]. For phylogenetic analysis, we trimmed extra and ambiguous positions from the studied sequences and the GenBank-retrieved sequences to make a homogeneous length of 410 bp dataset. To visualize the genetic relationships among different sequences, median-joining networks were constructed in PopART ver. 1.7 (ref. 18).

Results

In the colony of 60 pigeons, the morbidity rate was 31.66%, with a mortality rate of 16.66%. The affected birds had nodular lesions on their eyelids, beak and face. The affected eyes were closed (Figure 1). The clinical symptoms observed were loss of appetite, dyspnoea, gasping and dullness. In an organized poultry farm in South Andaman, the morbidity in chickens was 20.34%, and mortality was 2.77%. To rule out the possibility of infection with avian influenza, serum samples from infected birds were screened for detection of bird flu antibodies using a Bird Flu antibody ELISA test kit (Life Technologies, New Delhi). All the samples were found to be negative for avian influenza.

DNA extracted from the nodular lesions after amplification with *P4b* gene fragment-specific primer set yielded an amplicon of 574 bp (Figure 2). Subsequently, sequence information generated in the present study was submitted to GenBank with accession numbers OK483026–27 for pigeonpox and OK483028 for fowlpox.

Further, analysis on pairwise nucleotide variation revealed that the Andaman isolates of pigeon pox (OK483026–27) showed a variation of 0–1.22 with clade A, 0.311–0.323 with clade B, 0.317–0.320 with clade C, and 0.344 with a single isolate of clade D and 0.326 with a single isolate of clade E (Figure 3). When compared with different subclades of clade A, the Andaman isolates of pigeonpox exhibited no nucleotide variation with clade A2 and the highest nucleotide variation with clade A4 (Figure 4).

Phylogenetic analysis of data revealed that the Andaman isolates of Avipox virus (OK483026–28) belonged to clade A. The Andaman isolates of pigeonpox (OK483026–27) were homologous to the Indian isolates of Avipox reported elsewhere (DQ873811, MF496043, HM481408-9,

Figure 1. Pigeon showing lesion on the eye, beak and inside the buccal cavity.

NC_043178), and Avipox isolated from ostrich of unknown geographical location (AY530305). On the contrary, the Andaman isolate of fowlpox virus shared a cluster with turkeypox and sparrowpox characterized from Germany (AY530304 and AY530307 respectively) and fowlpox reported from China (KX196452) and had complete homology with Avipox virus reported from Germany (AY530304, AY530307; Figures 5 and 6). Further analysis of data revealed that the Andaman isolates of pigeonpox (OK483026–27) belonged to subclade A2 while the Andaman isolate of fowlpox was under subclade A1 (Figures 7 and 8).

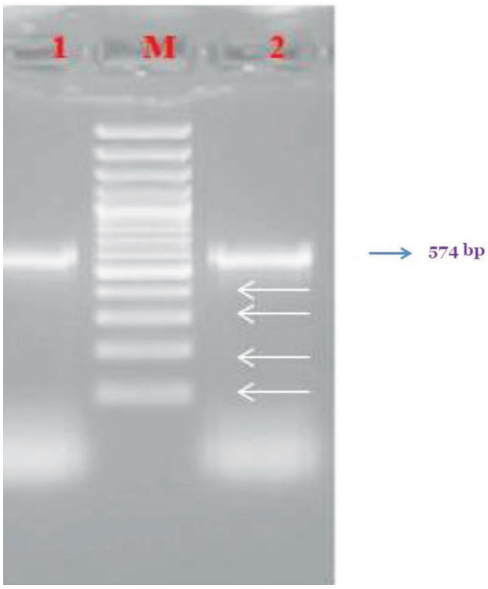

Figure 2. Amplification of partial fragment of P4b core protein of pox virus (lanes 1 and 2, amplified products of pigeon pox and fowl pox respectively, lane M, 100 bp DNA ladder; arrows from the bottom – 100, 200, 300 and 400 bp).

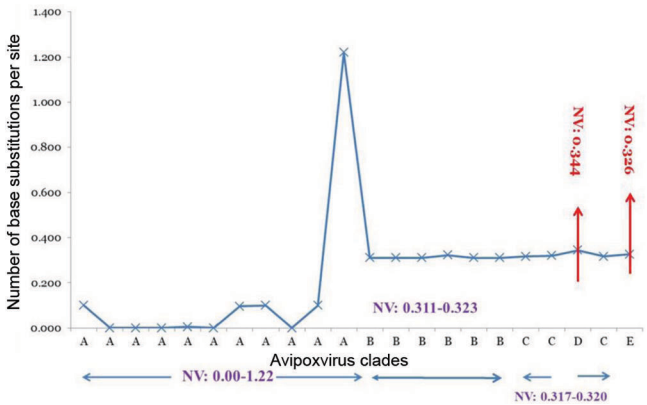

Figure 3. Pairwise nucleotide sequence variation of Andaman isolates of pigeon pox with A–E clades of Avipoxvirus (NV, nucleotide variation). The prototype strains used are as follows: Clade A – Fowlpox (KX196452, OK483028), quailpox (NC_043178), turkeypox (AY530304), ostrichpox (AY530305), falconpox (AY530306), sparrowpox (AY530307), Indian little brown dovepox (HM481408), common wood pigeonpox (HM481409) and pigeonpox (MF496043, DQ873811). Clade B – canarypox (AY318871, AY530309), creat titpox (AY453173, AY453174, AY453175), stone curlewpox (AY530310). Clade C – agapornispox (AY530311), macawpox (AM050382), fowlpox in parrot (AM050383). Clade D – fowlpox in quail (GQ180200). Clade E – turkeypox (KP728110).

Discussion and conclusion

Avian pox is a deadly viral disease that affects 374 avian species from 23 orders[1,19]. The infection causes huge economic loss to the poultry industry as it impairs growth and egg production, and causes blindness and significant mortality to the poultry birds[5]. It also reduces mating success and is therefore considered a risk factor for conserving endangered bird species[20]. Thus, the genetic diversity of Avipoxviruses needs to be understood to design and implement

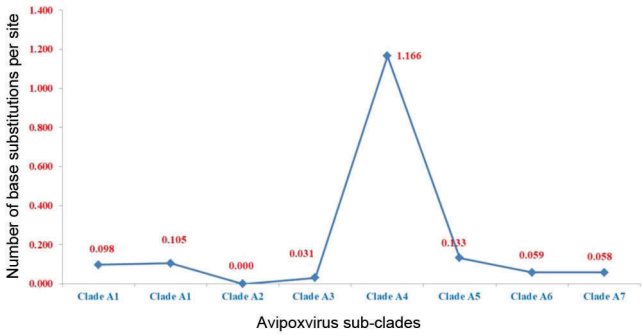

Figure 4. Pairwise nucleotide sequence variation of Andaman isolates of pigeon pox with A1–A7 subclades of Avipox virus (NV, nucleotide variation). The prototype strains used are as follows: Subclade A1, fowlpox (AM050377, GQ180207); subclade A2, fowlpox (GQ180204); subclade A3, great bustardpox (KC017974); subclade A4, woodpecker finchpox (KC017949); subclade A5, wood duckpox (KC017996); subclade A6, mourning dovepox (KC018000) and subclade A7, Goshawkpox (KC018008).

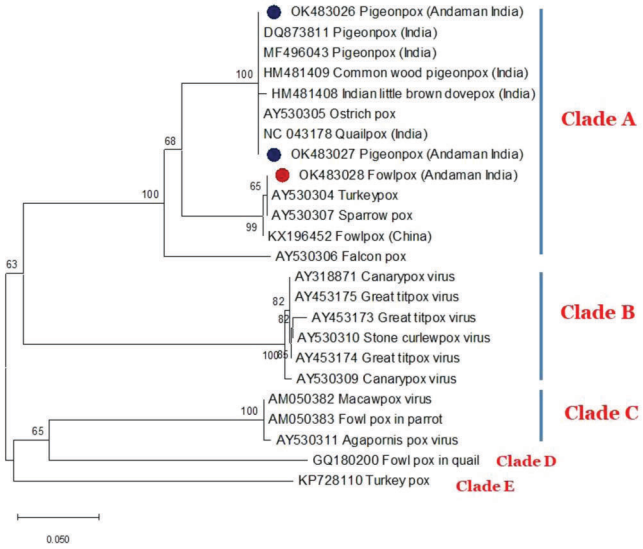

Figure 5. Phylogenetic analysis of clades of Avipoxvirus based on the nucleotide sequence on *P4b* gene fragment for the phylogenetic signalling of Andaman isolates of pox virus isolated from pigeon and fowl. The evolutionary distances were estimated using neighbor-joining method with 1000 bootstrap replications.

adequate control strategies[21]. In the present study, the genetic lineage of Avipoxvirus has been determined with the aid of *Pan* genus 4b core protein using primers described elsewhere[15], with minor modifications in the sense primer.

Avian pox is known to occur in two forms: the dry or cutaneous form and the diphtheric form. The latter form is less common, while the former is more common in birds[2]. The cutaneous form of pox is characterized by wart-like growth on the feather-free areas of birds like legs, feet, beaks or eyes. Wart-like growth is generally 1–5 mm in diameter, and birds may not exhibit any clinical signs and symptoms. However, in severe cutaneous lesions, there may be impairment of vision, loss of appetite and difficulty breathing[22,23]. In the present study, the birds suffered from loss of appetite and lost their vision since lesions were seen on the eyes. The mortality rate was high (16.66%) due to the severity of the lesions.

In this study, we have characterized pigeonpox and fowlpox on the basis of sequence information and phylogenetic signalling of the *Pan*-genus P4b locus, which is a well-known pre-requisite for the characterization of Avipox virus[24,25]. On the basis of sequence information, a total of five genetic lineages of the Avipox virus have been reported as clades A–E. Fowlpox, turkeypox, albatrosspox, pigeonpox, ostrichpox, sparrowpox and falconpox viruses come under clade A. Clade B includes canarypox, Great titpix, stone curlewpox and houbarapox viruses. Clade C comprises macawpox, parrotpox and agapornispox viruses[8,26]. Bányai *et al.*[21] and Manarolla *et al.*[27] have described two unique clades designated as clades D and E. The findings of the present study on the clustering of pigeonpox and fowlpox in clade A are in agreement with the findings of Jarmin *et al.*[8].

Clade A has been subdivided into seven subclades (A1–A7) and clade B into three subclades (B1–B3)[8,27,28]. Subclade A1 has been reported in chicken (China, Tanzania, Portugal, Nigeria, UK, France, Europe, Hungary, Egypt and USA), Turkey (Egypt Iran, the USA and Italy), parrot (Chile), MacQueens bustard (UAE), blue-eared pheasant (Hungary), and paradise shelduck, variable oystercatcher, black robin and shore plover (New Zealand)[9]. Subclade A2 has been reported in pigeon (Egypt, UK and South Africa), turkey (Mozambique, UK and Italy), dove (Hungary), Indian peafowl (Hungary), MacQueens bustard (UAE), grey partridge (Italy), gyrfalcon (Italy), as well as booted eagle, red kite and red-legged partridge (Spain)[9]. Subclade A3 has been reported in feral pigeons (South Africa), oriental turtle dove (South Korea) and great bustard (Spain), while subclade A4 in red-footed falcon and peregrine falcon (Hungary), subclade A5 in red head duck and trumpeter swan (USA), subclade A6 in goose and mourning dove (USA), and subclade A7 has been reported in hawk and red kite (Spain)[9]. Subclade B1 has been reported in canary (USA) and golden eagle (Spain), subclade B2 in European starling (USA), and subclade B3 has been reported in American crow and house finch (USA)[9].

Several studies from India have described Avian pox infection, including the circulation of the virus in pigeons. Reports are available from the northern, eastern, western and southern parts of the country[9–12,14]. Sahu *et al.*[9] studied

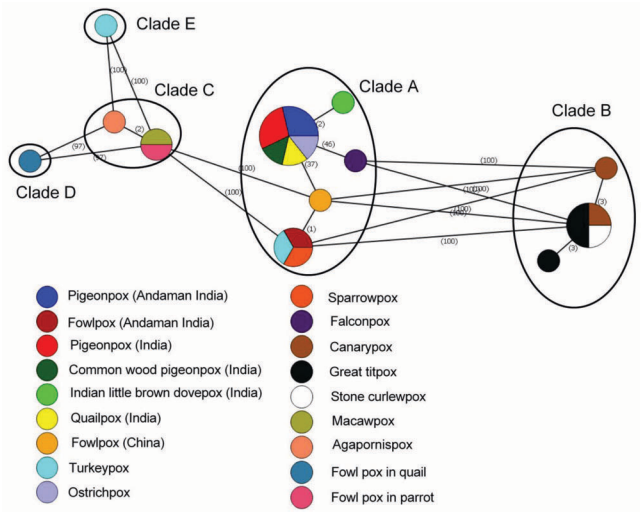

Figure 6. Network analysis of clades of Avipoxvirus based on the nucleotide sequence on *P4b* gene fragment to determine the positioning of Andaman isolates of pox virus isolated from pigeon and fowl.

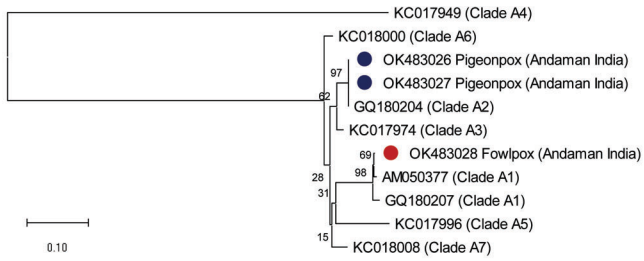

Figure 7. Phylogenetic analysis of subclades of Avipoxvirus based on the nucleotide sequence on *P4b* gene fragment for the phylogenetic signalling of Andaman isolates of pox virus isolated from pigeon and fowl. The evolutionary distances were estimated using neighbor-joining method with 1000 bootstrap replications.

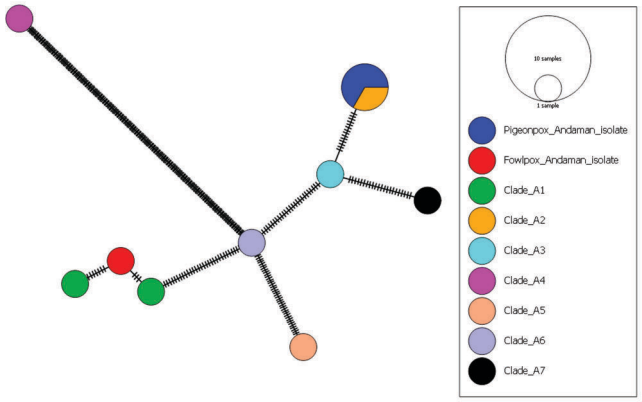

Figure 8. Network analysis of subclades of Avipoxvirus based on the nucleotide sequence on *P4b* gene fragment to determine the positioning of Andaman isolates of pox virus isolated from pigeon and fowl.

the circulation of subclades of clade A in pigeons and chickens and reported the circulation of subclades A1 and A3 of *Avipox* respectively[8]. However, the present study reports the circulation of the A2 subclade of the Avipox virus in pigeons from India. The difference in genotype may be due to the natural geographical barrier between the Andaman Islands and mainland India (Supplementary Figure 1).

During the present study, morbidity and mortality due to pigeonpox were higher than fowlpox in chickens. This may be attributed to the immediate isolation of infected chicken from the rest of the flock and palliative treatment with oral antibiotics and topical application of antiseptic ointment to check for secondary infection. However, such approaches cannot be implemented in semi-domesticated pigeons.

Animals and humans play no role in the spread of pigeonpox and fowlpox infection. For controlling pox infection in pigeons and chickens, regular vaccination is advised. During an outbreak, segregation of the infected bird and its palliative treatment with oral and topical antibiotics is advocated, along with disinfection of the premises to prevent the spread of the virus. This is extremely important considering the recent outbreak of monkeypox in humans in several countries.

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