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Research Article

INVESTIGATING FOWLPOX VIRUS IN CHICKENS: EXTRACTION, MOLECULAR PROFILING AND PHYLOGENETIC ANALYSIS

Sanganagouda K[1*], Nagaraja K[1], Basavaraj Sajjanar[2], Sabha Kounin[1], Muniyellappa H.K[1], Pavithra B.H[3], Sumathi BR[1], Shivaraj Murag[1], Shivashankar B P[1], Arun Kharate[4], Mukartal S.Y[5], Raveendra Hegde[1]

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ABSTRACT: Fowlpox is a highly contagious viral disease that is endemic worldwide, caused by the *Avipoxvirus* genus within the *Poxviridae* family. To diagnose and study the evolution of this virus, PCR amplification combined with sequencing and phylogenetic analysis are effective methodologies. In this study, nodular tissue from a suspected infected bird was collected and tested for the presence of the Fowlpox virus. The virus was identified using conventional PCR, targeting the virion core protein (P4b) gene with specific primers, resulting in an amplicon of 415 bp. The virus was subsequently isolated from chicken embryonated eggs and confirmed through conventional PCR. The partial P4b gene was sequenced and the sequence was submitted to GenBank with the accession number OQ469498. Phylogenetic analysis of the Fowlpox virus outbreak isolates revealed that it formed a distinct clade with isolates from Germany, the United States, China, South Africa, New Zealand, and Australia, all exhibiting 100% homology. This analysis indicated minimal genetic diversity within the Fowlpox virus, suggesting its stability. Additionally, the investigation into the outbreak highlighted issues such as vaccine failure, changes in tissue tropism, and variations in disease outbreak patterns. Therefore, it is essential to conduct focused and comprehensive molecular epidemiological studies and research on host-pathogen interactions to understand better these factors, which will aid in the effective control and eradication of Fowlpox disease.

Keywords: Fowlpox virus, P4b gene, Phylogeny, Skin nodule, Virus isolation.

INTRODUCTION

Fowlpox (FP) is a highly contagious disease transmitted by vectors, primarily affecting chickens, and caused by the Fowlpox virus (FPV) [1]. The virus was first discovered by Bollinger in 1873 and a vaccine was introduced in 1918. In recent years, FP outbreaks in India and other regions have led to considerable socio-economic repercussions [2, 3]. Due to its rapid spread and economic significance, FP is classified as a list-B notifiable disease by the World Organization for Animal Health (WOAH) [4]. Fowlpox has been reported globally, manifesting as a mild to severe disease in poultry. Notably, there has been a rise in fowlpox outbreaks in poultry flocks, particularly in countries such as China, Nepal, Bangladesh, Egypt, and various nations in Africa and Kenya [5, 6, 7]. This increase is attributed to the emergence of a novel strain of the Fowlpox virus. To combat Fowlpox virus infections, live attenuated vaccines have been utilized. However, many strains of FPV are found to contain nearly complete genomes of the reticuloendotheliosis virus (REV). This genetic incorporation is believed to enhance the virulence of FPV and is associated with outbreaks resulting from REV-induced immunosuppression. Consequently, the recurrence of FP in flocks that have previously been vaccinated poses an ongoing challenge and concern for poultry health management [8]. Addressing these issues

[1]*Institute of Animal Health and Veterinary Biologicals (IAH&VB), KVAFSU, Hebbal, Bengaluru Karnataka, India.*
[2]*Indian Veterinary Research Institute (IVRI), Hebbal, Bengaluru, Karnataka, India.*
[3]*Veterinary College, Hebbal, KVAFSU, Bengaluru, Karnataka, India.*
[4]*Veterinary College, Bidar, KVAFSU, Bidar, Karnataka, India.*
[5]*Veterinary College, Athani, KVAFSU, Athani, Karnataka, India.*
[*]*Corresponding author. e-mail: drpatilvet@gmail.com*

requires continuing research, monitoring to improve vaccine efficacy and understand the dynamics of host-pathogen interactions in the context of Fowlpox outbreaks.

The Fowlpox virus belongs to the *Chordopoxvirinae* subfamily within the *Poxviridae* family [9, 10]. Among the genera of Chordopoxvirus (ChPV), the Avipoxvirus (APV) genus is notable for having the largest and most genetically diverse genome [11]. This genus includes several species, such as the Fowlpox virus (FWPV), Turkeypox virus (TKPV), Pigeonpox virus (PGPV), Canarypox virus (CNPV), Ostrichpox virus (OSPV), Penguinpox virus (PEPV), Falconpox virus (FLPV) and Sparrowpox virus (SRPV), along with various newly identified poxviruses affecting different avian species [12]. Avipoxviruses can be transmitted from an infected host to a susceptible one primarily through direct contact with broken skin. Additionally, indirect transmission can occur via mosquitoes, particularly those of the *Culex* and *Aedes* species, which serve as mechanical vectors for these viruses [13, 14, 15]. Infection with avianpox viruses typically develops slowly and can present in two forms: cutaneous and diphtheritic [3, 5]. The cutaneous form is the more common type, generally self-limiting and characterized by wart-like nodules that appear around the eyes, beak, and areas of non-feathered skin [16]. The diphtheritic form also referred to as "wet" pox, leads to lesions on the mucosal membranes of the mouth and respiratory tract, including the throat, trachea, and lungs of infected birds. This form can result in breathing difficulties and challenges with feeding [2]. The size and number of nodular growths vary according to the stage and severity of the infection [9, 17].

Despite over 150 years of extensive research and numerous studies, many questions regarding Fowlpox (FP) remain unanswered. One significant challenge is the large genome size of the Fowlpox virus (FPV), which is approximately 300 kbp, leaving many aspects of its genetic composition unresolved [18, 19]. Since the completion of the first FPV genome sequence in 2000, a few additional strains have been sequenced. Among these, the P4b gene, which encodes a 75.2 kDa virion core protein, is highly conserved across all poxviruses [20, 21]. This gene is crucial not only for understanding the pathogenicity and diagnosis of FPV but also for genetic characterization and determining the virus's origin through sequencing and phylogenetic analysis. Traditional laboratory diagnosis of APVs relies on clinical signs, histopathological examinations, electron microscopy, molecular analyses, and serological methods [22]. Polymerase chain reaction (PCR) has emerged as the most sensitive molecular technique for routine diagnosis, aiding in the understanding of host range, epidemiology, and pathogenesis. Phylogenetic analysis helps determine the evolutionary relationships between different avian poxvirus strains, providing insights into their origins and transmission patterns. It also aids in identifying genetic variations that can influence virus behavior, such as virulence and host specificity [23]. In the current study, avian poxvirus was isolated from cutaneous lesions in chickens, and these isolates were genetically characterized using sequencing and phylogenetic approaches. This study aimed to identify genetic variations and any molecular mutations present in the isolates associated with the outbreak being investigated.

MATERIALS AND METHOD

Samples collection and processing

During investigations conducted on eight organized poultry farms in the Koppal district of Karnataka, farmers reported that 165,200 layer birds had been vaccinated with a commercial live attenuated FPV vaccine at three months of age. Despite this vaccination, symptoms of FPV were observed in 400 birds. During the visit, 40 diseased chickens were presented for post-mortem examination, revealing nodular skin lesions in the interdigital web. The farmers noted that these nodular lesions had previously appeared in unfeathered areas such as the comb, wattle, eyelids, and beak. However, in the recent episodes, the lesions were more prominently located in the interdigital web and on the shank region.

Pooled samples were collected aseptically and stored in viral transport media (VTM) containing 1% antibiotic and antimycotic solution. Nodular tissue from the affected birds was grounded using a sterile mortar and pestle along with sterile sand. A 10% (w/v) suspension was then prepared in sterile phosphate-buffered saline (PBS) (pH 7.4), also containing the antibiotic and antimycotic solution to prevent bacterial and fungal contamination (cat no: A5955, Sigma-Aldrich, USA). This homogenized tissue suspension was frozen at -80°C for 30 minutes and then thawed at 37°C up to five times to facilitate the release of viral particles from the cells. Afterward, the mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting clear supernatant was carefully transferred to a clean, sterile microcentrifuge tube and stored at -20°C for future use.

Histopathology

Skin nodules were collected at different stages of disease progression and subsequently tested positive for the Fowlpox virus using conventional PCR technique. The clinical specimens were initially preserved in 10% neutral buffered formalin for 48 hours to ensure proper fixation. Following this fixation period, the samples were processed for histological examination by cutting the specimens and embedding them in paraffin wax. Thin sections, measuring 3 to 4 micrometers in thickness, were then prepared from the paraffin blocks. These sections were stained with hematoxylin and eosin (H&E) to facilitate detailed examination under a bright field microscope (Leica DMi8, Germany).

Isolation of Fowlpox virus in embryonated chicken eggs

Nine-day-old embryonated chicken eggs were sourced from a poultry facility and assessed for viability through candling, with the air cavity marked for reference. Approximately 1 ml of a filtered inoculum was then injected into the Chorioallantoic membrane (CAM) of each egg. To prevent contamination and maintain the integrity of the inoculum, the openings created in the air sac and the shell of the inoculated eggs were sealed using melted paraffin. The inoculated eggs were placed in an incubator (Incucell, Germany) and maintained at a temperature of 37°C for a period of 5 to 6 days. During this incubation period, the eggs were candled twice daily to monitor their development and viability. After the incubation period, the eggs were subjected to freezing at 4°C for 20 minutes to facilitate the collection of the CAM.

Following the freeze-thaw process, the thickened CAM was carefully harvested for the preparation of the inoculum. To enhance the concentration of the virus, multiple passages were performed using the harvested CAM. The CAM exhibiting pock lesions, indicative of viral replication, was collected for subsequent DNA extraction. This extracted DNA was then subjected to PCR for confirmation of the presence of the virus, thereby ensuring the reliability of the inoculum for further studies.

Genomic DNA extraction

Genomic DNA from the Fowlpox virus was isolated from the supernatant of homogenized nodular tissue using the DNeasy Blood & Tissue kit (Cat no-69504, QIAGEN, Germany), adhering to the manufacturer's protocol with slight modifications. In summary, 200 µl of the nodular tissue supernatant was first filtered through a 0.45 µm syringe filter (Millipore). Then the filtered supernatant underwent a lysis step at 56°C for 10 minutes to facilitate the breakdown of cellular components and release the viral DNA. Following this lysis phase, the sample was subjected to a series of washing steps as outlined in the kit instructions. Finally, the DNA was eluted in 50 µl of the elution buffer provided with the kit.

To assess the quality and concentration of the extracted DNA, measurements were taken using a micro volume UV spectrophotometer (Eppendorf, USA). The absorbance was recorded at wavelengths of 260 nm and 280 nm, using the elution buffer as a blank control. This analysis allowed for the determination of the purity and concentration of the DNA samples.

Fowlpox virus confirmation

The presence of Fowlpox virus in samples collected during the outbreak investigation was confirmed using specific primers: the forward primer 5'-CGTACATCCAAGGTCCCATTT-3' and the reverse primer 5'-TTCGATAGTACCACGGGTAGAG-3'. To amplify the P4b gene, polymerase chain reaction (PCR) was set up in a total reaction volume of 25 µL. This mixture included 12.5 µL of 2x DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 5.5 µL of nuclease-free water (NFW), 5.0 µL of the extracted DNA template, and 1 microliter (µL) of each primer of around 10 micro molar concentration. The PCR amplification was performed under a series of thermal cycling conditions: an initial denaturation step at 94°C for 2 minutes was followed by 35 cycles consisting of denaturation at 94°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds. The final elongation step was conducted at 72°C for 2 minutes to ensure complete amplification of the target gene. After the PCR process, the resulting amplicons were analyzed by electrophoresis on a 1.5% agarose gel that contained 0.5 µg/mL of SYBR Safe DNA gel stain (Invitrogen, USA). The gel was run in Tris-Borate-EDTA (TBE) buffer at a voltage of 100 volts for 45 to 60 minutes. To determine the size of the PCR products, a 100 bp DNA ladder (APS LABS, Cat No- MAGSPIN-21, India) was included in the gel. Following electrophoresis, images of the gel were captured and analyzed using the GelDoc Go Imaging System (Bio-Rad, USA), allowing for the visualization and confirmation of the presence of the Fowlpox virus in the tested samples.

Sequencing of P4b gene of Fowlpox virus

Following agarose gel electrophoresis, the PCR amplicon reaction mixture was scaled up to a total volume of 50 µL to facilitate further analysis. The amplicons corresponding to the P4b gene were then purified using a PCR purification kit (Cat no: 69504, QIAGEN, Germany), meticulously following the manufacturer's instructions to ensure optimal recovery of the target DNA. Subsequently, Sanger sequencing was performed to determine the nucleotide sequence of the purified P4b gene amplicons. This sequencing was conducted by M/S Barcode Bioscience Private Limited, located in Bangalore.

Phylogenetic analysis

The P4b gene sequences obtained from M/S Barcode Bioscience Private Limited were trimmed for quality and assembled into consensus sequences using Molecular Evolutionary Genetics Analysis (MEGA version 11) and Serial Cloner software (version 2.6.1). The sequence data for the isolate identified in this study has been submitted to GenBank. Subsequently, the trimmed sequences were compared with other P4b gene sequences of Fowlpox and avian pox available in the NCBI database. To confirm the specificity of the Fowlpox P4b nucleotide sequences, they were translated into amino acid sequences using the ExPASy online tool, followed by a multiple sequence alignment performed with MEGA 11 software. Finally, a phylogenetic tree was constructed using the Neighbor-Joining method to visualize the evolutionary relationships among the analyzed sequences, offering insights into the genetic diversity and evolution of the Fowlpox virus by comparing it with other avian poxviruses [24].

RESULTS AND DISCUSSION

The Fowlpox disease is a slow-spreading, vector-borne, non-zoonotic, viral infection of domestic and wild birds caused by the Fowlpox virus [13, 16]. The genome, which contains 260 to 309 Kbp of double-stranded DNA, is larger than the earlier identified *Chordopoxvirus* (ChPV) genome [18]. The clinical symptoms of FPV varied according to the severity of the disease. The disease is characterized by proliferative lesions in the skin that progress to thick scabs (cutaneous form) and by lesions in the upper gastrointestinal tract and respiratory tracts (diphtheritic form). Virulent strains may lead to cause lesions in internal organs (systemic form). In commercial poultry, FP is a frequent and economically serious disease that can have adverse impacts on flock performance, including reduced egg production and growth, blindness, and an increase in mortality [25, 26]. This disease spreads slowly, however, it spreads faster if insects like mosquitos and mites are present in the flock [27].

Fowlpox is recognized as one of the earliest documented diseases affecting poultry with comprehensive studies dating back to the 1870s by Von Bollinger, who conducted microscopic examinations of eosinophilic cytoplasmic inclusion bodies [28, 29]. The clinical manifestations observed during outbreaks were characterized by distinct epithelial lesions located on the heads of infected birds. These lesions correlated with notable epithelial hyperplasia, accompanied by cell ballooning and degeneration, as well as the presence of cytoplasmic inclusion bodies, commonly referred to as Bollinger bodies. This has been previously documented in cases of cutaneous Fowlpox [2].

In addition to the typical lesions, birds infected with Fowlpox virus (FPV) exhibited nodular growths that ranged from small, localized yellowish eruptions to more pronounced, spherical wart-like masses found on unfeathered skin areas, including the comb, wattle, eyelids, and snood in turkeys, where relevant data were available [30]. In the present study, the affected bird displayed multifocal, raised nodules that were grey or tan, presenting as crusty and verrucous irregular formations on the feet and the interdigital web region (Fig. 1). Histopathological analysis of the epidermis revealed hyperkeratosis, with occasional discontinuities observed in the keratin layer (Fig. 2). Diffuse areas of the epidermis exhibited hyperplasia and ballooning degeneration of keratinocytes (Panels A & B). Many keratinocytes displayed cytoplasmic vacuolation and eosinophilic intra-cytoplasmic inclusions (Bollinger bodies), which resulted in the displacement of nuclei towards the periphery (Panel C). The dermis demonstrated signs of fibrous connective tissue proliferation, along with congested blood vessels and infiltration of inflammatory cells (Panel D).

Embryonated eggs provide a potential host system for the primary isolation and propagation of various viruses, such as avian coronaviruses, lumpy skin disease virus, Fowlpox virus, and many others used in the commercial production of vaccines [31]. In the present study, the Fowlpox virus was propagated and isolated by inoculation of the Fowlpox virus in 9-day-old embryonated chicken eggs. Subsequently infected

Fig. 1. The clinical signs of FPV affected chicken showing skin nodule on the leg toes and interdigital web region (shown in arrows).

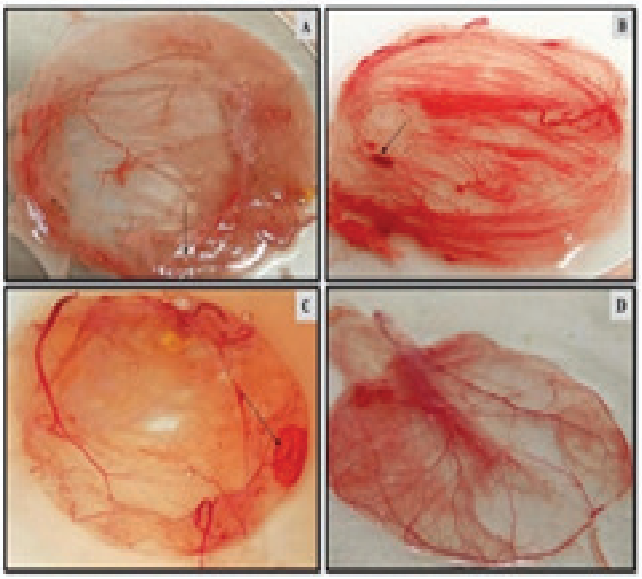

Fig. 3. Fowlpox virus infected chorioallantoic membrane (CAM). [Panel A: More distinct pocks were produced by field strain of Fowlpox virus on CAM; Panel B: Haemorrhagic necrosis of CAM; Panel C: Oedematous thickening CAM; Panel D: Control CAM].

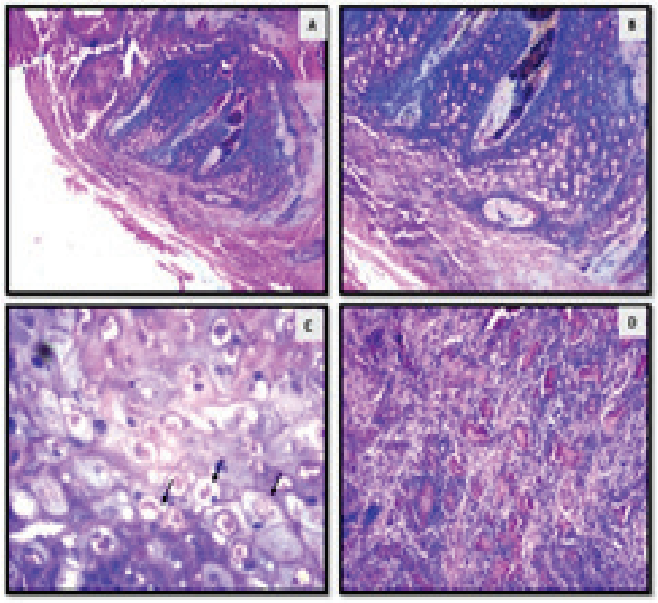

Fig. 2. Histopathological manifestation in skin nodules of FPV infected birds. [Panel A: Thickened keratin and ballooning degeneration of keratinocytes; Panel B: Ballooning degeneration of keratinocytes and multifocal areas showed bacterial colonies cocci; Panel C: swollen keratinocytes having Bollinger bodies (shown in arrow mark); Panel D: Dermis showing fibroblast proliferation, congested vessels and infiltration of inflammatory cells].

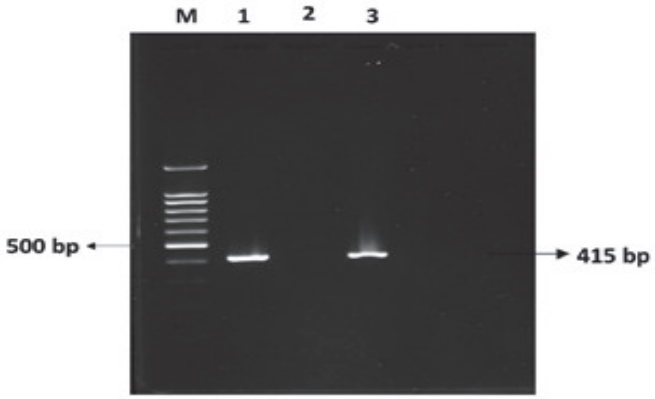

Fig. 4. PCR amplification of P4b gene. [Lane M: 1.5 kbp DNA ladder; Lane 1: Fowlpox live attenuated vaccine (positive control); Lane 2: Negative control (NTC); Lane 3: Fowlpox field isolate].

embryos, on first passage resulting into the opaque, thick, necrotic, and proliferative CAM were observed. During the third passage, small, round, raised circular pock lesions of varying morphologies were distinctly visible (Fig. 3). Size of lesions approximately ranged from 2.0 mm to 4.5 mm in diameter. Whereas, the lyophilized vaccine strain did not produce such symptoms as hemorrhage, or thickening of the membrane at the first passage, second, and third passages respectively. The findings of the current investigation supported the findings of Zhao *et al.* 1996 [32] and observed similar findings like thickening of CAM upon inoculation with Fowlpox and Pigeonpox viruses. Pandey and Mallick 1975 [33] also reported large and small secondary pock, necrosis, and edematous lesions on CAM of chick embryos upon inoculation.

PCR assay is a precise, sensitive, and preferable technique for detecting the Fowlpox virus in the affected nodular tissue of infected birds [34, 38, 39]. In the present study, the Fowlpox virus was isolated from infected nodular tissue. Further isolated and live

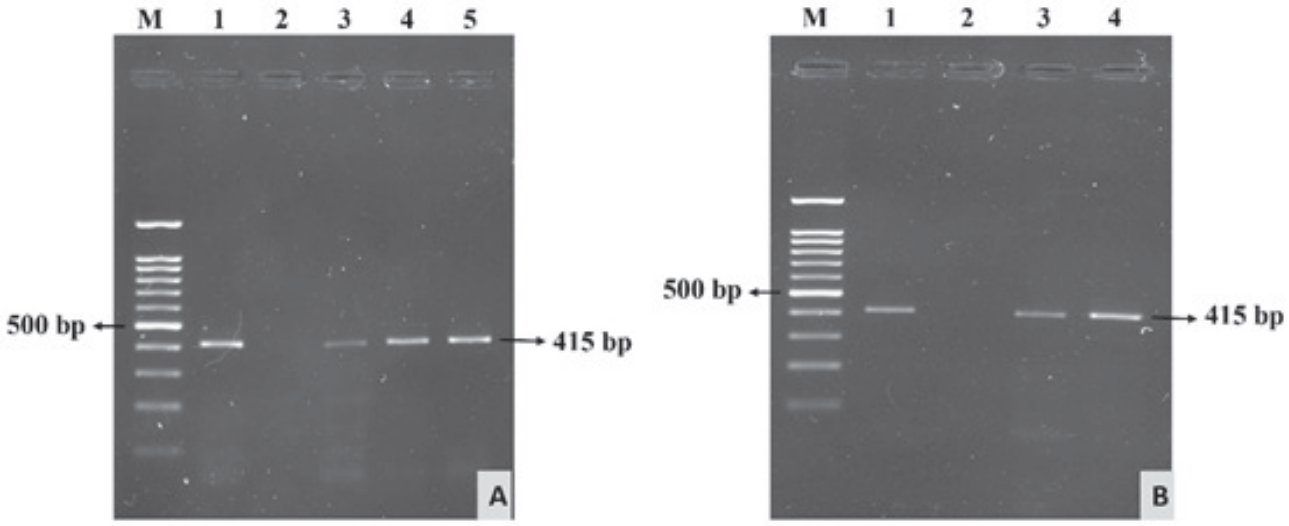

Fig. 5. Agarose electrophoresis gel image depicting CAM passages for propagation of virus. [(Panel A: Fowlpox field isolate; Panel B: Fowlpox live attenuated vaccine), Lane M: 1.5kbp DNA ladder; Lane 1: Positive control; Lane 2: Negative control; Lane 3: Passage 1; Lane 4: Passage 2; Lane 5: Passage 3].

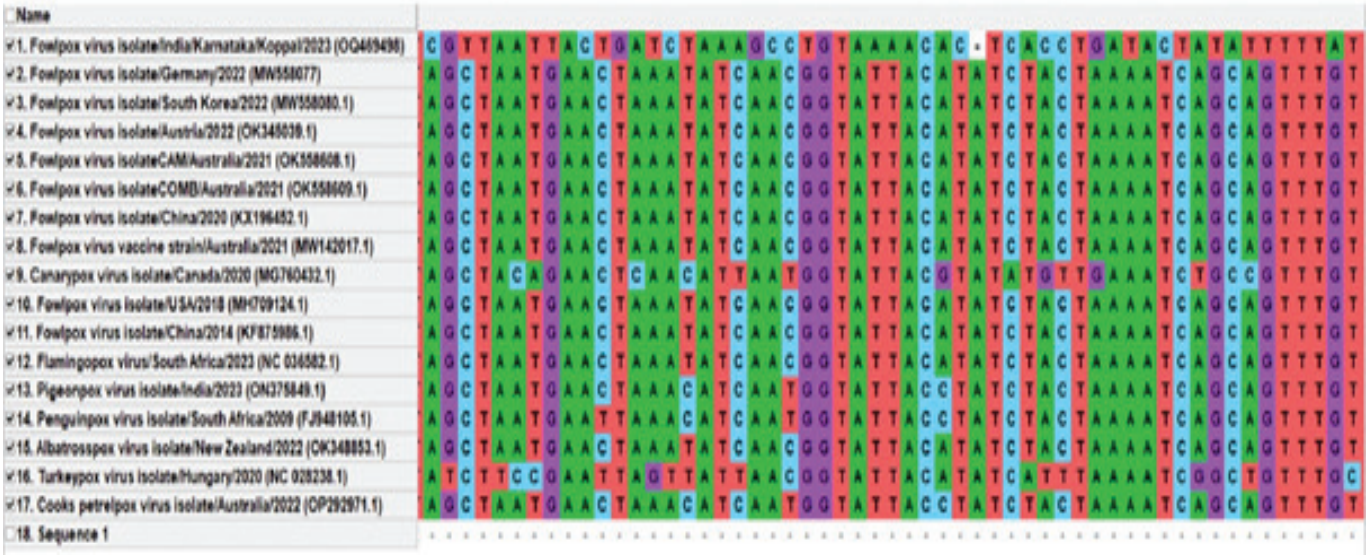

Fig. 6. Multiple sequence alignment of P4b gene among avianpoxvirus isolates using MEGA 11 software.

attenuated Fowlpox vaccine strains were propagated in the Chorioallantoic membrane (CAM) and confirmed by specific PCR assay with an amplicon size of 415 bp (Fig. 4 and 5). Similar findings were also reported by previous researchers [34, 35, 38] for the encoding of P4b gene, producing a 578 bp product in Canarypox and Pigeonpox viruses isolated from Iran. The phylogenetic study of currently obtained Fowlpox isolate provides very significant information on molecular epidemiology, the origin of the outbreak, and the appropriate selection of the most effective vaccine strain for disease control [36, 37, 38]. In this context, the current study of the P4b gene targeted Fowlpox virus isolate was sequenced, trimmed, and submitted to the GenBank database (NCBI accession number: OQ469498). The Multiple sequence alignments of the P4b gene revealed that Fowlpox virus isolate showed single nucleotide polymorphism (SNP) with other field and vaccine strains of FPV, such as Canarypox virus, Flamingopox virus, Pigeonpox virus, Penguinpox virus, Cooks petrelpox virus, Albatrosspox virus and Turkeypox virus stains retrieved from the NCBI database (Fig. 6).

The phylogenetic tree was constructed using the partial P4b gene sequence from our outbreak isolate, along with 16 additional FPV sequences retrieved

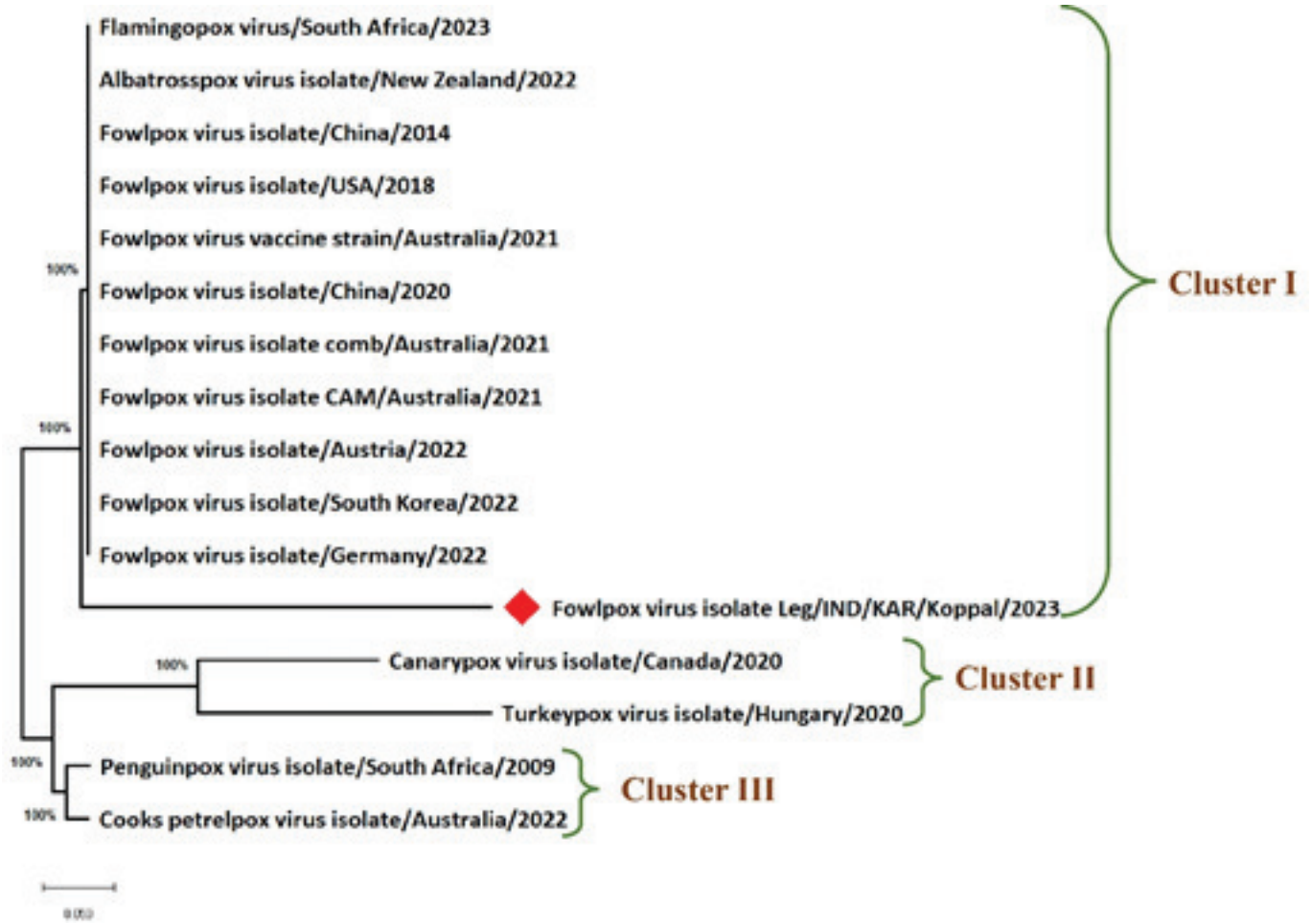

Fig. 7. Molecular phylogenetic analysis by Neighbourhood joining method. [Phylogenetic tree of partial P4b gene sequences. Field isolate of our study compared with other avian sequences obtained from Gen-Bank database].

from the GenBank database (NCBI). Before the construction of phylogeny, all the FPV and avian poxvirus sequences were trimmed to a length of 322 bp. Followed by, a phylogenetic analysis of the P4b gene revealed that the Fowlpox virus identified in our epidemic investigation formed separate clusters with 100% homology. Cluster I included Fowlpox virus isolates from Germany, the United States, China, South Africa, New Zealand, and Australia. Cluster II contained our FPV isolate, which was identical to Canarypox virus and Turkeypox virus isolates from Canada and Hungary. Cluster III grouped our FPV isolate with Penguinpox virus and Cooks petrel poxvirus isolates from South Africa and Australia. (Fig.7). On the other hand, Norouzian & Farjanikish (2017) [36] also reported that the isolated Fowlpox virus was classified in a different subclade than other Iranian isolates. This indicates a close relationship with the FPV isolates from Tanzania, Egypt, and Germany. Similar results were previously reported and a phylogenetic tree revealed distinct sequence clusters. The sequencing research revealed that the Iranian isolates belong to a gene cluster of highly conserved P4b virion core proteins found in several nations [37].

CONCLUSION

The present study was based on the phylogenetic analysis of the P4b gene for the Fowlpox virus isolate from the outbreak which disclosed 100% homology and established a separate clade with isolates from Germany, the United States, China, South Africa, and Australia from 2014 to 2022. Our research revealed that there is relatively little genetic diversity in FPV, indicating that it is stable. The investigation also evidenced the variation in tissue tropism, disease outbreak pattern, and vaccination failure despite being vaccinated with live attenuated Fowlpox vaccine during the field investigation. This could be due to the

evolution of the Fowlpox strain over a period which is not giving complete protection. On the other hand some attributes like host-pathogen interaction, host epigenetics, and immunological status. Consequently, focused and vigorous molecular epidemiological and host-pathogen interaction studies are required to investigate the above-mentioned attributes. Such research will facilitate improved control and eradication strategies for Fowlpox disease.

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