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Genotyping of Local Isolates of Parvoviruses Causing Short Beak and Dwarfism Syndrome in Young Ducklings and Goslings Using PCR and RFLP Technique

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Abstract

GOOSE parvovirus (GPV) is a highly contagious pathogen of waterfowl, responsible for short beak and dwarfism syndrome (SBDS), leading to significant economic losses in the poultry industry. The virus spreads through both horizontal and vertical transmission, in addition to contamination of eggs and hatcheries facilitating its persistence and wide distribution. Despite its global presence, including recurrent outbreaks in Egypt, the molecular epidemiology and evolutionary dynamics of GPV remain incompletely understood. In the present study, molecular analysis of the VP1 capsid protein gene revealed complete nucleotide homology (100%) among the 2023 Egyptian field isolates, despite being recovered from two geographically distinct regions—Gharbia Governorate (Northern Egypt) and Beni Suef (Upper Egypt) suggesting the circulation of a single predominant strain during the 2023 outbreaks. In contrast, the 2018 vaccine strain demonstrated 98.1% identity with the 2023 isolates, differing by more than 16 single nucleotide polymorphisms (SNPs). Phylogenetic analysis further demonstrated that the Egyptian isolates in general rather than the isolates of 2023 are clustered closely together within the novel Goose parvovirus (N-GPV) lineage, while maintaining a distinct but related position to the Chinese reference strain QH15. This close genetic relationship indicates possible shared ancestral origins between Egyptian and Chinese N-GPV strains, while also reflecting local viral evolution under regional epidemiological pressures. To address the need for rapid molecular differentiation, the study developed a novel PCR–restriction fragment length polymorphism (PCR-RFLP) assay targeting an 855 bp fragment of the VP1 gene. Diagnostic digestion with HinfI and AlwI revealed unique and reproducible restriction patterns that enabled clear discrimination between classical GPV (C-GPV) and novel GPV (N-GPV). This method provides a rapid, cost-effective, and reliable genotyping tool for field and laboratory investigations. In summary, In conclusion the present findings confirm the predominance of a single C-GPV strain in Egypt during 2023, highlight its phylogenetic relatedness to Chinese isolates, and introduce a practical PCR-RFLP method for differentiating GPV genotypes. These insights strengthen the understanding of GPV epidemiology and provide critical data for vaccine development and outbreak control strategies.

Keywords: SNPs, C-GPV, N-GPV, HinfI, AlwI, RFLP, SBDS, PCR, Digestion, QH15.

Introduction

Goose parvovirus (GPV) infection poses a significant threat to young goslings and ducklings, resulting in severe economic losses in poultry production due to high mortality rates, which can reach 70–100% in immunologically naïve flocks."[1] , "Also referred to as Derzsy's disease, goose parvovirus (GPV) was first described by

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Domokos Derzsy 1967, who identified it as an infectious condition affecting waterfowl [2]. The disease is characterized by feather abnormalities in older goslings, oculonasal discharge, growth retardation, and watery diarrhea, the latter resulting from viral replication within the intestinal epithelium following primary infection." [3], Young goslings and Muscovy ducks are highly vulnerable to goose parvovirus (GPV) infections, which are associated with severe clinical disease and elevated mortality rates, often ranging from 70% to 100% in naïve populations [4]. Infections with Muscovy duck parvovirus (MDPV), a related parvovirus, have been shown to cause significant morbidity and mortality in Muscovy ducks, primarily due to profuse watery diarrhea and neurological impairment [5],A novel genetic variant of GPV, designated novel goose parvovirus (nGPV), has recently been identified and is currently known to infect only mule ducks in Egypt [6]. This variant has been implicated in the emergence of short beak and dwarfism syndrome (SBDS), a condition characterized by shortened beak development and stunted growth. The syndrome has been documented in Pekin ducks in both China and Egypt [7], GPV is a non-enveloped, single-stranded DNA virus with an approximate genome length of 5100 base pairs. It encodes three capsid proteins (VP1, VP2, and VP3), in addition to non-structural proteins involved in viral replication and pathogenesis[8].

The emergence of nGPV and the concurrent appearance of SBDS have been hypothesized to result from viral recombination events [9]. This hypothesis gained further support with the detection of recombinant MDPV (rMDPV) strains across several provinces in China [10], In Egypt, outbreaks of Angel Wing Syndrome (AWS) were first observed in Muscovy duck flocks in the Behira governorate in 2017. Initially presumed to be of environmental or hereditary origin, the etiology of AWS was later associated with GPV infection following its identification in affected flocks in 2022. Phylogenetic analysis of circulating strains revealed nucleotide identities ranging from 95.7% to 96.6% with nGPV strains linked to SBDS, and from 96.8% to 97.4% with classical GPV strains responsible for Derzsy's disease [11],In 2023, efforts by Egyptian researchers focused on molecular characterization of the VP1 gene, a major capsid protein, to compare the antigenic profiles of newly isolated strains with those from the 2018 outbreaks, which were utilized in the formulation of the local monovalent vaccine. Sequence analysis revealed 100% nucleotide identity among the 2023 isolates, and 98.1% identity when compared to the 2018 strain, indicating a high degree of genetic conservation with minor antigenic drift [12].

The pathological impact of the virus characterized by extensive necrosis of the thymic cortex, accompanied by disintegration of Hassall's corpuscles. In the bursa of Fabricius, normal histoarchitecture was lost, with marked follicular atrophy and pronounced lymphocytic depletion. Additionally, apoptosis of B-lymphocytes was observed within the lymphoid follicles of the spleen. Immunohistochemical analysis demonstrated strong parvoviral antigen expression in cortical thymic lymphocytes, bursa-associated medullary lymphoid follicles, and diffusely throughout the splenic tissue [13]. The GPV capsid exhibited notable thermal stability under physiological pH conditions, whereas its stability was significantly reduced in more acidic environments.[14], therefore, restriction fragment length polymorphism (RFLP) analysis of PCR-amplified products can serve as a reliable method for differentiating between GPV and MDPV. The molecular findings confirm the presence of both GPV and MDPV in avian populations in Thailand [15], A PCR-based restriction fragment length polymorphism (PCR–RFLP) assay was developed to differentiate between goose parvovirus (GPV) and Muscovy duck parvovirus (MDPV), utilizing sequence differences identified within the NS gene through comparative genomic analysis[16].

The aim of the work of the present study aimed to employ a restriction fragment length polymorphism (RFLP) assay based on PCR-amplified fragments of the VP1 gene as a rapid and reliable molecular tool to differentiate between novel goose parvovirus (NGPV) and classical goose parvovirus (CGPV). This approach was designed to complement sequencing and phylogenetic analysis, providing an accessible method for distinguishing circulating field isolates and assessing their epidemiological and genetic relationships.

Material and Methods

Isolates used in this study

The three isolates used in this study were characterized by sequence analysis and designed as AM/KH//Gooseparvovirus/VSVRI/Perma/Egypt/2018 Accession Number # OR854265 which is previously characterized as Novel Gooseparvo virus(nGPV) strain, this strain has been utilized in the formulation of a locally produced inactivated Derzsy's disease vaccine, which is commercially available and manufactured by the Veterinary Serum and Vaccine Research Institute (VSVRI), AME/Gooseparvovirus/VSVRI/Tanta/Egypt/2023 accession number # PP058119 and AME/ Gooseparvovirus/VSVRI/ Beni Suef/Egypt/2023 accession number # OR878549 which have isolated in 2023 , the liver homogenate and allantoic fluid of each of the previously characterized isolates of 2023 was kindly supplied by Prof.Dr Amani Ali Saleh for further DNA extraction and PCR while the isolate of 2018.
AM/KH//Gooseparvovirus/VSVRI/Perma/Egypt/201

8)used in local vaccine preparation was supplied as cell suspension of Primary Duck Embryo Fibroblasts cells (DEF) [6] and [17].

Viral nucleic acid extraction

Genomic DNA was extracted from 300 µL of the supernatant obtained from liver tissue homogenate, allantoic fluid, and virus propagated in duck embryo fibroblast (DEF) cells. Extraction was performed using the Genomic DNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's instructions, with minor modifications. Specifically, during the lysis step, 100 µL of 10% SDS and 20 µL of proteinase K were added to enhance cell disruption and protein digestion. The extracted DNA was eluted in 50 µL of TE buffer and stored at −20 °C until further use.

Polymerase Chain Reaction (PCR)

PCR amplification of the VP1 gene was carried out using Thermo Scientific DreamTaq Green PCR Master Mix (2X) (Cat. No. K1081). The reaction utilized 50 Picomol of each forward primer (5′-GTGGGTAATGCCTCGGGAA-3′) and reverse primers (5′-GACACAGGTCCGGGTTGTAG-3′) and 50 Nano gram of DNA designed to amplify an 885 bp fragment. Amplification was performed using a Applied Biosystems™ ProFlex™ PCR System, 96-well The PCR thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s; with a final extension step at 72 °C for 10 min. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. A 50 bp DNA ladder NEB # (New England BioLabs) Catalog # N3236S and GeneDirex 50bp DNA Ladder Cat no# M1800 were used as a molecular size marker to estimate amplicon sizes.

Restriction Fragment Length Polymorphism (RFLP)

Single Digestion using HinfI

Restriction digestion of the PCR products was performed using the HinfI restriction enzyme (10 U/µL; Thermo Scientific, Cat. No. FD0804), which recognizes the nucleotide sequence 5′-G↓ANTC-3′. The enzymatic digestion was carried out in a total volume of 30 µL following the manufacturer's protocol. The reaction mixture comprised 10 µL of PCR product (~0.1–0.5 µg DNA), 17 µL of nuclease-free water, 2 µL of 10× Buffer R (composition: 10 mM Tris-HCl, pH 8.5; 10 mM $MgCl_2$; 100 mM KCl; 0.1 mg/mL BSA), and 2 µL of HinfI enzyme.The mixture was gently mixed and briefly centrifuged to collect the contents at the bottom of the tube. Digestion was performed at 37 °C for 3 hours, followed by enzyme inactivation at 65 °C for 1 hour. The digested PCR products were mixed with loading dye (to a final concentration of 20 mM), and electrophoresed on a 1% agarose gel. DNA fragment sizes were estimated by comparison to a GeneDirex 50bp DNA Ladder Cat no# M1800. DNA bands visualized using a UV transilluminator as shown in Fig. 1

Single Digestion using AlwI

Restriction digestion of the PCR products was performed using the Alw**I** restriction enzyme (10 U/µL; Thermo Scientific, Cat. No. FD0804), which recognizes the nucleotide sequence 5' G G A T C N4↓ 3' ′. The enzymatic digestion was carried out in a total volume of 30 µL following the manufacturer's protocol The reaction mixture comprised 10 µL of PCR product (~0.1–0.5 µg DNA), 17 µL of nuclease-free water, 2 µL of 10× Tango Buffer and 3 µL of AlwI enzyme. The mixture was gently mixed and briefly centrifuged to collect the contents at the bottom of the tube. Digestion was performed at 55°C for 30 minutes, followed by enzyme inactivation at 80 °C for 1 hour. The digested PCR products were mixed with loading dye (to a final concentration of 20 mM), and electrophoresed on a 1% agarose gel. DNA fragment sizes were estimated by comparison to a 50 bp DNA ladder NEB # (New England BioLabs) Catalog # N3236S (Thermo Scientific, Cat. No. SM0311). DNA bands were visualized using a UV transilluminator.

Double Digestion using AlwI and HinfI

Digestions were performed sequentially for each restriction enzyme at different incubation temperatures: 55°C for AlwI and37°Cfor HinfI. As Incubation of AlwI at 37 °, C results in 30% activity. So the optimal amounts of enzymes to be added in this double digestion reaction according to the manufacturer's instructions using the DoubleDigest Calculator is using 1X Tango buffer AlwI and 2-fold excess of HinfI, The enzymatic digestion was carried out in a total volume of 40 µL following the manufacturer's protocol The reaction mixture comprised 10 µL of PCR product (~0.1–0.5 µg DNA), 11 µL of nuclease-free water, 2 µL of 1× Tango Buffer and 3 µL of AlwI enzyme and 4 µL HinfI The mixture was gently mixed and briefly centrifuged to collect the contents at the bottom of the tube. Digestion was performed at first at 55°C °C for 30 minutes for AlwI and 37°C for HinfI for 1 hour, followed by enzyme inactivation at 80 °C for 1 hour. The digested PCR products were mixed with loading dye (to a final concentration of 20 mM), and electrophoresed on a 1% agarose gel. DNA fragment sizes were estimated by comparison to a 50 bp DNA ladder NEB # (New England BioLabs) Catalog # N3236S (Thermo Scientific, Cat. No. SM0311). DNA bands were visualized using a UV transilluminator.

Results

Based on the published sequence data of the three local isolates of 2018 and 2023 and presence of 16

SNPs between them [12] VSVRI/Perma/Egypt/2018 and the two 2023 isolates, AME/GooseParvoVirus/ VSVRI/BeniSuef/Egypt/2023 and AME/GooseParvo Virus/VSVRI/Tanta/Egypt/2023 the current study used these variations along the sequenced 855 bp of the Vp1 Coding gene part of the three isolate to distinguish between as the local isolate of 2018.

AM/KH//GooseParvovirus/VSVRI/Perma/Egypt/ 2018) was characterized and designed according to the previously published data [6] to be divergent from C-GPV isolates.and considered as N-GPV we have found that HinfI enzyme 5' G G A T C N4↓ 3' and AlwI enzyme 5' G G A T C N4↓ 3' could be used to distinguish between the three local isolate as the digestion of the 855 bp partial coding sequence of the Vp1 either when we used single digestion or double digestion give specific pattern of bands could be used as a novel method to distinguish between the C-GPV and N-GPV as shown in Fig. 1 and Table 1.

Where the HinfI enzyme has only one cutting site in the isolate of 2018 accession number # OR854265 at 203/206 which is characterized as N-GPV and 2 sites at the isolates of 2023 accession number # PP058119 and OR878549 at *146/149, 203/206 which are considered as C-GPV while the AlwI enzyme showing 3 cutting sites at #275/276, #620/621, #740/741 in the isolate of 2018 which is considered as N-GPV accession number # OR854265 while showing only 2 cutting sites at #275/276, #740/741 in the isolates of 2023 which are considered as C-GPV as shown in Fig. 2 and Table 1.

The amplified 855 bp PCR products from each isolate were subjected to electrophoresis alongside the respective restriction digestion profiles. Fig. 3 depicts the digestion pattern obtained with the HinfI enzyme. The AM/KH//Gooseparvovirus/VSVRI/Perma/Egypt/2018 isolate (classified as N-GPV; GenBank accession no. OR854265) exhibited two fragments of 652 bp and 203 bp. In contrast, the two 2023 isolates (GenBank accession nos. PP058119 and OR878549) displayed three distinct fragments measuring 652 bp, 146 bp, and 57 bp .Similarly, digestion with AlwI as shown in Fig. 4 revealed a unique restriction profile for the AM/KH//GooseParvovirus/VSVRI/Perma/ Egypt/2018 isolate, generating four fragments of 345 bp, 275 bp, 120 bp, and 115 bp The 2023 isolates, however, yielded only three fragments of 465 bp, 275 bp, and 115 bp, lacking the 120 bp fragment observed in the 2018 a shown in isolate ,Both restriction enzymes, HinfI and AlwI, were individually capable of differentiating between N-GPV and C-GPV. This distinction was also evident when both enzymes were applied sequentially in a double-digestion assay. In the latter case, digestion was performed strictly in accordance with the manufacturer's recommended protocol, including optimal incubation temperatures for each enzyme and the prescribed digestion order, as determined using the DoubleDigest™ Calculator The two isolates obtained in 2023 were found to be genetically identical, exhibiting 100% nucleotide sequence homology despite being collected from different geographical locations. In contrast, the 2018 isolate shared 98.1% sequence homology with the 2023 isolates. Double digestion of the VP1 amplicon from the 2018 local isolate *AM/KH/GPV/VSVRI/ Perma/Egypt/2018* produced five fragments of 72, 115, 120, 203, and 345 bp. In contrast, double digestion of the two 2023 isolates *AME/Gooseparvovirus/VSVRI/BeniSuef/Egypt/2023* and ME/Gooseparvovirus/VSVRI/Tanta/Egypt/2023*)* yielded highly similar profiles of 57, 72, 115, 146, and 465 bp**.** A shown in table (2) and Fig. (6) The RFLP pattern of the two 2023 isolates was indistinguishable under the conditions used, consistent with their near-identical VP1 sequences. The isolate 2018 displayed a distinct digestion pattern with changes in fragment sizes that reflect gain/loss or repositioning of restriction sites relative to the 2023 isolates.so Bands present in 2023 but not in 2018 are: 57 bp, 146 bp, 465 bp and Bands present in 2018 but not in 2023: 120 bp, 203 bp, and 345 bp while Bands shared between both: 72 bp and 115 bp.

Multiple Sequence Alignment

The alignment shows very high similarity across the VP1 coding region. Only a few point mismatches are observed, which may represent single nucleotide polymorphisms (SNPs). Restriction enzyme (HinfI) recognition sites as shown in Fig. 1 showing that OR854265 (2018 isolate) has a HinfI site at nucleotide 204, but not at 147. While OR878549 (2023 isolate) and PP058119 (2023 isolate) both carry two HinfI sites at positions 147 and 204.This indicates that the 2023 isolates share a restriction site gain (mutation introducing a HinfI site) that is absent in the 2018 strain. The appearance of the HinfI (147) site in the 2023 isolates reflects a genetic variation that can be used as a molecular marker to differentiate between older (2018) and recent (2023) circulating strains. Since these changes occur within the VP1 gene, which is antigenically important, they may also have functional or antigenic consequences**.**

While in Fig. 2 OR854265 (Egypt/2018 isolate): contains two AlwI recognition sites at nucleotide positions 276 and 621, with an additional site at **7**41. This generates three cleavage events, producing four fragments upon digestion. This pattern is unique compared to the 2023 isolates. OR878549 and PP058119 **(**Possesses two AlwI recognition sites at positions 276 and 741. This yields two cleavage events, producing three fragments**.** Lacks the site at 621 that was present in the 2018 strain. Confirms the clonal relationship between these two recent isolates the loss of the AlwI site at position 621 in the 2023 isolates is the key differentiating marker from the

2018 strain. OR878549 and PP058119 are genetically indistinguishable by AlwI digestion, suggesting they belong to the same circulating lineage. which assures that the 2018 strain (OR854265) is more divergent, consistent with your earlier HinfI analysis, where it also showed a different digestion profile. Fig. 3 showing restriction map and sequence alignment for 3 Egyptian GPV isolates (OR854265, OR878549, PP058119) digested with two enzymes: HinfI and AlwI.

HinfI sites

OR878549 and PP058119 → sites at 147 bp & 204 bp. While OR854265 → only at 204 bp (missing the 147 bp site).This difference provides **a** useful RFLP marker to distinguish OR854265 from the other two isolates. AlwI sites: OR878549 and PP058119 → at 276 bp & 741 bp. OR854265 → at 276 bp, 621 bp, and 741 bp (extra site at 621 bp). The additional cut site in OR854265 makes its digestion pattern unique and more complex**.** Fig. 4 showing multiple sequence alignment and restriction analysis of three local goose parvovirus (GPV) isolates characterized in the present study (OR878549**,** PP058119**,** and OR854265) compared with the five additional Egyptian isolates available in GenBank. The VP1 gene employed as a molecular marker to assess sequence variability and restriction profiles among the isolates.

OR878549 and PP058119: Both share identical restriction sites at positions 147 & 204 bp and nearly identical sequence patterns → confirms their close genetic relationship (previously noted in your phylogenetic analysis).

OR416225 and OR416226: Both show conserved HinfI (204 bp) and nearly identical sequence tracks → strong evidence they are a distinct but closely related subgroup.

OR416224: Similar to OR416225/226 but with slight variations (minor divergence within the same clade).

PV256247 & PV256248: Both have the HinfI (204) site, but their overall sequence tracks differ more noticeably, especially toward the 3′ end (~700–850 bp), marking them as more divergent strains**.**

OR854265**:** Stands apart, showing unique sequence variations compared to the rest → matches your earlier phylogenetic tree result (unique antigenic profile).

Phylogenetic analysis:

Isolates of 2023 (PP058119 & OR878549) these two recent field isolates **(**Tanta/2023 and Beni Suef/2023) form a tight, terminal cluster with no intermediate branches, indicating very high genetic identity (likely >99.5% in VP1).This suggests a single dominant lineag**e** is circulating in Egypt in 2023 across different locations. While EgyArmy_ZU_202_2022 (OR416224) This 2022 military isolate sits basal to the 2023 field isolates, implying it is an immediate ancestor or very close progenitor to the current circulating 2023 strain. This suggests that the 2023 outbreak viruses may have evolved from strains circulating in the Egyptian Army flocks in early 2022. While EgyArmy_ZU_239_2022 & EgyArmy_ZU_274_2022 (OR416225 & OR416226) These two 2022 military isolates cluster together but on a different branch from the main 2023 field isolates, showing that more than one GPV lineage was present in Egypt in 2022**.** In addition, they share a closer relationship to each other than to the 2023 field clade. And 2018 Field Isolate (OR854265 – AM/KH/Perma/Egypt/2018) This older isolate is placed mid-tree, forming a bridge between the 2022 army isolates and the Egyptian vaccine strains. As shown in Fig. 8, this indicates that at least part of the Egyptian GPV population has been genetically stable over several years**,** possibly due to persistence in local flocks or reintroduction from stored vaccine seeds. While Vaccine valley Strains (PV256248 – SA203/2023 & PV256247 – SA204/2024) these two strains form their own closely related sub-clade, separate from the 2023 field clade. This genetic separation suggests that the vaccines are based on an older lineage rather than the currently circulating 2023 field strain.

For broader phylogenetic analysis to show the antigenic diversity of the local isolates and their relations to the other isolates around the world as shown in Fig.9 AM/KH/GPV/VSVRI/ Perma/ Egypt/2018; Vaccine-Valley-SA203/2023; Vaccine-Valley-SA204/2024; EgyArmy_ZU_239/2022; EgyArmy_ZU_274/2022. Closest Chinese isolates: FJ/15 (often referred to as QH/15 in some datasets) sits as the nearest Chinese reference on the sister branch; additional near neighbors (slightly deeper) are the JS lineage (JS1/JS1603) and Shandong/Anhui strains (SDLY1512, SDLY1602, SDDY1605, SDHZ1604, AH1605, AH1606). While 2023 Egyptian field isolates AME/Gooseparvovirus/ VSVRI/Tanta/2023; AME/GPV/VSVRI/BeniSuef/ 2023 and the Closest Chinese isolates the adjacent sister clade is the JS/SD/Anhui block**—**JS1, JS1603, SDLY1512, SDLY1602, SDDY1605, SDHZ1604, AH1605, AH1606**.** While EgyArmy_ZU_202/2022 (single Egyptian leaf near the Chinese clade) Closest Chinese isolates: AH1605 / AH1606 / SDHZ1604 (immediate neighbors within the compact Chinese clade).

Discussion

RFLP–PCR assay provides a rapid, specific, and cost-effective diagnostic tool for distinguishing GPV from MDPV, facilitating reliable genotypic differentiation of field isolates and supporting epidemiological investigations [15, 16] in the current study Restriction Fragment Length Polymorphism

(RFLP) analysis of the VP1 gene has proven to be a reliable molecular approach for the discrimination of goose parvovirus (GPV) variants. In the present study, the use of *HinfI* and *AlwI* endonucleases generated distinct restriction patterns that permitted differentiation between divergent Egyptian field isolates. The ability of RFLP to resolve such intra-species variation highlights its value as a rapid, cost-effective screening tool that complements nucleotide sequencing, particularly in resource-limited diagnostic settings. Moreover, the restriction signatures generated here provide important molecular markers for epidemiological surveillance and vaccine strain selection. A clear distinction was observed between the older Egyptian isolate from 2018 (OR854265) and the two more recent field isolates from 2023 (OR878549 and PP058119). The 2018 isolate exhibited a unique restriction profile, with a single *HinfI* recognition site and three distinct *AlwI* cleavage positions, producing multiple smaller fragments. By contrast, the 2023 isolates showed identical restriction patterns, characterized by two *HinfI* sites and only two *AlwI* sites, which resulted in a more conserved fragment distribution as shown in Tables (1&2). These findings emphasize both the genetic stability of the 2023 isolates and possibility of their divergence from the earlier 2018 strain. The close similarity between the two 2023 isolates, despite their isolation from different geographic governorates (Beni Suef and Tanta), further suggests recent circulation of a genetically homogeneous viral subpopulation within Egyptian waterfowl populations [17].

When compared with reference goose parvovirus strains from China, the Egyptian isolates revealed restriction patterns consistent with their phylogenetic clustering. The 2023 isolates (OR878549 and PP058119) demonstrated closer similarity to the Chinese isolate QH15, reinforcing the notion of shared evolutionary origins or possible introduction events through regional trade or migratory bird routes [18]. Conversely, the 2018 Egyptian isolate displayed a more divergent restriction profile, underscoring its relative genetic distance from both the local 2023 isolates and the Chinese counterparts. Together, these data confirm that while Egyptian GPV strains remain broadly related to Chinese lineages, intra-country variability persists, reflecting local evolutionary pressures and ongoing viral adaptation. The 2018 Perma isolate (OR854265) and the 2023 isolates (OR878549 – Beni Suef, PP058119 – Tanta) show very high nucleotide similarity (>98%), but their RFLP digestion patterns differ because of distinct SNPs within the VP1 gene.

The difference in restriction fragment sizes between the 2018 and 2023 isolates indicates sequence changes that alter HinfI/AlwI cutting sites (or whichever two enzymes were used), and therefore point to nucleotide substitutions, small insertions/deletions, or local rearrangements in the amplified VP1 region and shows a different RFLP pattern, suggesting it is a close relative rather than an identical strain — consistent with limited sequence evolution (few nucleotide changes) between 2018 and 2023. The distinct pattern of the Vaccine-Valley isolates (PV256247/PV256248) further indicates they are genetically separable from the circulating field lineage and may represent vaccine-related or lab-maintained strains. Which is very clear due to gained and lost restriction sites for both enzymes in 2018 and 2023 isolates as follows: Sites gained in 2023: HinfI at 146 (creates the 146 bp + 57 bp fragments).Sites lost in 2023**:** AlwI at 620 (this site is present in 2018 but absent in 2023).

In addition, Sites conserved HinfI at 203**,** AlwI at 275, and AlwI at 740 (all present in both sets). As shown in Fig. (7) and Table (2&3). For broader analysis to find the relationship and epidemiology of the field isolates of interest and the local Egyptian isolates the 8 Egyptian isolates including the 2018 and 2023 isolates through showing restriction site map and sequence alignment. The RFLP analysis reveals two distinct genotypes among the eight Egyptian GPV isolates based on the HinfI restriction enzyme which are the Classical Goose Parvovirus (C-GPV): Isolates 1 (OR878549) and 2 (PP058119). They show a restriction pattern with two HinfI sites, located at positions 147 and 204. In addition, Novel Goose Parvovirus (N-GPV): Isolates 3 through 8 are classified as the novel type. They are characterized by a different RFLP pattern with only one HinfI site, located at position 204. as shown in Fig. 4.

Phylogenetic analysis based on the deduced amino acid sequence of the local Egyptian isolates including the isolates of interest (2018&2023) in the current study as shown in Fig. 8 showing that The two 2023 Egyptian field isolates (Tanta & Beni Suef) are genetically identical, confirming a common outbreak strain. These 2023 isolates are closely related to the 2022 EgyArmy strains, showing a clear evolutionary lineage in Egypt. The 2018 Egyptian isolate related but more distant, suggesting progressive viral evolution over the past 5 years (2018-2023). While the vaccine valley strains are genetically distinct from the circulating field strains, which may explain potential differences in protection or breakthrough infections.

To find out the relation between the local Egyptian field isolates and other goose parvoviruses around the world phylogenetic analysis of the deduced amino acid sequence of the local Egyptian isolates as a whole compared against 64 isolate around the world as shown in Fig. 9, using the Vp1 protein as important structural immunogenic protein and is considered as a common target for classifying Parvoviruses [19]. The Egyptian GPV isolates appear to have multiple introductions from different Chinese lineages: Some from a vaccine-like ancestor (FJ/15-

related) protein Accession NO # ANX99768.1 and others from field-related ancestors (JS/SD/Anhui-related).

The scattered positions of Egyptian isolates suggest independent evolution after introduction, leading to unique Egyptian sub-lineages. The closeness of ZU_202/2022 to Chinese field strains indicates ongoing exchange or recent introduction, while the divergence of other Egyptian isolates points to local adaptation and diversification. The diversity among Egyptian isolates suggests significant antigenic variability, which could explain differences in vaccine protection and possible immune escape. While Vaccine valley derived, Egyptian strains remain closer to FJ/15**,** while recent field strains diverge toward Chinese field-like profiles**,** Phylogeographer analysis indicated China as a major source of GPV dissemination, with trade likely acting as a transmission route. Despite limitations in genomic data, the findings provide new insights into GPV evolution and spread, supporting future control strategies [20].

Conclusion

The restriction fragment length polymorphism (RFLP) assay provided a rapid, reproducible, and cost-effective molecular tool for distinguishing between classical and novel GPV lineages based on distinct restriction profiles. The obtained digestion patterns were in full agreement with the phylogenetic clustering of the VP1 gene, confirming that RFLP can serve as a reliable genotyping method that mirrors evolutionary relationships inferred from sequence-based analyses. This strong concordance highlights the utility of RFLP not only as a diagnostic tool for routine differentiation of circulating field isolates but also as a complementary approach to phylogenetic analysis in molecular epidemiological investigations of goose parvovirus. The deduced amino acid tree demonstrates that Egyptian GPV isolates are polyphyletic, reflecting multiple introductions from China and subsequent local diversification**.** Vaccine-valley Egyptian strains cluster near FJ/15 amino acid Accession number ANX99768.1, while recent field isolates branch closer to Chinese JS/SD/Anhui lineages precisely the 2019-HB23 strain. amino acid Accession No# UUJ75089.1

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

TABLE 1. Showing Restriction Fragment Length Polymorphism (RFLP) Analysis of the VP1 Gene of Three Egyptian Goose Parvovirus Isolates (OR878549, PP058119, and OR854265) Using HinfI and AlwI Restriction Endonucleases demonstrating the single digestion pattern of each enzyme used, recognition sequence, number of cuts, Cut positions, cutting interval from 5` to 3` prime ends, coordinates and the size of each of the produced fragments of each enzyme per isolate

#	Enzyme	Specificity	Cuts	Cut positions (blunt - 5' ext. - 3' ext.)	Ends	Coordinates	Length (bp)
OR878549	HinfI	G▼ANT▲C	2	*146/149, 203/206	(Left End)-HinfI	1-146	146
					HinfI-HinfI	147-203	57
					HinfI-(Right End)	204-855	652
	AlwI	GGATCNNNN▼N▲	2	#275/276, #740/741	(Left End)-AlwI	1-275	275
					AlwI-AlwI	276-740	465
					AlwI-(Right End)	741-855	115
PP058119	HinfI	G▼ANT▲C	2	*146/149, 203/206	(Left End)-HinfI	1-146	146
					HinfI-HinfI	147-203	57
					HinfI-(Right End)	204-855	**652**
	AlwI	GGATCNNNN▼N▲	2	#275/276, #740/741	(Left End)-AlwI	1-275	**275**
					AlwI-AlwI	276-740	465
					AlwI-(Right End)	741-855	**115**
OR854265	HinfI	G▼ANT▲C	1	203/206	(Left End)-HinfI	1-203	203
					HinfI-(Right End)	204-855	**652**
	AlwI	GGATCNNNN▼N▲	3	#275/276, #620/621, #740/741	(Left End)-AlwI	1-275	**275**
					AlwI-AlwI	276-620	345
					AlwI-AlwI	621-740	120
					AlwI-(Right End)	741-855	**115**

TABLE 2. Showing Fragment Profiles Generated by Sequential Digestion of the VP1 Gene of Egyptian Goose Parvovirus Isolates (OR854265, OR878549, and PP058119) with HinfI and AlwI Restriction Endonucleases indicating the precise cleavage sites within the local isolates and the corresponding fragment sizes for each isolate.

isolate	Ends	Coordinates	Fragment Length (bp)
OR854265	(Left End)-HinfI	1-203	203
	HinfI-AlwI	**204-275**	**72**
	AlwI-AlwI	276-620	345
	AlwI-AlwI	621-740	120
	AlwI-(Right End)	741-855	115
OR878549	(Left End)-HinfI	1-146	146
	HinfI-HinfI	147-203	57
	HinfI-AlwI	**204-275**	**72**
	AlwI-AlwI	276-740	465
	AlwI-(Right End)	741-855	115
PP058119	(Left End)-HinfI	1-146	146
	HinfI-HinfI	147-203	57
	HinfI-AlwI	**204-275**	**72**
	AlwI-AlwI	276-740	465
	AlwI-(Right End)	741-855	115

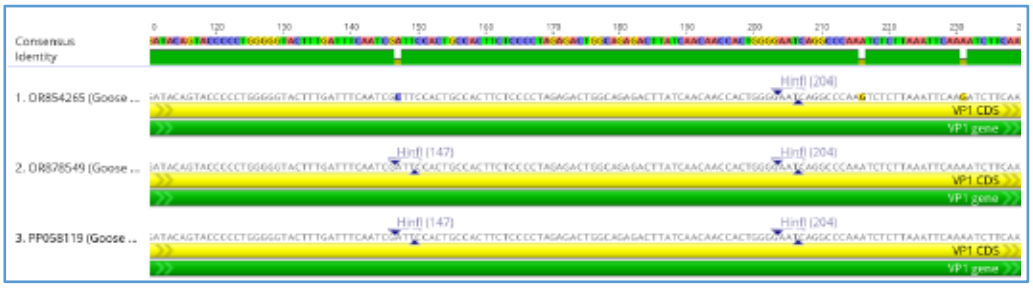

Fig. 1. Schematic representation of the restriction cleavage sites generated by HinfI **enzyme in three Egyptian goose parvovirus (GPV) isolates. The analysis includes the 2018 isolate (Perma/Egypt/2018) and the two field isolates obtained in 2023 (BeniSuef/Egypt/2023 and Tanta/Egypt/2023). by Meg Alignment software**

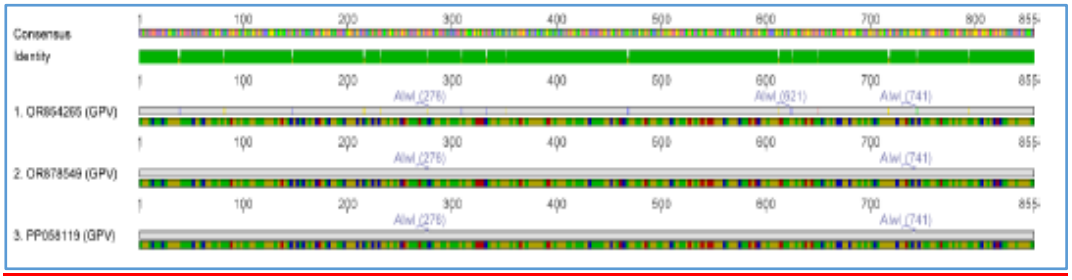

Fig.2. Schematic representation of the *AlwI* restriction sites in the three local isolates: AM/KH//Gooseparvovirus/VSVRI/Perma/Egypt/2018, AME/GooseParvovirus/VSVRI/BeniSuef/Egypt/2023, and AME/GooseParvovirus/VSVRI/Tanta/Egypt/2023. The comparative restriction maps demonstrate the distribution and positions of *AlwI* cleavage sites, highlighting conserved and variable patterns among the 2018 isolate and the two field isolates recovered during the 2023 outbreaks

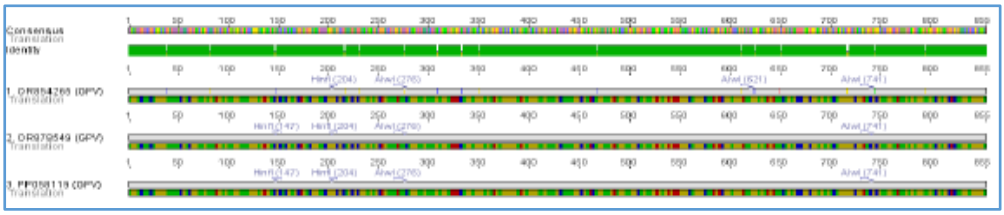

Fig. 3. Schematic representation of alignment and restriction map showing the cleavage sites generated by AlwI **and** HinfI **across three Egyptian goose parvovirus (GPV) isolates. The analysis includes the 2018 isolate (AM/KH//GooseParvovirus/VSVRI/Perma/Egypt/2018) and the two field isolates recovered in 2023 (AME/GooseParvovirus/VSVRI/BeniSuef/Egypt/2023** and **AME/GooseParvovirus/VSVRI/Tanta/Egypt/2023**). by **Meg Alignment** software.

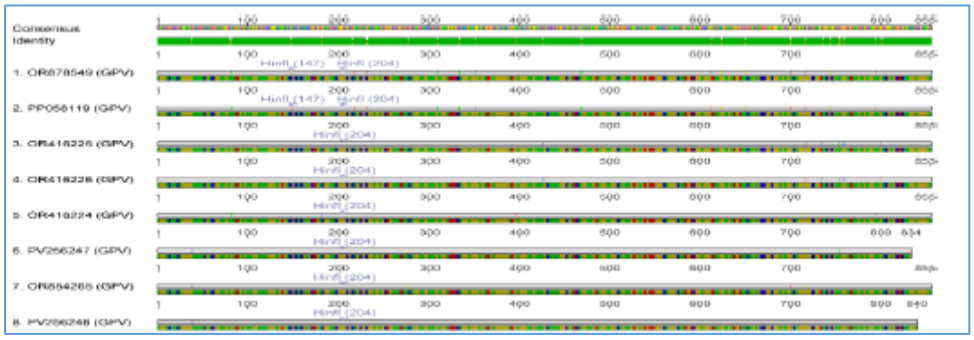

Fig. 4. showing restriction site map and sequence alignment showing 8 local Egyptian GPV isolates across an ~850 bp fragment, with predicted HinfI restriction enzyme recognition sites indicated.by Meg Alignment software.

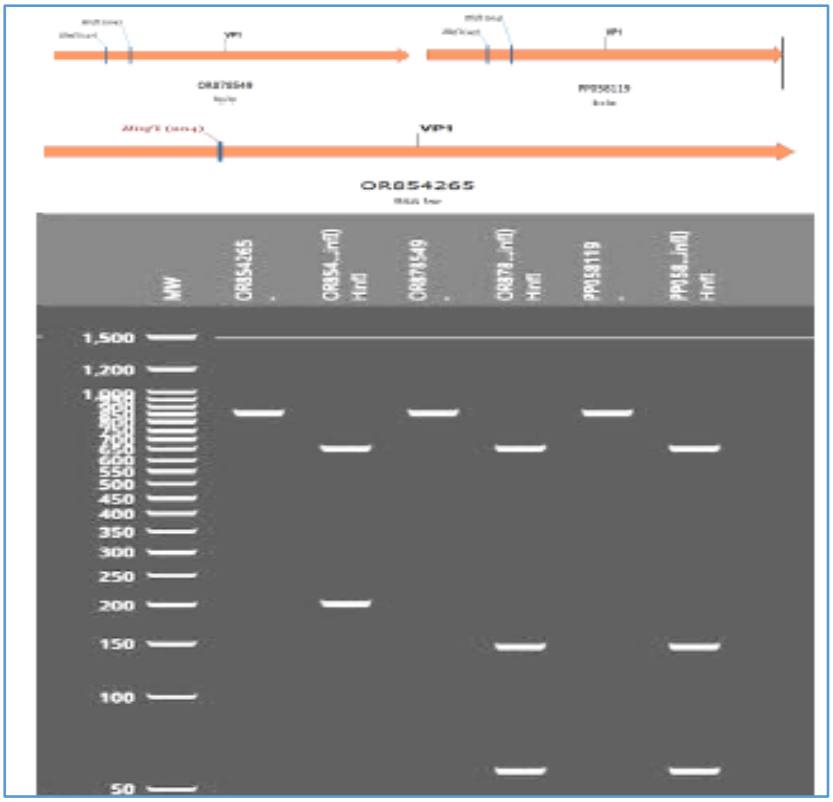

Fig. 5. Electrophoretic analysis of PCR products and *HinfI* restriction digestion patterns. Lane 1: 50 bp DNA ladder; Lane 2: 855 bp PCR amplicon from the 2018 local isolate (GenBank accession no. OR854265); Lane 3: 855 bp PCR amplicon from the 2023 local isolate (GenBank accession no. OR878549); Lane 4: *HinfI* digestion pattern of the 2023 isolate (OR878549); Lane 5: 855 bp PCR amplicon from the second 2023 local isolate (GenBank accession no. PP058119); Lane 6: *HinfI* digestion pattern of the 2023 isolate (PP058119).

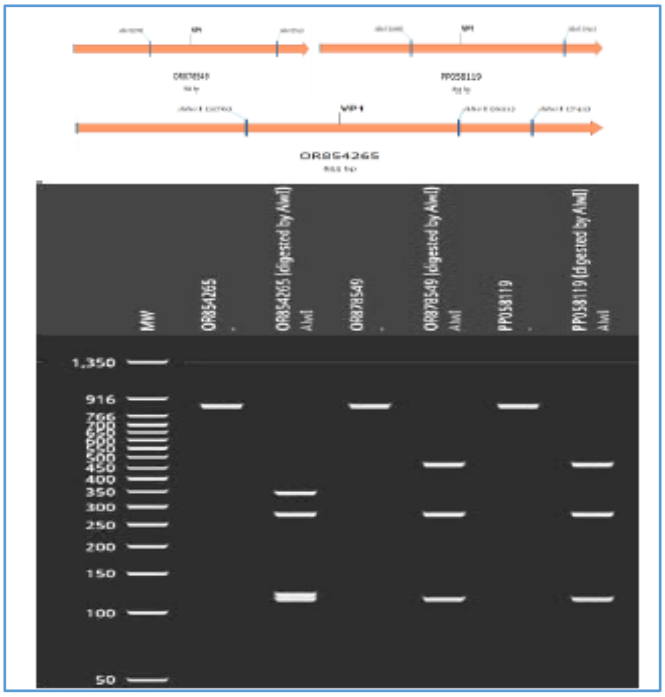

Fig. 6. Electrophoretic analysis of PCR products and *AlwI* restriction digestion patterns. Lane 1: 50 bp DNA ladder; Lane 2: 855 bp PCR amplicon from the 2018 local isolate (GenBank accession no. OR854265); Lane 3: 855 bp PCR amplicon from the 2023 local isolate (GenBank accession no. OR878549); Lane 4: *AlwI* digestion pattern of the 2023 isolate (OR878549); Lane 5: 855 bp PCR amplicon from the second 2023 local isolate (GenBank accession no. PP058119); Lane 6: *AlwI* digestion pattern of the 2023 isolate (PP058119).

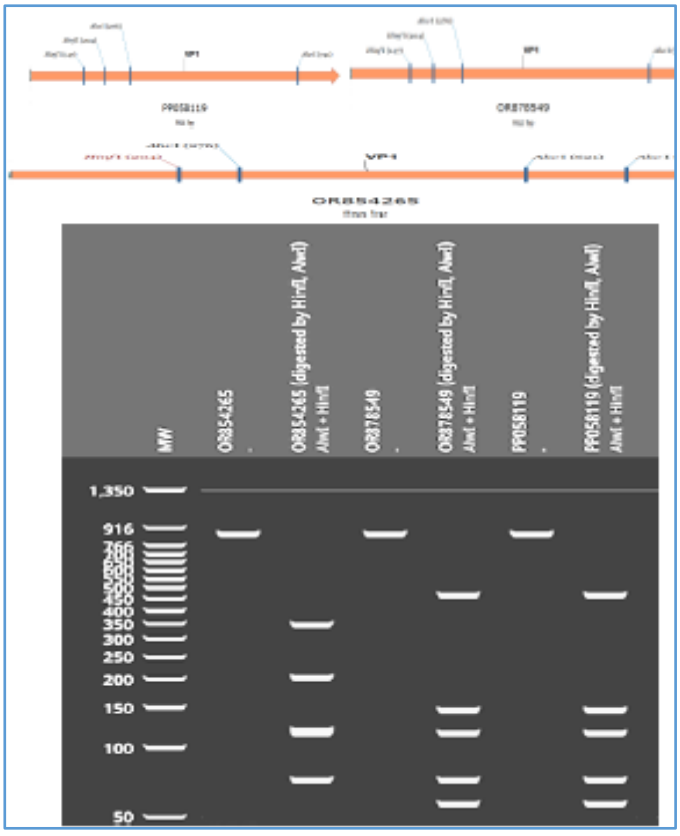

Fig. 7. Electrophoretic analysis of PCR products and restriction digestion patterns of *AlwI* and *HinfI* enzymes. Lane 1: 50 bp DNA ladder; Lane 2: 855 bp PCR amplicon from the 2018 local isolate (GenBank accession no. OR854265); Lane 3: 855 bp PCR amplicon from the 2023 local isolate (GenBank accession no. OR878549); Lane 4: corresponding restriction digestion pattern; Lane 5: 855 bp PCR amplicon from the second 2023 local isolate (GenBank accession no. PP058119); Lane 6: corresponding restriction digestion pattern.

Fig. 8. Phylogenetic analysis of deduced amino acid sequences of the VP1 gene showing antigenic relationships among Egyptian Goose Parvovirus solates including the isolates of interest for the current study which are designed as (N-GPV) and (C-GPV).

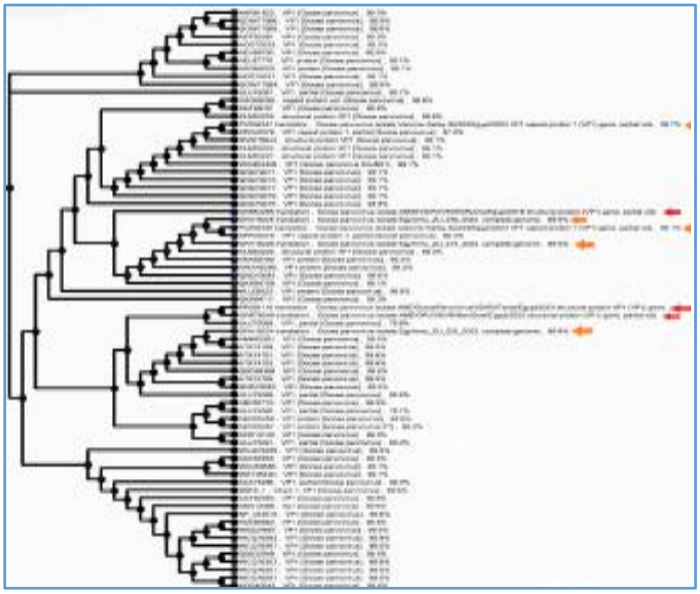

Fig. 9. Phylogenetic tree based on the deduced amino acid sequences of the VP1 gene showing the evolutionary relationships between Egyptian Goose Parvovirus isolates and representative Chinese strains. The tree illustrates a precise, sequence-based mapping of the closest Chinese neighbors to each Egyptian subgroup. Red arrows indicate the focal Egyptian isolates of interest in this study (OR878549, PP058119, and OR854265), while yellow arrows denote the remaining local Egyptian isolates, highlighting their distribution across distinct Chinese clades. Bootstrap values shown at the nodes.

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التصنيف الجيني للمعزولات المحلية لفيروسات البارفو في صغار البط و الأوز باستخدام تفاعل البلمرة المتسلسل وانزيمات القطع المحددة للتفرقة بينهم

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الملخص

يُعد فيروس بارفو الإوز (GPV) من المسببات الفيروسية شديدة العدوى في الطيور المائية، حيث يؤدي إلى متلازمة المنقار القصير والتقزّم، وما يترتب عليها من خسائر اقتصادية كبيرة في صناعة الدواجن. ينتقل الفيروس بطرق متعددة تشمل الانتقال الأفقي والعمودي من الأمهات المصابة للصغار عن طريق البيض الملوث بالفيروس، بالإضافة إلى تلوث البيض والمفاقس، مما يسهم في استمراريته وانتشاره الواسع. وعلى الرغم من انتشاره عالميا، وظهور موجات وبائية متكررة في مصر، فإن علم الوبائيات الجزيئي والديناميكية التطورية لفيروس بارفو الإوز GPV ما تزال غير مفهومة بشكل كامل.في هذه الدراسة أظهر التحليل الجزيئي لجين البروتين الكبسيدي VP1 تجانساً نووياً كاملاً (100%) بين العترات الحقلية المصرية المعزولة خلال عام 2023، رغم اختلاف مواقع عزلها جغرافياً بين محافظة الغربية (شمال مصر) ومحافظة بني سويف (جنوب مصر)، مما يشير إلى تداول سلالة واحدة سائدة خلال وبائيات عام 2023. وعلى النقيض من ذلك، أظهرت السلالة المستخدمة كلقاح في عام 2018 نسبة تماثل بلغت 98.1% مع معزولات عام 2023، مع وجود أكثر من 16 اختلافاً بنيوكليوتيدياً مفرداً .(SNPs) كما أوضح التحليل الوراثي الشجري أن المعزولات المصرية بشكل عام – وليس معزولات عام 2023 فقط – تجمعت في سلالة فيروس بارفو الإوز المستجد(N-GPV) ، مع احتفاظها بموقع مميز لكنه وثيق الصلة بالعترة المرجعية الصينية .QH15 وتدل هذه القرابة الوراثية الوثيقة على أصول سلفية مشتركة محتملة بين معزولات الـ N-GPVالمصرية والصينية، كما تعكس في الوقت ذاته تطوراً فيروسياً محلياً تحت تأثير الضغوط الوبائية الإقليمية.وللتغلب على الحاجة إلى التمييز الجزيئي السريع، تم تطوير اختبار جديد يعتمد على تفاعل البلمرة المتسلسل–تعدد أشكال طول الجزء المحدود (PCR-RFLP) مستهدفاً قطعة طولها 855 زوج قاعدة من جين .VP1 وقد أظهر الهضم التشخيصي بإنزيمي HinfI وAlwI أنماط قطع فريدة وقابلة لإعادة الإنتاج، مكّنت من التمييز الواضح بين فيروس بارفو الإوز الكلاسيكي (C-GPV) وفيروس بارفو الإوز المستجد .(N-GPV) ويُعد هذا النهج وسيلة سريعة وموفرة للتكاليف وموثوقة لتصنيف الأنماط الجينية سواء في الدراسات الحقلية أو المعملية.وباختصار، تؤكد النتائج سيادة سلالة واحدة من-C GPV في مصر خلال عام 2023، وتبرز قربها الوراثي من العترات الصينية، كما تقدم طريقة عملية باستخدام-PCR RFLP للتمييز بين أنماط فيروس بارفو الإوز الجينية. وتُسهم هذه المعطيات في تعزيز الفهم الوبائي لفيروس بارفو الإوز، وتوفر أساساً علمياً مهماً لتطوير اللقاحات واستراتيجيات السيطرة على الوباء.

الكلمات الدالة: SNPs، C-GPV، N-GPV، HinfI، AlwI، تفاعل البلمرة المتسلسل، الهضم، QH15