



Preliminary Studies on The Virus Causing Outbreak of Duckling Short Beak and Dwarfism Syndrome (SBDS) in Egypt

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ABSTRACT

Many Mule, Muscovy, and Bikini duck flocks in different duck-producing regions in Egypt have shown signs of a new disease designated "short beak and dwarfism syndrome" (SBDS) since 2015. The ducks with SBDS showed strong growth retardation with beak atrophy, enteritis, and paralysis. Although the mortality rate was 2%–8% in affected flocks, the morbidity rate was 20%–50% and even 80% in some regions. The disease is characterized by dyspraxia, weight loss, a protruding tongue, and high morbidity and low mortality rates. To characterize the etiological agent, a virus was isolated from the allantoic fluid following serial passage in embryonated duck eggs. This virus causes a cytopathic effect in duck embryo fibroblast (DEF) cells. Using enzyme-linked immunosorbent assay (ELISA), the isolate was positive for the antigen of goose parvovirus (GPV). The bovine sperm agglutination test indicated that the virus was most closely related to GPV strain. Together, these data indicated that the isolated virus from mule ducks with SBDS disease is a GPV-related parvovirus causing and that it is divergent from classical GPV isolates.

Keywords: Bikini duck, Dwarfism, ELISA, SBDS, Syndrome.

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INTRODUCTION

Waterfowl parvovirus infections are generally divided into goose parvovirus (GPV) and Muscovy duck parvovirus (MDPV). GPV infects both goslings and Muscovy ducks, causing Derzsy's disease (Derzsy *et al.*, 1970) while MDPV infects Muscovy ducklings, causing a disease known as "three-week disease". Both types of infection are fatal to waterfowls.

Derzsy's disease or gosling plague was first reported in 1969 due to high morbidity and mortality in young Muscovy ducklings and goslings (Dressy, 1967; Jansson *et al.*, 2007). Short beak and dwarfism syndrome (SBDS) was first reported in France in the early 1970s in mule duck flocks (mule duck, a sterile intergeneric hybrid produced by crossing Peking and Muscovy duck). The diseased flocks showed retarded growth with short beaks (Palya *et al.*, 2009). In 2017, SBDS was reported in Egypt in Gharbya and Sharqya governments. The etiological agent of SBDS is a novel goose parvovirus (NGPV), which shared the highest homology with goose parvovirus (GPV) and was regarded as a variant of GPV (Palya *et al.*, 2009; Chen *et al.*, 2015). NGPV was not a lethal etiological agent

in ducks, but farmers suffered from great economic losses due to reduced sales of NGPV-infected ducks.

NGPV infects 1-day-old Cherry ducks and Mule ducks and demonstrates a high rate of infection with low mortality. Most ducks develop asymptomatic infections, but those with significant clinical symptoms showed growth retardation, atrophied beaks, a protruding tongue outside of the beak, swelling and hemorrhage of the thymus, and fractured feathers and legs (Palya *et al.*, 2009; Chen *et al.*, 2015 and Chen *et al.*, 2016).

N novel goose parvovirus (NGPV), a waterfowl parvovirus, along with GPV and MDPV, belongs to *Anseriform depend on parvovirus* but is distinguished from other depend on viruses by its ability to replicate autonomously in vitro without helper-viruses (Kisary, 1979, Brown *et al.*, 1995). In the family Parvoviridae, the waterfowl parvoviruses are small non-enveloped viruses with a linear, single-stranded DNA genome of approximately 5.1 kb in length (Zadori *et al.*, 1994, Wozniakowski *et al.*, 2009; Ji *et al.*, 2010 and Wang *et al.*, 2016).

The full-length genome contains two open reading frames (ORFs): the left ORF codes for the non-structural proteins NS1 and NS2 (Smith *et al.*, 1999) and the right ORF codes for the structural proteins VP1, VP2 and VP3 (Zádori *et al.*, 1995). Additionally, there are two inverted terminal repeats (ITRs) at both ends of the genome (Zádori *et al.*, 1995; Poonia *et al.*, 2006). In general, parvoviruses agglutinate erythrocytes, but the goose parvovirus (GPV) is an exception. The GPV cannot agglutinate red blood cells but agglutinates bovine sperm (Brown *et al.*, 1995).

Based on the serological investigation and phylogenetic analysis, it was suggested that the syndrome was caused by a distinct GPV that was very difficult to isolate from clinical samples. Palya *et al.* (2009) first isolated this distinct GPV and successfully reproduced this disease by experimental infection. In 1989, a similar disease designated "duckling atrophic beak with protruding tongue and stunted growth disease" was reported with significantly higher morbidity and mortality than that reported in France (Palya *et al.*, 2009).

As a waterfowl parvovirus, NGPV can propagate autonomously. In previous reports, the viruses isolated by Palya *et al.* (2009) were adopted in Muscovy duck eggs after being blindly propagated for one or two passages. The other strains were adapted in ducks after three- or four-blind propagations and could only cause a cytopathic effect (CPE) in duck embryo fibroblasts (DEFs) (Brown *et al.*, 1995, Chen *et al.*, 2015). It was collectively demonstrated that NGPV could replicate in duck eggs and DEFs, but the details about the adaption process and growth curve of NGPV were not reported. During propagation, waterfowl parvovirus shows both autonomous and dependent virus parvoviruses (Palya *et al.*, 2009; Ji *et al.*, 2010). Certain parvoviruses such as H-1 can cause cytopathic effects (CPE) and induce apoptosis in infected cells through the process of replication (Poonia *et al.*, 2006). Still, it is unclear whether NGPV has apoptosis effect.

In this study, Mulard (mule) duckling flocks in different duck-producing areas of Egypt develop SBDS disease was reported. SBDS disease mostly affects young Mulard, Muscovy and Bikini ducklings, characterized by a notably atrophic beak and protruded swollen tongue, stunted growth, fractured feathers and legs, high morbidity, and low mortality. No diagnostic histopathologic lesions other than enteritis were observed. Resistance against the disease increased with age. This disease was not found to be present in adult ducks. Due to the striking loss of weight and size caused by SBDS, the disease causes much economic loss to the Egyptian duck industry. The NGPV local strain was isolated, and its growth characteristics were investigated, laying a foundation for further vaccine and viral molecular research.

MATERIALS AND METHODS

1. Sample collection

To diagnose this epidemic disease, liver, spleen, and intestine samples from infected birds identified as NGPV positive infection were collected from five different duck farms and used for isolation of the virus. The samples were homogenized in phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 6000 rpm for 15 min. The supernatant was filtered through a 0.22- μ m filter then the filtered supernatant was stored at -80°C .

2. Virus isolation and adaption in embryonated duck eggs

Muscovy embryonated duck eggs (9-11-day-old) were inoculated in the allantoic fluid with the filtered supernatant (previously prepared) (0.2 ml/egg) and the inoculated eggs were checked daily till the death of the embryo for survival. If the embryos survived, allantoic fluid was collected, pooled, and used to inoculate embryonated duck eggs for further passages. If the embryo died within days, the allantoic fluid and internal organs were tested for ELISA's waterfowl parvovirus antigens.

3. Virus isolation and adaption in duck embryo fibroblast cells (DEFs)

Duck embryo fibroblasts (DEF_s) cells were prepared from 11-day-old Muscovy duck embryos as described previously (Shahsavandi *et al.*, 2013). The DEFs were inoculated with the filtered supernatant (previously prepared) at a 1:10 dilution and incubated at 37°C in 5% CO_2 . At the same time, the DEFs in the control group were inoculated with PBS. The infected cells underwent three freeze-thaw cycles seven days after inoculation prior to harvest and storage at -80°C for the next infection and detecting CPE. The virus was then serially passaged five times in DEFs. After the virus adaptation to the cells, the CPE was observed daily for up to 7 days. An apparent cytopathic effect (CPE) was considered positive for virus replication.

4. Enzyme-Linked Immunosorbent Assay (ELISA KIT)

Confirmation can be obtained in infected flock tissues and post isolation of the parvovirus in cell cultures or embryonated eggs derived from susceptible Muscovy ducks. The virus's presence can be confirmed by the Geese parvovirus antigen ELISA kit (Shanghai Coon Koon Biotech Co., Ltd. The optical density (OD) values were observed at 450 nm using an automated enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo, Waltham, MA, USA).

5. Bovine sperm agglutination test

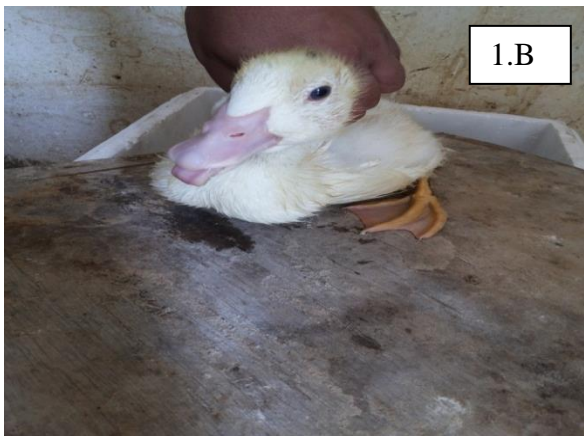
GPV can agglutinate bovine sperm, and this feature can be used for virus identification (Yakun and Shangjin, 2015). The virus to be tested is diluted in saline (ratio 1:5) and 0.25 ml was added in a small test

tube. In comparison, in a second tube, 0.25 ml of physiological saline was added as a control tube, then 0.25 ml buffered cattle semen (semen was added to 1 ml of 2.9% tri-sodium citrate solution at 42°C) was added to each tube, mixed, and set at room temperature for six h before observation. Animal Reproduction Research Institute, El-Giza supplied bovine semen.

RESULTS

1. Clinical symptoms

Several cases of a new disease in commercial meat ducks were reported in the Gharbya government (Byrma) and the outbreaks have since spread to several primary duck production regions. The cases were typically characterized clinically by tongue protrusion accompanied by a shortened beak (Fig.1 A and B), with approximately 10 to 30% morbidity and 2 to 6% mortality at the end of the growth period. The final beak size was approximately 10 to 30% of the normal beak of a healthy duckling. Poor beak development resulted in protruded tongues in previously infected ducklings, significantly affecting their ability to move, eat and drink (Fig. 1 C and D). As a result, the diseased ducklings continued to have poor growth and increased mortality.



In most cases, the affected ducklings had fragile and easily broken legs, potentially caused by the progressive loss of skeletal muscle mass in the legs. Initial symptoms, including the shortened beak and tongue protrusion, began at the age of one week and peaked at three weeks. Infection rates were much lower in adult ducks above four weeks, and symptoms likely became subclinical. All major duck species (Mulard, Muscovy and Bikini) in this study were found to be susceptible to this new disease, and both males and females were represented among the cases. The morbidity rate was variable among affected duck flocks, ranging from 30% to 60%. Although the low mortality rate observed for affected farms was not high, ducks' production performance that survived the infection was significantly reduced due to abnormal feathering and growth retardation.

2. Virus isolation and adaptation in embryonated duck eggs and duck embryo fibroblast cells (DEFs)

After five blindly passages by the allantoic cavity route through embryonated duck eggs, 60% of duck embryos died at 72 hours post-inoculation (HPI) with systemic hemorrhage (Fig.2). The isolate generated

Fig.1: Typical clinical symptoms of SBDS in mule duckling with a short beak, protruding tongue (A&B). Mule duckling with a movement disorder and broken leg (C&D).

little or no cytopathic effect (CPE) in DEF cell cultures at 96 HPI and on microscopic examination, significant cytopathic effects have occurred 72 HPI after four passages. Infected DEF cells became rounded and shrunken, and by 96-120 hours, the entire monolayer was destroyed (Fig.3). In each passage, the inoculated cells were harvested, and the virus was detected using ELISA. Overall, the ELISA kit assay demonstrated that the infected cells were positive for GPV.



Fig.2: GPV-infected duck embryo showed generalized spots of hemorrhage.

3-Enzyme-Linked Immunosorbent Assay (ELISA KIT)

The positive and negative cut-off value for optical density (OD) 450 was calculated and the negative cut-off value was rounded off to ≤ 1.0 to ensure accurate results. Among the tissue samples from infected ducklings, all the five flocks were identified as positive for GPV infection. These positive flocks, whose offspring often manifested SBDS symptoms on the epidemic investigation, were also identified by viral isolation. All the duck egg and cell culture samples of all passages were identified as positive for GPV infection.

4.Titration of the GPV in embryonated duck eggs and DEFs

At the fifth passage in duck eggs and seven passages in DEF cell, the allantoic fluids and infected cell cultures of the isolated strain had infectivity titers of 10^7 EID₅₀/ml and $10^{6.3}$ TCID₅₀/ml, respectively, calculated by the Reed–Muench method (**Reed and Muench, 1938**).

5,Bovine sperm agglutination test

As the first tube virus agglutinates bovine semen in 2.9% trisodium citrate solution and the

second one does not agglutinate, the virus can be judged as GPV (Fig.4).

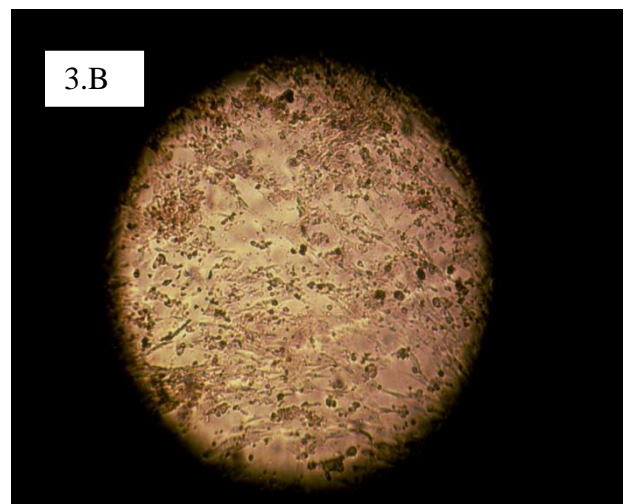
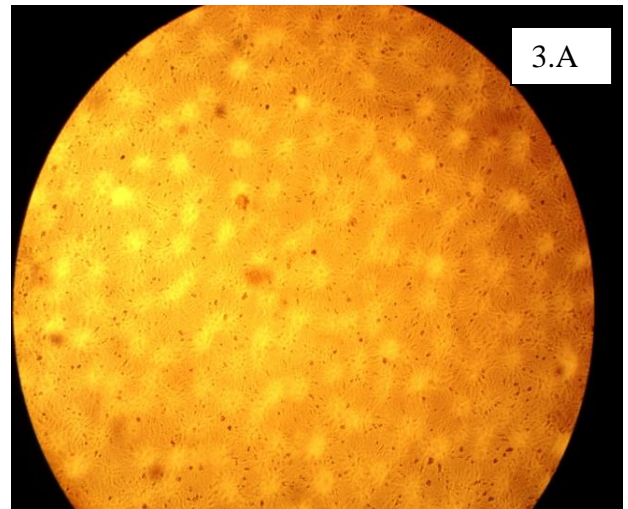


Fig. (3.A) showed normal DEF cells and fig (3.B) showed CPE caused by GPV isolate in infected DEF cells observed at 96 - 120 HPI showing cells rounding and shrunken, and by 96-120 hours, the entire monolayer was destroyed.



Fig.4: Positive bovine sperm agglutination by GPV isolate

The pyrazole derivatives with bis-tolyl groups in positions three and five were revealed hydrogen bond acceptor from sidechain between Arg 68 and oxygen of methanone derivative with bond length 2.87 °A and strength (14%) (Fig. 4). In addition to one arene-cation interaction through the tolyl group at position five and Lys 51 and hydrophobic interaction that arises due to the presence of amantadine and *p*-tolyl moiety.

Furthermore, pyrazole derivatives 6c that containing phenyl and *p*-chlorophenyl as two aryl derivatives observed binding energy $S = -18.02$ kcal/mol with sidechain hydrogen bond acceptor between Arg 68 and oxygen of carbonyl of the methanone derivative with bond length 2.51 °A and (30%) strength. Besides two arene-cation interaction between Arg 68 with the *p*-chlorophenyl group and Lys 51 with phenyl group at the position three and five in pyrazole core respectively, (Fig.4).

Finally, the pyrazole derivatives hybrid with Amantadine core 6a-c suggested being 3C protease inhibitor and exhibited binding energy higher than the two standard drugs Amantadine ($S= 6.15$ kcal/mol) and Ribavirin ($S= 10.77$ kcal/mol) with different binding mode. All the docked pyrazole derivatives 6a-c showed favorable binding interactions with hydrophobic interaction and energy scores from -15.41 to -20.0 kcal/mol in comparison to Amantadine ($S) = -6.15$ kcal/mol, and Ribavirin ($S) = -10.77$ kcal/mol. These highest binding energy and binding interactions suggest that these compounds may act as 3C protease inhibitors and thus may participate as antiviral drugs.

DISCUSSION

Mule duck SBDS was reported in France in the 1970s and Hungary in 2009 (Palya *et al.*, 2009). The causative agent was a distinct lineage of goose parvovirus that was very difficult to isolate. A more severe SBDS disease was also reported in Taiwan in 1989, but the causative agent was diagnosed as a coinfection of duck parvovirus and duck viral hepatitis (Chen *et al.*, 2015). However, since 2015, a disease similar to the SBDS was observed in young Chinese Cherry Valley duck flocks (Lu *et al.*, 1993).

In this study, SBDS disease was observed in Mule, Muscovy, and Bikini ducks in Egypt, and this is the first report of SBDS disease existing in both mule ducks and bikini ducks. The viral strain was isolated from ducks with SBDS based on the pathogenic differential diagnosis, disease reproduction. It was found that SBDSV M15 is the causative agent of SBDS disease in China and related to GPV but not MDPV (Chen *et al.*, 2016). In 2015, the NGPV outbreak in a Northern China coastal city resulted from a novel

goose parvovirus, which was closely related to the goose parvovirus (GPV) (Lu *et al.*, 1993).

The virus was isolated from the liver and intestinal tract tissues obtained from infected ducks in the current study. After blind-propagation for five passages in vitro (embryonated duck eggs), it was found that the isolate strain infection of duck embryos resulted in death at 72 HPI with systemic hemorrhage (Fig.2). In inoculated DEFs, cytopathic effects were only observed in DEFs after blind-replication for four passages characterized by cells rounding and shrunken. By 96-120 hours, the entire monolayer was destroyed (Fig.3.B), and viral infectivity titers across 5 and 7 passages were 10^7 EID₅₀/ml and $10^{6.3}$ TCID₅₀/ml, respectively. These results appear similar to the previous reports showing that NGPV could be isolated and propagated in duck embryos and DEFs are permissive to NGPV, while GEFs are not (Palya *et al.*, 2009; Chen *et al.*, 2015).

However, NGPV can replicate in duck embryos and cause DEF cell death. It was exciting that NGPV cannot induce apoptosis, which was different from the most parvovirus. Future studies focusing on vaccine development and identifying the mechanisms of NGPV infection are necessary to address this problem going forward (Zhang *et al.*, 2019).

ELISA is a common diagnostic test for detecting various infectious pathogens, and different versions are available, including direct ELISA, antigen capture ELISA, sandwich ELISA, and dipstick ELISA. Recently, Wang *et al.* (2014) developed a competitive ELISA using a monoclonal antibody to detect GPV virus-like particles and vaccine immunization in goose sera (Wang *et al.*, 2014). The procedural details for ELISA and competitive ELISA are available in published reports (Baxi *et al.*, 2006; Cheng *et al.*, 2009). The obtained results were confirmed by ELISA parvovirus antigen kit and bovine sperm agglutination test for virus identification in all tissue specimens and infected allantoic and tissue cultures fluid.

CONCLUSIONS

The isolated virus from diseased duck flocks might be a novel Muscovy duck infecting GPV (MDGPV) circulating in Egypt and it appears that variant goose parvoviruses do exist in nature. The high prevalence of GPV and MDPV infection does provide the opportunity for coinfection, the first step in generating recombinant viruses. Genetic data indicate that the MDGPV/ strain has adapted to infect Muscovy ducks, and antigenic variation has resulted in inconsistent responses to traditional GPV vaccines (Wang *et al.*, 2013). Future studies will characterize the isolated GPV sequence, molecular epidemiology of

virus infections and pathogenesis studies. However, it is important to have a vaccine against duck origin GPV in Egypt.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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